Selected Papers by Tongji Medical College Overseas Alumni

Tongji Medical College Overseas Alumni Association

2014

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To Tongji Medical College.
Foreword

Tongji Medical College Overseas Alumni Association (TJMCOAA) is a U.S. Federal-registered non-profit organization dedicated to strengthening the professional ties among our members, and between our members and alma mater. The annual collection of publications, which was initiated by Drs. Guohua Li, Steven (Sijiu) Shen, Huiyun Xiang, Marilyn (Mengrong) Li, Henry (Henyi) Liu in 2007, was an ongoing project organized by the TJMCOAA. Since 2013, the collection has been kindly sponsored by Medjaden Bioscience Limited, and XIA&HE Publishing Limited. We are very proud that all the previous collections were well received and appreciated by our Alma Mater, Tongji Medical University and now Huazhong University of Science and Technology.

Here, we are pleased to present the selected scholarly work published by our fellow alumni around the world. The 2014 edition is composed of 38 articles authored by 14 alumni published from late 2012 to July 2014. The publications not only exemplify the academic achievements of our overseas alumni, but also provide a snapshot of the international research trends in various biomedical fields.

We hope this collection of publications will serve as an intellectual bridge between our fellow overseas alumni and Tongji faculties and students. Please feel free to contact us if you have any comments and suggestions. Your feedback is much appreciated.

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September 30, 2014
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Prognostic value of circulating tumor cells in patients with pancreatic cancer: a meta-analysis

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Lu Han and Wei Chen are to be considered equal contributors and co-first authors


Increasing scientific evidences suggest that circulating tumor cells (CTC) in peripheral blood may be a powerful predictor of survival in patients with pancreatic cancer. However, many existing studies have yielded inconclusive results. This meta-analysis aims to assess the prognostic value of CTC in patients with pancreatic cancer. An extensive literary search for relevant studies was conducted on PubMed, Embase, Web of Science, Cochrane Library, CISCOM, CINAHL, Google Scholar, CNKI, and CBM databases from their inception through July 1, 2013. The meta-analysis was then performed using the Stata 12.0 software. Crude hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated under a fixed or random effect model. Nine cohort studies were included in this meta-analysis with a total of 623 pancreatic cancer patients. This number included 268 CTC-positive patients and 355 CTC-negative patients. Our meta-analysis revealed that patients in the CTC-positive group were significantly associated with poor progression-free survival (PFS) (HR = 1.89, 95% CI = 1.25-4.00, P < 0.001). Furthermore, pancreatic cancer patients in the CTC-positive group also showed worse overall survival (OS) than those in the CTC-negative group (HR = 1.23, 95% CI = 0.88-2.08, P < 0.001). Subgroup analysis by ethnicity indicated that CTC-positive patients had poor OS among both Asian and Caucasian populations (all P < 0.05). Further subgroup analyses by detection and treatment methods also suggested that CTC-positive patients showed worser OS than CTC-negative patients in the majority of subgroups (all P < 0.05). No publication bias was detected in this meta-analysis. In conclusion, our meta-analysis suggests that CTC-positive pancreatic cancer patients may have worser PFS and OS than CTC-negative patients. Detection of CTC in peripheral blood may be a promising biomarker for the detection and prognosis of pancreatic cancer.

PubMed 链接:

论文全文链接:
OBJECTIVE: This paper aimed to investigate the molecular mechanisms associated with angiotensin-converting enzyme (ACE)-inhibitory peptidase activity involved in vascular extracellular matrix (ECM) remodeling. Therefore, changes in collagen fibers, elastic fibers and laminin were assessed in the left common carotid artery (LCCA).

METHODS: We selected 10-week-old male spontaneously hypertensive rats to study the expression levels of matrix metalloproteinases (MMPs), transforming growth factor, angiotensin (Ang) II and nuclear factor (NF)-p65 in the wall of carotid arteries.

RESULTS: Compared to the control group, laminin expression was significantly increased (p < 0.05) in the vascular endothelium of the LAP (a homemade ACE-inhibitory peptide, named by ourselves) group, whereas the percentage of elastic/collagen fibers in the LCCA vascular area was significantly decreased (p< 0.0001) in the LAP group. Immune blots of MMP-2, MMP-9, NF-p65 and AngII were significantly reduced in the LCCA wall in the LAP group.

CONCLUSION: Vascular ECM remodeling may be related to the inhibitory action of LAP on ECM deposition.


论文全文链接: http://www.karger.com/Article/FullText/356951
The efficacy and safety of arotinolol combined with a different calcium channel blocker in the treatment of Chinese patients with essential hypertension: a one-year follow-up study

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*Hong Fang and Wei Chen contributed equally as joint first authors


Background: Combined treatment of a calcium antagonist and α/β-adrenoreceptor blocker is expected to offer some advantages in the management of hypertension; however, their antihypertensive efficacy and safety remain relatively under-explored. Methods: The current study addresses the 24-h antihypertensive efficacy and safety of arotinolol combined with a different calcium channel blocker. One-hundred fifty-two patients were randomly divided into three groups: nifedipine, amlodipine and felodipine group. In each group, the antihypertensive treatment dose was 30 mg/d, 5 mg/d, 5 mg/d long acting nifedipine, amlodipine, felodipine plus 20 mg/d arotinolol, respectively. Blood pressure was measured in ABPM devices and mercury manometer. Results: The result showed that the effective rate of one year antihypertensive treatment of arotinolol combined with nifedipine was 51 of 53, significantly effective (p < 0.05) among three combinations. It also showed that there was no statistical significant difference (p > 0.05) in controlled rate of morning peak blood pressure between treatment of arotinolol combined with amlodipine and arotinolol combined with nifedipine, but there was a significant difference (p < 0.01) in controlled rate of morning peak blood pressure between arotinolol combined with nifedipine vs. felodipine and arotinolol combined with amlodipine vs. felodipine. Conclusions: The therapy approached of arotinolol combined with nifedipine or amlodipine could be effective and well-tolerated, and they can be used as the better chosen antihypertensive drug.

PubMed 链接:

论文全文链接:
Genetic Variation in 15-Hydroxyprostaglandin Dehydrogenase and Colon Cancer Susceptibility

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Background: 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a metabolic antagonist of COX-2, catalyzing the degradation of inflammation mediator prostaglandin E2 (PGE2) and other prostanoids. Recent studies have established the 15-PGDH gene as a colon cancer suppressor.

Methods: We evaluated 15-PGDH as a colon cancer susceptibility locus in a three-stage design. We first genotyped 102 single-nucleotide polymorphisms (SNPs) in the 15-PGDH gene, spanning ~50 kb up and down-stream of the coding region, in 464 colon cancer cases and 393 population controls. We then genotyped the same SNPs, and also assayed the expression levels of 15-PGDH in colon tissues from 69 independent patients for whom colon tissue and paired germline DNA samples were available. In the final stage 3, we genotyped the 9 most promising SNPs from stages 1 and 2 in an independent sample of 525 cases and 816 controls (stage 3).

Results: In the first two stages, three SNPs (rs1365611, rs6844282 and rs2332897) were statistically significant (p<0.05) in combined analysis of association with risk of colon cancer and of association with 15-PGDH expression, after adjustment for multiple testing. For one additional SNP, rs2555639, the T allele showed increased cancer risk and decreased 15-PGDH expression, but just missed statistical significance (p-adjusted = 0.063). In stage 3, rs2555639 alone showed evidence of association with an odds ratio (TT compared to CC) of 1.50 (95% CI = 1.05–2.15, p = 0.026).

Conclusions: Our data suggest that the rs2555639 T allele is associated with increased risk of colon cancer, and that carriers of this risk allele exhibit decreased expression of 15-PGDH in the colon.

PubMed 链接:

论文全文链接：(详见 A1-A8 页)
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0064122
A proliferative burst during preadolescence establishes the final cardiomyocyte number

It is widely believed that perinatal cardiomyocyte terminal differentiation blocks cytokinesis, thereby causing binucleation and limiting regenerative repair after injury. This suggests that heart growth should occur entirely by cardiomyocyte hypertrophy during preadolescence when, in mice, cardiac mass increases many-fold over a few weeks. Here, we show that a thyroid hormone surge activates the IGF-1/IGF-1-R/Akt pathway on postnatal day 15 and initiates a brief but intense proliferative burst of predominantly binuclear cardiomyocytes. This proliferation increases cardiomyocyte numbers by ~40%, causing a major disparity between heart and cardiomyocyte growth. Also, the response to cardiac injury at postnatal day 15 is intermediate between that observed at postnatal days 2 and 21, further suggesting persistence of cardiomyocyte proliferative capacity beyond the perinatal period. If replicated in humans, this may allow novel regenerative therapies for heart diseases.
PubMed 链接:

论文全文链接:
http://www.cell.com/cell/abstract/S0092-8674(14)00410-3
The anti-oxidant effects are not the main mechanism for glutamine’s protective effects on acute kidney injury in mice.

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This study was support by the Seed grant from University of Pittsburgh School of Medicine (ZP); and R01HL080926; R01DK070910 (JAK). The content is solely the responsibility of the authors and does not necessarily represent the official views of NIDDK, NHLBI, or the National Institutes of Health. Electronic address: pengz@upmc.edu.


Acute kidney injury (AKI) is a common problem characterized by an inflammatory response in the kidney and oxidative stress. However, there are no interventions to prevent AKI. Glutamine is an important precursor of glutathione and has also been shown to induce heat shock proteins (HSP). Thus, glutamine may affect both oxidative stress and inflammation. This study was to explore the effects of glutamine pretreatment on nephrotoxic AKI and to investigate the underlying mechanisms. First, the effects of alternate doses of glutamine were compared in CD-1 mice with AKI induced with folic acid intra-peritoneal injection. Then the effects of glutamine quercetin (an HSP inhibitor), and quercetin+glutamine, were compared in the same AKI model. AKI were assessed with plasma creatinine, urine neutrophil gelatinase-associated lipocalin, and renal histology. Inflammatory response was monitored with renal tumor necrosis factor (TNF-α), chemkines (CXCL1 and CCL2) contents, and neutrophil infiltration. Oxidative injury was detected with reduced glutathione,
malondialdehyde, and protein thiol. Glutamine provided dose-dependent renal protection. Pretreatment with quercetin, which was showed to inhibit HSP-70 expression, abolished glutamine's renal-protective effects. Quercetin also abrogated glutamine's beneficial effects on renal TNF-α, chemokines, and neutrophil infiltration. However, quercetin did not affect glutamine's anti-oxidative effects. These results suggest that glutamine's renal-protective effects are mainly related to its activation of HSP-70, which mitigates inflammatory response, renal neutrophil infiltration and subsequent AKI. Regulating neutrophil infiltration might be a potential therapeutic target for AKI.

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PubMed 链接:

论文全文链接:
ID: PZY-2

Development of venovenous extracorporeal blood purification circuits in rodents for sepsis.

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BACKGROUND:
Unlike pharmacologic interventions in sepsis, extracorporeal blood purification, which is widely used in septic patients, is not typically studied in experimental rodents. Most of the previous studies have performed extracorporeal blood purification in larger animals and typically use arteriovenous (AV) vascular access. We developed a venovenous (VV) purification model in the rat as an adjunct for the treatment of sepsis.

METHODS:
Using adult male Sprague-Dawley rats, we cannulated the femoral artery or vein and the jugular vein with P50 tubing and created an AV or VV circuit. Blood flow was maintained by arterial pressure in the AV circuit, whereas in the VV circuit the blood flow was regulated using a rotary pump. The safety of this circuit was evaluated using the changes of blood interleukin 6, rectal temperature, and 7-d survival with sham extracorporeal circulation (circuit connection without treatment) compared with the control (without circuit). The main side complications of this VV circuit were compared with those of the AV circuit.

RESULTS:
The differences in interleukin 6, body temperature, and cumulative survival were not statistically significant after extracorporeal circulation. The main complications of extracorporeal circulation occurred less often with VV compared with AV therapy: massive bleeding (2.5% versus 15%, P = 0.04); clot formation (2.5% versus 15%, P = 0.04). This VV circuit has been successfully used in different septic rodent models with different techniques (hemoadsorption and hemofiltration).

**CONCLUSIONS:**

VV blood purification in a rodent model appears to be effective and is safer than AV circuit.

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**PubMed 链接:**


**论文全文链接:**

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**ISSN:**

**ABSTRACT:**

**PURPOSE OF REVIEW:**
There is significant controversy for perioperative fluid management. This review discusses the evidence from clinical studies, basic research, and systematic reviews to provide a summary of the current best practice in this area.

**RECENT FINDINGS:**
Recent evidence has challenged the long-held contention that use of colloids results in substantially less fluid volumes to achieve resuscitation endpoints. Meanwhile, evidence that hydroxyethyl starch does carry a risk of renal toxicity is now strong. Mounting evidence also points to a hazard, especially for the kidney, when large volumes of saline are used. A patient's clinical condition may also determine the deposition of infused fluids in the body. Total positive fluid balance is an indicator of adverse clinical outcomes, though a cause-effect relationship has not been firmly established. The optimal perioperative fluid management requires a balance of the beneficial and adverse effects of intravenous fluid.

**SUMMARY:**
Although potentially life-saving, evidence points to significant hazards associated with various types and use-strategies for intravenous fluids. Like other drugs, intravenous fluids should be used with...
caution for specific indications, in specific amounts, and with careful attention to potential adverse
effects associated with various products. An individualized approach to perioperative fluid therapy is
recommended.

PubMed 链接:

论文全文链接:
Clear cell papillary renal cell carcinoma (CCP-RCC) has recently been recognized as a distinct subtype of renal cell carcinoma (RCC) due to its unique morphologic, immunohistochemical, and genetic features and indolent clinical behavior. However, the incidence of this tumor in a nephrectomy series for renal mass has not been fully investigated. Twelve cases of CCP-RCC were identified from a total of 290 consecutive partial (n = 137) or radical nephrectomies (n = 153) for RCC from 2010 to 2012 in our hospital. In this series, CCP-RCC was the fourth most common (4.1%) kidney tumor following clear cell (conventional) (70%), papillary (16.6%), and chromophobe (5.9%) RCCs. The average age of the CCP-RCC patients was 58.2 years (range, 18-81 years), with an equal sex distribution. Four cases (33.3%) were associated with end-stage renal disease. Of the 12 CCP-RCCs, 9 presented as solitary tumors; 2 coexisted with clear cell RCC; and 1 with papillary RCC. The average size of tumors was 2.5 cm (range, 0.8-6.0 cm). All tumors were pT1 (10 pT1a and 2 pT1b). Two cases were initially misclassified as clear cell RCC. Strong positive cytokeratin 7 stain and negative stains with α-methylacyl-CoA racemase and RCC marker differentiate CCP-RCC from low-grade clear cell RCC with similar histologic features. We conclude that CCP-RCC is a common renal neoplastic entity, representing the fourth most common (4.1%) RCC. It can be easily misclassified due to its overlapping features with low-grade clear cell RCC. In equivocal cases, immunohistochemical stains with a small panel of markers (cytokeratin 7, α-methylacyl-CoA racemase, RCC marker, or CD10) are warranted in making the correct histologic classification.
论文全文链接：(详见 A9-A14 页)
http://www.humanpathol.com/article/S0046-8177(13)00337-7/abstract
Extrusion of amyloid fibrils to the extracellular space in experimental mesangial AL-amyloidosis: transmission and scanning electron microscopy studies and correlation with renal biopsy observations

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In vitro studies have provided much information regarding the process of glomerular AL-amyloidogenesis. Research efforts have been successful in deciphering how glomerulopathic light chains interact with mesangial cells. The sequential steps involved in the genesis of amyloid fibrils include interactions with surface caveolae in mesangial cells and internalization of the monoclonal light chains through a clathrin-mediated process followed by trafficking in the mesangial cells to the mature lysosomal compartment where fibrils are formed. This manuscript focuses on how mesangial cells, once amyloid has been formed, deliver the fibrils to the extracellular matrix. The delivery of amyloid fibrils to the outside of the cells is carried out by lysosomes, which abut the mesangial cell membranes and extrude their contents into the extracellular space. This final step responsible for the fibrils to be present predominantly in the extracellular space is well demonstrated with scanning electron microscopy.


An animal model of glomerular light-chain-associated amyloidogenesis depicts the crucial role of lysosomes

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Kidney International advance online publication, 30 April 2014; doi:10.1038/ki.2014.122.

In vitro and ex vivo studies have elucidated the step-by-step process whereby some physicochemically abnormal light chains are processed by mesangial cells to form amyloid fibrils. Although crucial steps in the cascade of events have been determined, these findings have not been reproduced in vivo. This has led to some doubts as to the significance and clinical application of the information that has been deciphered. Here, we developed an animal model which uses mice injected with amyloidogenic light chains purified from the urine of patients with biopsy-proven, light-chain-associated glomerular amyloidosis which validated in vitro/ex vivo findings. This animal model showed internalization of the light chains utilizing caveolae followed by trafficking to the mature lysosomal compartment where fibrils were formed. This model permits evaluation of mesangial amyloidogenesis for prolonged periods of time, is potentially useful to test maneuvers to modulate events that take place, and can be used to design novel therapeutic interventions.

PubMed 链接:

论文全文链接:
http://www.nature.com/ki/journal/vaop/ncurrent/full/ki2014122a.html
ID: WHM-1

论文题目：
The CUL7/F-box and WD repeat domain containing 8 (CUL7/Fbxw8) ubiquitin ligase promotes degradation of hematopoietic progenitor kinase 1

作者及其单位和联系信息：
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杂志及论文出版信息：

论文摘要：
HPK1, a member of mammalian Ste20-like serine/threonine kinases, is lost in >95% pancreatic cancer through proteasome-mediated degradation. However, the mechanism of HPK1 loss has not been defined. The aims of this study are to identify the ubiquitin ligase and to examine the mechanisms that targets HPK1 degradation. We found that the CUL7/Fbxw8 ubiquitin ligase targeted HPK1 for degradation via the 26 S proteasome. The ubiquitination of HPK1 required its kinase activity and autophosphorylation. Wild-type protein phosphatase 4 (PP4), but not the phosphatase-dead PP4 mutant, PP4-RL, inhibits the interaction of Fbxw8 with HPK1 and Fbxw8-mediated ubiquitination of HPK1. In addition, we showed that Thr-355 of HPK1 is a key PP4 dephosphorylation site, through which CUL7/Fbxw8 ubiquitin ligase and PP4 regulates HPK1 stability. Knockdown of Fbxw8 restores endogenous HPK1 protein expression and inhibits cell proliferation of pancreatic cancer cells. Our study demonstrated that targeted degradation of HPK1 by the CUL7/Fbxw8 ubiquitin ligase constitutes a negative-feedback loop to restrain the activity of HPK1 and that CUL7/Fbxw8 ubiquitin ligase promotes pancreatic cancer cell proliferation. CUL7/Fbxw8 ubiquitin ligase-mediated HPK1 degradation revealed a direct link and novel role of CUL7/Fbxw8 ubiquitin ligase in the MAPK pathway, which plays a critical role in cell proliferation and differentiation.

PubMed 链接：

论文全文链接：
http://www.jbc.org/cgi/pmidlookup?view=long&pmid=24362026
Loss of phosphatase and tensin homolog expression is associated with recurrence and poor prognosis in patients with pancreatic ductal adenocarcinoma

Phosphatase and tensin homolog (PTEN) is a tumor suppressor in the AKT/mTOR pathway. Animal model studies have shown that loss of PTEN function is involved in the progression of pancreatic cancer. However, the prognostic significance of loss of PTEN expression in pancreatic cancer is unclear. PTEN expression was evaluated by immunohistochemistry on tissue microarrays consisting of multiple cores of 133 resected stage II pancreatic ductal adenocarcinomas. A PTEN expression score was calculated as the product of the percentage of positive tumor cells and the intensity of PTEN staining. We categorized PTEN expression for each tumor as retained (PTEN score >5) or lost (PTEN score ≤5). Thirty-four (25.6%) patients had tumors with loss of PTEN expression, and 99 (74.4%) had tumors with retained PTEN expression. Recurrence/Metastasis was observed in 88.2% (30/34) of patients whose tumors showed loss of PTEN compared with 68.7% (68/99) of patients whose tumors showed retained PTEN (P = .03). Patients whose tumors showed loss of PTEN had a shorter overall survival (median, 19.9 ± 3.6 months) than did patients whose tumors had retained PTEN (32.7 ± 5.0 months, P = .03). In a multivariate analysis, loss of PTEN expression was an independent prognostic factor for poor overall survival in patients with stage II pancreatic ductal adenocarcinoma. No significant correlations between loss of PTEN expression and other clinicopathologic parameters were observed (P > .05). Assessment of PTEN expression may be used as a prognostic marker for patients with resected pancreatic ductal adenocarcinoma.
Solid pseudopapillary neoplasm of the pancreas with prominent atypical multinucleated giant tumour cells

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AIMS: Solid pseudopapillary neoplasm of the pancreas (SPN) is a rare low-grade malignant neoplasm. To our knowledge, SPN with prominent atypical multinucleated giant tumour cells (MNGTCs) has not yet been reported.

METHODS AND RESULTS: We identified four cases of SPN with prominent atypical MNGTCs in a cohort of 62 cases of SPN (6.5%). The MNGTCs contained multiple enlarged, hyperchromatic, irregular nuclei with ample eosinophilic cytoplasm, typically present in the solid area of the tumour. The MNGTCs had an immunohistochemical profile typical of the conventional SPN and were positive for vimentin, β-catenin, CD10 and progesterone receptor, but negative for pan-cytokeratin, chromogranin, synaptophysin, trypsin, Ki-67 and CD68 in all four cases. Patients of SPN with prominent MNGTCs were older than those with conventional SPN (P = 0.01); tumours were discovered incidentally by imaging studies for an unrelated disease in all four cases, and with a female to male ratio of 1:1. The proliferation index (Ki-67) was <1% in all four cases. None of the three patients for whom information was available developed recurrence during follow-up of 2.7, 3.8 and 5.0 years.

CONCLUSIONS: The presence of MNGTCs in SPN most probably represents degenerative change of the tumour cells and does not seem to affect the prognosis.


论文全文链接: (详见 A28-A37 页)
http://onlinelibrary.wiley.com/doi/10.1111/his.12023/abstract;jsessionid=449CBAE4317ECE8E2247FDB9C5B378D6.f02t01
Pancreatic intraepithelial neoplasia and histological changes in non-neoplastic pancreas associated with neoadjuvant therapy in patients with pancreatic ductal adenocarcinoma

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AIMS: To study the histological changes in non-neoplastic pancreas and the effects on pancreatic intraepithelial neoplasia (PanIN) after neoadjuvant chemoradiation therapy (NCRT) for pancreatic ductal adenocarcinoma (PDAC).

METHODS AND RESULTS: We reviewed the archival H&E slides from 218 patients with PDAC who completed NCRT and pancreaticoduodenectomy. Sixty-five patients who underwent pancreaticoduodenectomy for PDAC without NCRT were used as controls. Various histological features were reviewed and correlated with NCRT and survival. The NCRT group had lower densities of PanIN2 (P = 0.004) and PanIN3 (P = 0.02) than the control group. The extent of fibrosis, the frequency of neuroma-like nerve proliferation and the frequency of islet cell aggregation were significantly higher in the NCRT group than in the control group (P < 0.05). The intensity of inflammation was less in the NCRT group than in the control group (P = 0.02). In the NCRT group, patents with moderate to severe fibrosis or grade 2 inflammation had poorer survival than those with mild fibrosis (P = 0.04) or those with grade 0 or grade 1 inflammation (P = 0.003), respectively.

CONCLUSIONS: Non-neoplastic pancreatic tissue from patients who received NCRT had a reduced density of high-grade PanIN lesions, more pancreatic fibrosis, and higher frequencies of neuroma-like nerve proliferation and islet cell aggregation, but less inflammation, compared to tissue from those who did not receive NCRT.

PubMed 链接:

论文全文链接:
Clinicopathologic features and prognosis of duodenal adenocarcinoma and comparison with ampullary and pancreatic ductal adenocarcinoma

Because of the rarity of duodenal adenocarcinoma (DAC), the clinicopathologic features and prognostication data for DAC are limited. There are no published studies directly comparing the prognosis of DAC to that of ampullary adenocarcinoma (AA) and of pancreatic ductal adenocarcinoma (PDA) after resection. In this study, we examined the clinicopathologic features of 68 patients with DAC, 92 patients with AA, and 126 patients with PDA who underwent resection. Patient clinicopathologic and survival information were extracted from medical records. Statistical analysis was performed using Statistical Package for the Social Sciences with 2-sided significance level of .05. Patients with DAC had higher American Joint Committee on Cancer (AJCC) stage than AA patients (P = .001). Lymph node metastasis (P = .013) and AJCC stage (P = .02) correlated with overall survival in DAC patients. Patients with DAC or AA had lower frequencies of lymph node metastasis and positive margin and better survival than those with PDA (P < .05). However, no differences in nodal metastasis, margin status, or survival were observed between DAC patients and those with AA. Our study showed that lymph node metastasis and AJCC stage are important prognostic factors for overall survival in DAC patients. Patients with DAC had less frequent nodal metastasis and better prognosis than those with PDA. There was no significant difference in prognosis between DAC and AA.

PubMed 链接:

论文全文链接:
http://www.humanpathol.com/article/S0046-8177(13)00319-5/abstract
Phosphatase and tensin homolog (PTEN) is one of the most frequently inactivated tumor suppressor genes in sporadic cancers. Somatic mutations of PTEN occur in many tumors including those of the gastrointestinal and hepatobiliary tracts. Loss of PTEN expression is associated with poor prognosis in patients with metastatic colonic adenocarcinoma, gastroesophageal junction adenocarcinoma, gastric adenocarcinoma, and pancreatic ductal adenocarcinoma.

OBJECTIVE:
To study the expression of PTEN and its significance in ampullary adenocarcinoma (AA).

DESIGN:
We constructed tissue microarrays by using archival tissue from 92 patients (55 males, 37 females; median age, 63 years; age range, 37 to 87 years) with previously untreated AA who underwent pancreaticoduodenectomy at our institution. PTEN expression was evaluated by immunohistochemistry, scored semiquantitatively (based on staining intensity and percentage positive tumor cells), and correlated with clinicopathologic features and survival.

RESULTS:
Of 92 cases, 23 (25.0%) were PTEN negative. Loss of PTEN expression correlated with lymph node metastasis (P = .004), advanced American Joint Committee on Cancer (AJCC) stage (P = .02), and higher frequency of recurrence (P = .03). Patients with PTEN-negative tumors had shorter disease-free survival (DFS, mean: 89.0 ± 20.8 months) and overall survival (OS, mean: 93.1 ± 19.1 months) than those with PTEN-positive tumors (DFS, mean: 161.4 ± 11.7 months, P = .01; OS, mean: 175.4 ± 11.0 months, P = .001). In multivariate analyses, PTEN expression was a prognostic factor for both DFS and OS, independent of AJCC stage, lymph node status, pathologic tumor (pT) stage, and differentiation.

CONCLUSIONS:
Loss of PTEN expression is associated with poor DFS and OS in patients with AA after curative surgery. PTEN expression may be used as a prognostic marker for patients with resected AA.

PubMed 链接:

论文全文链接：（详见 A38-A45 页）
ID: WL-1

论文题目:
Longitudinal Alterations in the Dynamic Autoregulation of Optic Nerve Head Blood Flow Revealed in Experimental Glaucoma

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杂志及论文出版信息:

论文摘要:
Purpose: To use a novel dynamic autoregulation analysis (dAR) to test that the optic nerve head (ONH) blood flow (BF) autoregulation is disrupted during early stages of experimental glaucoma (EG) in nonhuman primates (NHPs). Methods: Retinal nerve fiber layer thickness (RNFLT, assessed by optical coherence tomography) and ONH BF (assessed by laser speckle imaging technique) were measured biweekly before and after unilateral laser treatment to the trabecular meshwork. Each NHP was followed until reaching either an early stage of damage (RNFLT loss <20%, n=6) or moderate-to-advanced stages of damage (RNFLT loss >20%, n=9). At each test, dAR was assessed by characterizing ONH BF changes during the first-minute of rapid manometrical IOP elevation from 10 to 40 mmHg. The dAR analysis extracted the following parameters: BFbl: average BF 10 seconds before IOP elevation. BFmax: maximum BF change from BFbl. Tr: time from BFbl to the BFmax. Kr: average descending BF rate. Results: Mean post-laser IOP was 20.2 ± 5.9 and 12.3 ± 2.6 mmHg in EG and control eyes, respectively (P<0.0001). Compared with baseline values, BFbl was higher in early EG, but lower in moderate-to-advanced EG (P=0.01). Tr was increased and Kr was reduced in both stages (P<0.01). BFmax was smaller in the early EG (P=0.05) and remained low in the moderate-to-advanced EG (P=0.15). No changes in the parameters were observed in control eyes. Conclusions: Chronic IOP elevation causes ONH autoregulation dysfunction in the early stages of EG, characterized by a disrupted BF response and delayed Tr, revealed by dAR analysis.

PubMed链接:

论文全文链接:
http://www.iovs.org/cgi/pmidlookup?view=long&pmid=24812551
论文题目:
Parametric transfer function analysis and modeling of blood flow autoregulation in the optic nerve head

作者及其单位和联系信息:
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杂志及论文出版信息:

论文摘要:
The aim of the study was to establish a parametric transfer function to describe the relationship between ocular perfusion pressure (OPP) and blood flow (BF) in the optic nerve head (ONH). A third-order parametric theoretical model was proposed to describe the ONH OPP-BF relationship within the lower OPP range of the autoregulation curve (< 80 mmHg) based on experimentally induced BF response to a rapid intraocular pressure (IOP) increase in 6 rhesus monkeys. The theoretical and actual data fitted well and suggest that this parametric third-order transfer function can effectively describe both the linear and nonlinear feature in dynamic and static autoregulation in the ONH within the OPP range studied. It shows that the BF autoregulation fully functions when the OPP was > 40 mmHg and becomes incomplete when the OPP was < 40 mmHg. This model may be used to help investigating the features of autoregulation in the ONH under different experimental conditions.

PubMed 链接:

论文全文链接：（详见 A46-A55 页）
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3961098/
PURPOSE: To characterize the static blood flow autoregulation in the optic nerve head (ONH), and to investigate its role in hemodynamic changes in experimental glaucoma (EG).

METHODS: Unilateral elevation of intraocular pressure (IOP) was induced in 15 adult rhesus macaques by laser treatment to the trabecular meshwork. Prior to and after laser treatment, retinal nerve fiber layer thickness (RNFLT) was assessed, biweekly, by spectral-domain optical coherence tomography. Optic nerve head static autoregulation was assessed by determining the percentage blood flow (BF) change after the IOP was acutely increased from 10 to 30, 40, or 50 mm Hg manometrically, utilizing a laser speckle flowgraphy device.

RESULTS: Postlaser IOP (measured during average 7.7 ± 2.6 months) was 20.2 ± 5.9 mm Hg in EG eyes and 12.3 ± 2.6 mm Hg in control eyes (P < 0.0001). Retinal nerve fiber layer thickness was reduced by 33 ± 22% of the baseline values (P < 0.001) on average in EG eyes and by 0.4 ± 2.3% in control eyes (P > 0.05). The ONH BF remained at a constant level within a range of ocular perfusion pressure (OPP), 41 mm Hg and above. The autoregulation curves, created by all 723 tests in control and 352 tests in EG, were not significantly different (P = 0.71).

CONCLUSIONS: Optic nerve head BF in normal nonhuman primate (NHP) eyes is effectively regulated within a range of OPP approximately 41 mm Hg and above. Chronic IOP elevation causes no remarkable change to the static autoregulation within the ONH of EG eyes.

PubMed 链接:

论文全文链接：（详见 A56-A63 页）
http://www.iovs.org/content/55/2/873.long
ID: WL-4

论文题目:
Longitudinal hemodynamic changes within the optic nerve head in experimental glaucoma

作者及其单位和联系信息:
Cull G, Burgoyne CF, Fortune B, Wang L.
Discoveries in Sight Research Laboratories, Devers Eye Institute, Legacy Research Institute Portland, Oregon.

杂志及论文出版信息:

论文摘要:
PURPOSE: To characterize longitudinal changes in basal blood flow (BF) of the optic nerve head (ONH) during progression of structural damage in experimental glaucoma (EG).

METHODS: Unilateral elevation of IOP was induced in 15 adult rhesus macaques by laser treatment to the trabecular meshwork. Prior to and after laser, retinal nerve fiber layer thickness (RNFLT) and ONH BF were measured biweekly by spectral-domain optical coherence tomography and a laser speckle flowgraphy device (LSFG), respectively.

RESULTS: Average postlaser IOP was 20.2 ± 5.9 mm Hg in EG eyes and 12.3 ± 2.6 mm Hg in control eyes (P < 0.0001). Longitudinal changes in basal ONH BF were strongly associated with changes in RNFLT as EG progressed from early through moderately advanced stages of damage, with Pearson correlation coefficients ranging from 0.64 to 0.97 (average = 0.81) and an average slope of 1.0. During early stage (RNFLT loss < 10%), basal ONH BF was mildly increased (9% ± 10%, P = 0.004) relative to baseline and compared with fellow controls (P = 0.02). Basal ONH BF declined continuously throughout subsequent stages in EG eyes reaching 25.0% ± 9.6% (P < 0.0001) below baseline at the final stage studied (RNFLT loss > 40%). In fellow control eyes, there was no significant change in basal ONH BF over time (P = 0.27).

CONCLUSIONS: In EG based on chronic mild-to-moderate IOP elevation, a two-phase pattern of ONH BF alteration was observed. ONH BF increased during the earliest stage (while RNFLT was within 10% of baseline) followed by a linear decline that was strongly correlated with loss of RNFLT.

PubMed 链接:

论文全文链接：（详见 A64-A70 页）
PURPOSE: To characterize the hemodynamic features and the association with structural damage in the optic nerve head (ONH) of idiopathic bilateral optic atrophy (BOA) in rhesus macaque monkeys.

METHODS: In five animals with BOA and nine healthy animals under general anesthesia (pentobarbital), intraocular pressure (IOP) was manometrically controlled. ONH blood flow was measured with a laser speckle flow graph device. Basal blood flow in global and quadrantal sectors was measured with IOP set at 10 mm Hg; autoregulation capacity was assessed by comparing blood flow changes before and after IOP was increased from 10 to 30 mm Hg. Spectral-domain optic coherence tomography was used to measure retinal nerve fiber layer thickness (RNFLT) by peripapillary circular scans.

RESULTS: Compared with control eyes, RNFLT in BOA eyes was significantly less in all sectors (P < 0.001) except the nasal (P = 0.25); the average global and sectoral blood flow in all quadrants was significantly lower (P < 0.001). These blood flow changes were significantly correlated with corresponding sectoral RNFLT (P < 0.01) except the nasal (P = 0.25). After IOP was increased to 30 mm Hg, global blood flow was significantly reduced (P < 0.001), but with no regional preferences despite prominent temporal RNFLT loss; no significant blood flow change was observed in control eyes (P = 0.24).

CONCLUSIONS: Basal blood flow and autoregulation capacity in the ONH of BOA were significantly compromised, with a close correlation to structural changes. The hemodynamic changes showed no regional preference across the ONH, which was consistent with postmortem histological observations.

PubMed 链接:
论文全文链接：（详见 A71-A78 页）
Anterior and posterior optic nerve head blood flow in nonhuman primate experimental glaucoma model measured by laser speckle imaging technique and microsphere method


PURPOSE: To characterize optic nerve head (ONH) blood flow (BF) changes in nonhuman primate experimental glaucoma (EG) using laser speckle flowgraphy (LSFG) and the microsphere method and to evaluate the correlation between the two methods.

METHODS: EG was induced in one eye each of 9 rhesus macaques by laser treatment to the trabecular meshwork. Prior to lasering and following onset of intraocular pressure (IOP) elevation, retinal nerve fiber layer thickness (RNFLT) and ONH BF were measured biweekly by spectral-domain optical coherence tomography and LSFG, respectively, until RNFLT loss was approximately 40% in the EG eye. Final BF was measured by LSFG and by the microsphere method in the anterior ONH (MS-BF(ANT)), posterior ONH (MS-BF(POST)), and peripapillary retina (MS-BF(PP)).

RESULTS: Baseline RNFLT and LSFG-BF showed no difference between the two eyes (P = 0.69 and P = 0.43, respectively, paired t-test). Mean (± SD) IOP was 30 ± 6 mm Hg in EG eyes and 13 ± 2 mm Hg in control eyes (P < 0.001). EG eye RNFLT and LSFG-BF were reduced by 42 ± 16% (P < 0.0001) and 22 ± 13% (P = 0.003), respectively, at the final time point. EG eye MS-BF(ANT), MS-BF(POST), and MS-BF(PP) were reduced by 41 ± 17% (P < 0.001), 22 ± 34% (P = 0.06), and 30 ± 12% (P = 0.001), respectively, compared with the control eyes. Interocular ONH LSFG-BF differences significantly correlated to that measured by the microsphere method (R² = 0.87, P < 0.001).

CONCLUSIONS: Chronic IOP elevation causes significant ONH BF decreases in the EG model. The high correlation between the BF reduction measured by LSFG and the microsphere method provides evidence that the LSFG is capable of assaying BF for a critical deep ONH region.

PubMed 链接:
论文全文链接：（详见 A79-A85 页）
Myeloid Cell-specific Disruption of Period1 and Period2 Exacerbates Diet-induced Inflammation and Insulin Resistance

The circadian clockworks gate macrophage inflammatory responses. Given the association between clock dysregulation and metabolic disorders, we conducted experiments to determine the extent to which over-nutrition modulates macrophage clock function and whether macrophage circadian dysregulation is a key factor linking over-nutrition to macrophage proinflammatory activation, adipose tissue inflammation, and systemic insulin resistance. Our results demonstrate that 1) macrophages from high fat diet-fed mice are marked by dysregulation of the molecular clockworks in conjunction with increased proinflammatory activation, 2) global disruption of the clock genes Period1 (Per1) and Per2 recapitulates this amplified macrophage proinflammatory activation, 3) adoptive transfer of Per1/2-disrupted bone marrow cells into wild-type mice potentiates high fat diet-induced adipose and liver tissue inflammation and systemic insulin resistance, and 4) Per1/2-disrupted macrophages similarly exacerbate inflammatory responses and decrease insulin sensitivity in co-cultured adipocytes in vitro. Furthermore, PPARγ levels are decreased in Per1/2-disrupted...
macrophages and PPARγ2 overexpression ameliorates Per1/2 disruption-associated macrophage proinflammatory activation, suggesting that this transcription factor may link the molecular clockworks to signaling pathways regulating macrophage polarization. Thus, macrophage circadian clock dysregulation is a key process in the physiological cascade by which diet-induced obesity triggers macrophage proinflammatory activation, adipose tissue inflammation, and insulin resistance. © 2014 by The American Society for Biochemistry and Molecular Biology, Inc.

PubMed 链接:

论文全文链接:
http://www.jbc.org/cgi/pmidlookup?view=long&pmid=24770415
ENDOTHELIAL PFKFB3 PLAYS A CRITICAL ROLE IN ANGIOGENESIS

OBJECTIVE: Vascular cells, particularly endothelial cells, adopt aerobic glycolysis to generate energy to support cellular functions. The effect of endothelial glycolysis on angiogenesis remains unclear. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3 (PFKFB3) is a critical enzyme for endothelial glycolysis. By blocking or deleting PFKFB3 in endothelial cells, we investigated the influence of endothelial glycolysis on angiogenesis both in vitro and in vivo.

APPROACH AND RESULTS: Under hypoxic conditions or after treatment with angiogenic factors, endothelial PFKFB3 was upregulated both in vitro and in vivo. The knockdown or overexpression of PFKFB3 suppressed or accelerated endothelial proliferation and migration in vitro, respectively. Neonatal mice from a model of oxygen-induced retinopathy showed suppressed neovascular growth in the retina when endothelial PFKFB3 was genetically deleted or when the mice were treated with a PFKFB3 inhibitor. In addition, tumors implanted in mice deficient in endothelial PFKFB3 grew more slowly and were provided with less blood flow. A lower level of phosphorylated protein kinase B was observed in PFKFB3-knockdown endothelial cells, which was accompanied by a decrease in
intracellular lactate. The addition of lactate to PFKFB3-knockdown cells rescued the suppression of endothelial proliferation and migration.

CONCLUSIONS: The blockade or deletion of endothelial PFKFB3 decreases angiogenesis both in vitro and in vivo. Thus, PFKFB3 is a promising target for the reduction of endothelial glycolysis and its related pathological angiogenesis.

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PubMed 链接:

论文全文链接:
http://atvb.ahajournals.org/content/34/6/1231.long
Metformin ameliorates hepatic steatosis and inflammation without altering adipose phenotype in diet-induced obesity

Non-alcoholic fatty liver disease (NAFLD) is closely associated with obesity and insulin resistance. To better understand the pathophysiology of obesity-associated NAFLD, the present study examined the involvement of liver and adipose tissues in metformin actions on reducing hepatic steatosis and inflammation during obesity. C57BL/6J mice were fed a high-fat diet (HFD) for 12 weeks to induce ...

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obesity-associated NAFLD and treated with metformin (150 mg/kg/d) orally for the last four weeks of HFD feeding. Compared with HFD-fed control mice, metformin-treated mice showed improvement in both glucose tolerance and insulin sensitivity. Also, metformin treatment caused a significant decrease in liver weight, but not adiposity. As indicated by histological changes, metformin treatment decreased hepatic steatosis, but not the size of adipocytes. In addition, metformin treatment caused an increase in the phosphorylation of liver AMP-activated protein kinase (AMPK), which was accompanied by an increase in the phosphorylation of liver acetyl-CoA carboxylase and decreases in the phosphorylation of liver c-Jun N-terminal kinase 1 (JNK1) and in the mRNA levels of lipogenic enzymes and proinflammatory cytokines. However, metformin treatment did not significantly alter adipose tissue AMPK phosphorylation and inflammatory responses. In cultured hepatocytes, metformin treatment increased AMPK phosphorylation and decreased fat deposition and inflammatory responses. Additionally, in bone marrow-derived macrophages, metformin treatment partially blunted the effects of lipopolysaccharide on inducing the phosphorylation of JNK1 and nuclear factor kappa B (NF-κB) p65 and on increasing the mRNA levels of proinflammatory cytokines. Taken together, these results suggest that metformin protects against obesity-associated NAFLD largely through direct effects on decreasing hepatocyte fat deposition and on inhibiting inflammatory responses in both hepatocytes and macrophages.

PubMed 链接:

论文全文链接：（详见 A86-A95 页）
http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0091111
Effects of selective COX-2 inhibitor and *Helicobacter pylori* eradication on precancerous gastric lesions

OBJECTIVE: *Helicobacter pylori* infection and overexpression of cyclo-oxygenase-2 (COX-2) are associated with gastric cancer and its precursors. To evaluate the effect of a selective COX-2 inhibitor alone and combined with *H pylori* eradication on the evolution of precancerous gastric lesions, a randomised, placebo-controlled trial was conducted in Linqu County, Shandong Province, China.

METHODS: A total of 1024 participants aged 35-64 years with *H pylori* infection and advanced gastric lesions were randomly assigned in a factorial design to two interventions or placebo: anti-*H pylori* treatment for 7 days, and a COX-2 inhibitor (celecoxib) for 24 months. The effects of the interventions were evaluated by the regression or progression of advanced gastric lesions.

RESULTS: Of the 1024 participants who received anti-*H pylori* treatment or placebo, 919 completed a subsequent 24-month treatment with celecoxib or placebo. The *H pylori* eradication rate by per-protocol analysis was 78.2%. Compared with placebo, the proportions of regression of gastric lesions significantly increased in the celecoxib treatment (52.8% vs 41.2%) and anti-*H pylori* treatment (59.3% vs 41.2%) group, and OR by per-protocol analysis was 1.72 (95% CI 1.07 to 2.76) for celecoxib and 2.19 (95% CI 1.32 to 3.64) for *H pylori* eradication. No statistically significant effect was found for *H pylori* eradication followed by celecoxib treatment on the regression of advanced gastric lesions (OR 1.48, 95% CI 0.91 to 2.40).

CONCLUSION: This population-based intervention trial revealed that celecoxib treatment or *H pylori* eradication alone had beneficial effects on the regression of advanced gastric lesions. No favourable effects were seen for *H pylori* eradication followed by celecoxib treatment.

Trial registration HARECCTR0500053 in accordance with WHO ICTRP requirements.

PubMed 链接:

论文全文链接:
http://gut.bmj.com/cgi/pmidlookup?view=long&pmid=21917649
ID: XHX-2

论文题目:
Noninvasive approach to assess coronary artery stenoses and ischemia (Letter)

作者及其单位和联系信息:
Dai N, Xia HH, Xu YW.

杂志及论文出版信息:

论文摘要:
No abstract available.

PubMed 链接:

论文全文链接:
ID: XHX-3

论文题目:
Liver diseases and autoimmunity (Editorial)

作者及其单位和联系信息:
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杂志及论文出版信息:

论文摘要:
No abstract available.

PubMed 链接:
No abstract available.

论文全文链接：（详见 A96 页）
Protective effects of losartan in mice with chronic viral myocarditis induced by coxsackievirus B3

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AIM: To investigate whether losartan has protective effects in mice with chronic viral myocarditis induced by coxsackievirus B3 (CVB3).

MAIN METHODS: Thirty two male Balb/c mice were intraperitoneally injected with CVB3 (10×TCID50) to induce chronic viral myocarditis (CVM). Losartan at 12.5mg/kg (n=16) or normal saline (n=16) were orally administered daily for 28 days to these mice. Uninfected mice (n=6) were used as controls. On day 29, all mice underwent anesthesia and echocardiography prior to sacrifice. Serum IL-17, IL-4, IFN-\(\gamma\) and TNF-\(\alpha\) levels were measured by enzyme-linked immunosorbent assay, and cardiac tissues were histologically examined after hematoxylin & eosin staining. In addition, the effect of losartan on the virus titers in primary cultured neonatal rat cardiomyocytes infected with CVB3 was measured on Hep-2 cells at 72 h post infection.

KEY FINDINGS: Mice infected with CBV3 had significantly increased mortality, heart/body weight ratios, necrosis and inflammatory scores and decreased cardiac ejection fractions, compared with the controls (all \(P<0.05\)). Losartan significantly decreased mortality from 40.0% to 12.5%, heart/body weight ratios from 7.08 ± 2.17 to 4.15 ± 0.99, and necrosis and inflammatory scores from 3.33 ± 0.50 to 2.50 ± 0.65 (all \(P<0.05\)), and increased ejection fractions from 55.80 ± 9.25 to 72.31 ± 12.15 (\(P<0.05\)). Losartan significantly enhanced IL-4, and decreased IFN-\(\gamma\), TNF-\(\alpha\) and IL-17 (all \(P<0.05\)). In the in vitro experiment, losartan had no influence on virus titers.

SIGNIFICANCE: Losartan protects mice against CVB3-induced CVM, most likely through upregulating Th2 responses, and down-regulating Th1 and Th17 responses.

PubMed 链接:

论文全文链接:
Antiviral therapy in patients with hepatitis B virus-related hepatocellular carcinoma: is it ready for universal application? (Letter)

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1Department of Hepatobiliary Oncology, State Key Laboratory of Oncology in Southern China, Sun Yat-sen University Cancer Center, Guangzhou, China.


No abstract available.

PubMed 链接:

论文全文链接:
Hepatic expression of interleukin 8 in diagnosing biliary atresia (Letter)

Dong R1, Chen G, Zheng S, Xia HH.
1Department of Pediatric Surgery, Children's Hospital of Fudan University, Key Laboratory of Neonatal Disease, Ministry of Health, Shanghai, China.


No abstract available.

PubMed link:

Full text link:
Perioperative complications in liver transplantation using donation after cardiac death grafts: A propensity-matched study

Donation after cardiac death (DCD) is an important source for expanding the donor pool for liver transplantation (LT). Although the long-term outcomes of LT using DCD grafts have been extensively studied, perioperative complications related to DCD grafts are rarely reported. The aim of this study was to determine whether DCD grafts were associated with a higher incidence of postreperfusion complications and worse outcomes in adult LT patients. After institutional review board approval, the medical records of all adult patients who underwent LT at our medical center between 2004 and 2011 were reviewed. Postreperfusion complications and posttransplant outcomes were compared between patients receiving DCD grafts and patients receiving donation after brain death (DBD) grafts. In all, 74 patients received DCD grafts during the study period, and 1369 patients received DBD grafts. An initial comparison showed that many preoperative, prereperfusion, and donor variables in the DCD group differed significantly from those in the DBD group. Propensity matching was chosen so that adjustments could be made for the differences. A postmatching analysis showed that the preoperative, prereperfusion, and donor variables no longer differed between the 2 groups. The postreperfusion requirements for blood products and vasopressors, the posttransplant ventilation times, the incidence of posttransplant acute renal injury, and the 30-day and 1-year patient and graft survival rates were comparable between the 2 groups. However, patients receiving DCD grafts experienced significantly higher rates of hyperkalemia (33.8% versus 18.9%, P < 0.05) and postreperfusion syndrome (PRS; 25.7% versus 12.3%, P < 0.05). In conclusion, after adjustments for preoperative and prereperfusion risks via propensity matching, DCD grafts remained a risk factor for postreperfusion hyperkalemia and PRS. A prophylactic regimen aimed at decreasing postreperfusion hyperkalemia and PRS is recommended for the management of LT using DCD grafts.

PubMed 链接:

论文全文链接:
An Independent Elderly Woman with Rapid Onset of Coma

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A 75-year-old woman was transferred from a local hospital because of rapid progression to coma preceded by lower back pain and recurrent falls. Cerebrospinal fluid analysis at the local hospital revealed increased protein with a slightly elevated white blood cell count. Our imaging studies revealed multiple punctate foci with nodular enhancement in the brain and multifocal cystic lesions on the chest and abdomen. The patient was empirically treated with antibiotics and corticosteroids without improvement. She died 3 days after transfer, and autopsy was performed. The differential diagnosis, pathologic findings, and final diagnosis are discussed.

PubMed 链接:

论文全文链接:
ID: ZLJ-1

论文题目:
Expression and diagnostic values of calretinin and CK5/6 in cholangiocarcinoma

作者及其单位和联系信息:
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杂志及论文出版信息:

论文摘要:
BACKGROUND: Mesothelin, a mesothelial marker, has been found expressed in and as a potential
treatment target of cholangioacarcinoma (CC). It is possible that CC may be derived from the cells
sharing mesothelial markers. However, the expression of other mesothelial markers in CC is largely
unknown.

METHODS: Thirty CC cases (10 extrahepatic and 20 intrahepatic) were retrieved from our
institutional archive. The immunohistochemical study of Calretinin (DC8), WT1 (6F-H2),
Lymphatic Endothelial Marker (D2-40), CK5/6 (D5/16 B4) and CK19 (b170) was done on formalin
fixed paraffin embedded sections for 2-3 blocks of each case. We compared the expression levels
between CC and normal bile duct (NBD) on the same block.

RESULTS: All of the CC and NBD are positive for CK19 (23/23) and negative for WT1 (0/23) and
D2-40 (0/23), except one CC positive for D2-40(1/30, 3.3%) and one NBD positive for WT1 (1/23,
4.3%). Calretinin immunoreactivity was detected in 52.2% (12/23) of CC, but none in NBD (0/23).
CK5/6 was also detectable in 73.3% (22/30) of CC and all NBD (30/30). Increased expression of
calretinin and reduced expression of CK5/6 were more likely associated with CC than NBD
(P < 0.001 and P = 0.002, respectively). The sequential staining pattern of positive calretinin and
negative CK5/6 in calretinin negative cases has a sensitivity of 69.57% and a specificity of 100% for
differentiating CC from NBD. CK5/6 expression was also more likely associated with well-
differentiated CC (7/7 versus 12/20 in moderately differentiated, and 9/10 in poorly differentiated,
P = 0.019) and extrahepatic CC (10/10 versus 12/20 in intrahepatic, P = 0.029), but there was no
association between the calretinin expression and the CC grade or location.
CONCLUSION: Calretinin and CK5/6 immunohistochemical stains may be useful for diagnosing a CC. Their immunohistochemical results should be interpreted with caution in the cases with differential diagnoses of mesothelioma and CC. A full mesothelioma panel, including WT1 and/or D2-40, is recommended to better define a mesothelial lineage. The biology of calretinin and CK5/6 expression in CC is unclear, but might shed light on identifying therapeutic targets for CC.

PubMed 链接：

论文全文链接：（详见 A97-A105 页）
http://www.ncbi.nlm.nih.gov/pmc/articles/pmid/24860692/
Characterization of the pathologic and endoscopic measurements of colorectal polyp sizes with a focus on sessile serrated adenoma and high-grade dysplasia

BACKGROUND: The characteristics of pathologically measured (PMS) and endoscopically measured sizes (EMS) of the colorectal polyps (CRPs) is poorly understood, particularly in polypoid unremarkable mucosa (PUM), sessile serrated adenoma (SSA), and high-grade dysplasia (HGD).

METHODS: To characterize the discordance and correlation between the PMS and EMS of CRPs including PUM, SSA, HGD, hyperplastic polyp (HP) and adenomas, we conducted this prospective observational study on the polyps collected between August 2012 and December 2013.

RESULTS: PMS was significantly smaller than EMS in the 497 qualified CRPs regardless of the sites (left, transverse and right colorectum) or EMS (≥1 cm and <1 cm) subgroups. The PMS and EMS discordance was associated with a diagnosis of HP and adenoma (versus PUM, SSA or HGD), single fragment (versus multiple), 3 of the 8 endoscopists and PMS<1 cm (versus ≥1 cm). Despite a good correlation between EMS and PMS in the adenomas (κ=0.626, 95% confidence intervals [CI], 0.505-0.746) and a moderate correlation in the serrated polyps (SPs) including HP and SSA, (κ=0.424, 95% CI, 0.244-0.604), 40.4% (23/57) of the adenomas and 63.6% (21/33) of the SPs with EMS≥1 cm might warrant longer follow-up intervals since their PMS were <1 cm. The PMS and EMS had linear correlations except in CRPs with HGD or EMS≥1 cm.

CONCLUSIONS: The discordance between PMS and EMS is associated with the pathologic diagnosis, fragment number, endoscopists and PMS, and may lead to different follow-ups in a considerable portion of adenomas and SPs.

PubMed 链接:

论文全文链接：（详见 A106-A114 页）
http://www.ncbi.nlm.nih.gov/pmc/articles/pmid/24817960/
OBJECTIVE: To characterize the clinicopathologic features of metastatic carcinomas at percutaneous endoscopic gastrostomy (PEG) tube sites.

METHODS: We reviewed the metastatic malignancies at PEG tube sites (2002-2011).

RESULTS: Five patients were identified, each with primary head and neck keratinizing squamous cell carcinoma. The metastases had a mean size of 6.08 cm (95% confidence interval [CI], 3.75-8.41). The time from PEG tube placement to metastasis diagnosis was 9.8 months (95% CI, 6.59-13.01). The survival times from PEG tube placement and from metastasis diagnosis were 23.5 (95% CI, 7.65-39.35) and 13.7 (95% CI, 0-31.08) months, respectively. Compared with a meta-analysis of the largest case series, our male patients were significantly older (mean, 73 years; 95% CI, 62.2-83.9 vs mean 59 years, 95% CI, 56.0-62.0) but had similar survival times.

CONCLUSIONS: Despite their older ages, our male patients had similar survival times to those reported previously. Larger series are needed to confirm our findings and explore the causes.

PubMed 链接:

论文全文链接:
http://ajcp.ascpjournals.org/cgi/pmidlookup?view=long&pmid=24619751
论文题目:
Histone deacetylase 2 (HDAC2) protein-dependent deacetylation of mortality factor 4-like 1 (MORF4L1) protein enhances its homodimerization

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Chen Y¹, Li J, Dunn S, Xiong S, Chen W, Zhao Y, Chen BB, Mallampalli RK, Zou C.
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杂志及论文出版信息:

论文摘要:
Histone acetyltransferase mortality factor 4-like 1 (MORF4L1) is a relatively new histone acetyltransferase component that exists as a homodimer to exert its epigenetic function. The mechanism of MORF4L1 self-assembly is unknown. Here we report that Lys-148 deacetylation is indispensable for facilitating MORF4L1 self-assembly into a homodimeric unit. Among a stretch of ∼10 amino acids in the NH2 terminus between the chromodomain and MORF4-related gene (MRG) domain within MORF4L1, Lys-148 is normally acetylated. Substitution of Lys-148 with arginine augments MORF4L1 self-assembly. However, acetylation mimics of MORF4L1, including K148L and K148Q, abolished its self-assembly of the histone acetyltransferase component. HDAC2, a deacetylase, interacts with and keeps MORF4L1 in a deacetylation status at Lys(148) that triggers MORF4L1 self-assembly. Knockdown of HDAC2 reduces MORF4L1 self-assembly. HDAC2-dependent deacetylation of MORF4L1 enhances MORF4L1 homodimerization, thus facilitating the functionality of complex formation to repress cell proliferation.

PubMed 链接:

论文全文链接:
http://www.jbc.org/cgi/pmidlookup?view=long&pmid=24451372
Regulation of histone modifying enzymes by the ubiquitin-proteasome system

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Histone post-translational modification is a key step that may result in an epigenetic mark that regulates chromatin structure and gene transcriptional activity thereby impacting many fundamental aspects of human biology. Subtypes of post-translational modification such as acetylation and methylation are executed by a variety of distinct modification enzymes. The cytoplasmic and nuclear concentrations of these enzymes are dynamically and tightly controlled at the protein level to precisely fine-tune transcriptional activity in response to environmental clues and during pathophysiological states. Recent data have emerged demonstrating that the life span of these critical nuclear enzymes involved in histone modification that impact chromatin structure and gene expression are controlled at the level of protein turnover by ubiquitin-proteasomal processing. This review focuses on the recent progress on mechanisms for ubiquitin-proteasomal degradation of histone modification enzymes and the potential pathophysiological significance of this process.

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PubMed 链接:

论文全文链接:
Genetic Variation in 15-Hydroxyprostaglandin Dehydrogenase and Colon Cancer Susceptibility

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Abstract

Background: 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is a metabolic antagonist of COX-2, catalyzing the degradation of inflammation mediator prostaglandin E2 (PGE2) and other prostanoids. Recent studies have established the 15-PGDH gene as a colon cancer suppressor.

Methods: We evaluated 15-PGDH as a colon cancer susceptibility locus in a three-stage design. We first genotyped 102 single-nucleotide polymorphisms (SNPs) in the 15-PGDH gene, spanning ~50 kb up and down-stream of the coding region, in 464 colon cancer cases and 393 population controls. We then genotyped the same SNPs, and also assayed the expression levels of 15-PGDH in colon tissues from 69 independent patients for whom colon tissue and paired germline DNA samples were available. In the final stage 3, we genotyped the 9 most promising SNPs from stages 1 and 2 in an independent sample of 525 cases and 816 controls (stage 3).

Results: In the first two stages, three SNPs (rs1365611, rs6844282 and rs2332897) were statistically significant (p<0.05) in combined analysis of association with risk of colon cancer and of association with 15-PGDH expression, after adjustment for multiple testing. For one additional SNP, rs2555639, the T allele showed increased cancer risk and decreased 15-PGDH expression, but just missed statistical significance (p-adjusted = 0.063). In stage 3, rs2555639 alone showed evidence of association with an odds ratio (TT compared to CC) of 1.50 (95% CI = 1.05–2.15, p = 0.026).

Conclusions: Our data suggest that the rs2555639 T allele is associated with increased risk of colon cancer, and that carriers of this risk allele exhibit decreased expression of 15-PGDH in the colon.

Introduction

Colon cancer is the end result of a multistep process of genetic and epigenetic changes resulting in the activation of oncogenic pathways as well as inactivation of tumor suppressor pathways [1]. A key event during the progression of colon cancer is the up-regulation of the cyclooxygenase-2 (COX-2) oncogene [2,3,4,5]. COX-2 catalyzes the conversion of arachidonic acid to PGH2, which is an intermediate substrate for a variety of bioactive prostaglandins, including PGE2 [6,7], the predominant prostaglandin found in colon cancer tissues [8]. Several lines of evidence suggest that the increased production of PGE2 mediates the oncogenic effect of COX-2 [7,9,10,11].

15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the rate-limiting enzyme in the degradation of prostaglandins, including PGE2, and directly antagonizes the COX-2 oncogenic pathway of prostaglandin production [12]. 15-PGDH is highly expressed in normal colon mucosa, is regulated through the TGF-β tumor suppressor pathway, and undergoes loss of expression in colon cancer [13,14]. We have previously demonstrated the tumor suppressor function of 15-PGDH, finding that re-expression of 15-PGDH in a colon cancer cell line blocks tumor growth following injection into athymic mice, and that knocking out murine 15-PGDH results in an increased development of colon tumors [13,15]. Moreover, in human studies we found a substantial 12-fold difference in levels of rectal 15-PGDH among individuals with lowest to highest 15-PGDH transcript levels, and that low levels of...
rectal 15-PGDH were associated with increased colorectal adenoma (a precursor to colon cancer) recurrences [16]. These findings prompted us to examine whether inherited genetic variation at the 15-PGDH locus would explain the wide population variation in levels of colon 15-PGDH, and would also be associated with risk of developing colon cancer. We evaluated the association with colon cancer risk of SNP markers spanning ~50 kb upstream to ~40 kb downstream of the 15-PGDH gene coding region in a population-based case-control study in two stages. We then tested these same SNPs for their associations with expression levels of 15-PGDH in colon tissues in a separate patient population.

**Materials and Methods**

**Study Design**

We employed a 3 stage study design for this project. In the first stage, we investigated all known SNPs in the 15-PGDH gene, spanning 50 kb upstream to 40 kb downstream, for association with risk of colon cancer in a population-based case-control study. In the second stage, we evaluated the association of these same SNPs with 15-PGDH expression in colonic epithelial tissues from an independent sample of patients. We then used a meta-analysis approach to combine the results from stages 1 and 2 in order to identify the most promising SNPs to move forward for stage 3. In stage 3, we genotyped these top SNPs in a second sample of colon cancer patients and population controls for validation.

**Patient Populations**

For the association analysis of 15-PGDH SNPs with risk of colon cancer, incident cases were identified from the state of Kentucky Surveillance, Epidemiology and End Results (SEER) registry. Controls were recruited through random digit dialing and friend referrals. The recruitment of this study population was described in more detail earlier [17]. Overall this study population is approximately 94% Caucasian [17]. In order to minimize the effect of population stratification and to increase homogeneity, we limited our analyses to only individuals self-reporting as Caucasians. For the discovery set (stage 1), subjects were recruited from February 2003 through December 2005, and included 464 cases and 393 controls self-reporting as Caucasian. For the replication set (stage 3) included 325 Caucasian cases and 816 Caucasian controls recruited from January 2006 to June 2010. All participants provided written informed consent, completed an extensive risk factor questionnaire and donated a sample of blood. Whole blood was shipped to the research laboratory at Case Western Reserve University overnight and processed immediately. DNA was isolated from buffy coats separated from whole blood collected in standard EDTA tubes.

To study the effect of SNPs on tissue gene expression (stage 2), normal colon tissue sections were collected from 69 Caucasian patients recruited at University Hospitals Case Medical Center (UHCMC). All participants provided informed consent. RNA and DNA from the tissue samples were prepared by extraction with guanidine isothiocyanate as previously described [18]. Total cellular RNA and genomic DNA were separated by ultracentrifugation of the extract through a cesium cushion. Both studies were approved by the UHCMC institutional review board.

**Genotyping**

We included all known SNPs from 50 kb upstream to 40 kb downstream of 15-PGDH that at the time of initiating our study were listed as Illumina Golden Gate validated, and for which ABI TaqMan assays were available. ABI TaqMan chemistry was employed to genotype these samples according to the manufacturer’s protocol. Specifically, 2 μl aliquots, containing 5–10 ng of DNA were transferred from 96-well reservoir plates to 384-well assay plates for each individual being genotyped. Multiple 384-well plates were generated; the DNA was dried down, the plates then sealed and frozen until assayed. A 5 μl aliquot of Master Mix, Probe & Primer was robotically added to each well of a 384-well plate previously plated with DNA. PCR [40 Cycles] was carried out on an ABI GeneAmp PCR System 9700 Dual Head Instrument and endpoint reads were carried out using the ABI 7900 Sequence Detection System (SDS). Since TaqMan Chemistry is a PCR-based procedure, all assay mixes were prepared in an ampiclon-free room to avoid contamination. To ensure data quality, each SDS file was individually reviewed before the data were exported to ensure the baseline is properly set. In the 384-well assay layout, the last column of the plate was reserved for water blanks to ensure no contamination occurred during plating. DNA samples, either from Coriell or from our own database, with known genotypes for the SNPs being interrogated in this study were included on each assay plate to serve as positive controls and to identify the 3 genotypes. Four replicate samples were included in the discovery sample (phase 1) and colon tissue expression sample (phase 2), which were genotyped at the same time, and 29 replicate samples were included in the validation sample (phase 3) to confirm accurate genotyping in the study. Genotype concordance of the replicates and control samples was confirmed. If 100% concordance was not observed, the primary data files were reviewed and typically the assay was repeated. The overall call rate was 94.6% (details in Table S1).

**Table 1. Demographics of Case-Control Sample Populations (Phases 1 and 3).**

<table>
<thead>
<tr>
<th></th>
<th>Phase 1</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cases (N = 464)</strong></td>
<td>Controls (N = 393)</td>
<td>p*</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>203 (51.7)</td>
<td>172 (37.1)</td>
</tr>
<tr>
<td>Female</td>
<td>190 (48.4)</td>
<td>292 (62.9)</td>
</tr>
<tr>
<td><strong>Age, mean (SD)</strong></td>
<td>64.6 (10.7)</td>
<td>58.1 (10.9)</td>
</tr>
<tr>
<td><strong>Age, range</strong></td>
<td>22–89</td>
<td>33–87</td>
</tr>
</tbody>
</table>

*p-value of differences between cases and controls within that phase.

doi:10.1371/journal.pone.0064122.t001
RNA using AMV Reverse Transcriptase (Roche, Indianapolis, IN) following the manufacturers recommended protocol. Real-time PCR measurement of 15-PGDH was performed using the human hydrolysis Probe/Primer set Hs00168359_m1 (HPGD, NM_000860) from Applied Biosystems (Foster City, CA). A 25 μl reaction mix contained 1 μl (40 ng) of cDNA template and a 1:20 dilution of an individual primer/probe set in 1X Supermix (Bio-Rad, CA) and was run on a CFX96 optical module (BioRad, Hercules, CA). Thermal cycling conditions for all assays was 95°C for 4 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Cytokeratin 20 (KRT20), a marker of colonic epithelial cell mass, was used as the reference gene for normalization and was amplified using the human KRT20 (NM_019010) hydrolysis primer/probe kit Hs00300643_m1 from Applied Biosystems following the reaction conditions above. KRT20 was selected because it is a specific marker for colonic epithelial mass [7,15,16], as well as having uniform expression by microarray analysis across 16 normal colon tissue biopsies and colonic crypt epithelial cells isolated from an additional 5 normal biopsy samples (Markowitz, unpublished data). For each reverse transcription reaction, 15-PGDH and KRT20 quantification cycle (Cq 15-PGDH and CqKRT20) values were determined as the average values obtained from three independent real-time PCR reactions. The overall level of 15-PGDH RNA expression was determined as the ratio of 15-PGDH/KRT20 = 2^(-ΔCq 15-PGDH−ΔCqKRT20). RNA that had not undergone the reverse transcrptase step as well as a water sample that was carried through the reverse transcrptase step were used as negative controls and were negative for all assays performed.

Statistical Analyses

For quality control, each SNP was tested for deviation from Hardy-Weinberg equilibrium (HWE) in the control population via a chi-square test of difference from expectation. SNPs that showed evidence of deviation from HWE (p<0.05) were excluded from further analyses.

Odds ratios (OR) and 95% confidence intervals (CI) for colon cancer were assessed via a logistic regression controlling for age and gender. In the logistic regressions, the allele more common in cases (compared to controls) was considered the risk allele. For each SNP, individuals were coded as 0, 1 or 2, representing the three possible genotypes for each SNP was evaluated using a one-way ANOVA with two degrees of freedom. Given that we were testing our a priori hypothesis that the risk allele is associated with decreased 15-PGDH expression, we reported one-sided p-values. The risk allele demonstrated higher expression, a p-value of 1 was assigned.

In order to determine which SNPs showed the most evidence of both association with 15-PGDH expression and risk of colon cancer, we used Fisher’s method to combine p-values [20] from the discovery SNP association and expression analyses. Fisher method allows for combining the p-values, especially in multi-stage analysis, to draw similar inference using different statistics calculated from the same samples. Each of the statistics combined tests a different aspect of the biological hypothesis under investigation. Power can be improved by combining the p-values

**Table 2. Association with Colon Cancer of SNPs Selected for Replication in the Discovery Sample (Phase 1).**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Controls</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n135611</td>
<td>&lt;0.0001</td>
<td></td>
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</tr>
<tr>
<td>CC 176 (48.0) 165 (38.9) 4.97 (2.73–9.05)</td>
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<tr>
<td>CT 174 (47.4) 188 (44.3) 4.59 (2.52–8.33)</td>
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<tr>
<td>TT 17 (4.6) 21 (6.8) 1.0 (ref)</td>
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<tr>
<td>n2253442</td>
<td>0.55</td>
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<tr>
<td>GG 204 (56.4) 231 (52.6) 1.29 (0.71–2.34)</td>
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<tr>
<td>AG 135 (37.3) 175 (39.9) 1.13 (0.61–2.07)</td>
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<tr>
<td>AA 23 (6.4) 33 (7.5) 1.0 (ref)</td>
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<tr>
<td>n2555639</td>
<td>0.038</td>
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<tr>
<td>TT 180 (46.5) 172 (37.5) 1.71 (1.09–2.69)</td>
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<tr>
<td>CT 163 (42.1) 213 (46.4) 1.28 (0.82–2.01)</td>
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<tr>
<td>CC 44 (11.4) 74 (16.1) 1.0 (ref)</td>
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<tr>
<td>n2555642</td>
<td>0.53</td>
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<tr>
<td>TT 219 (57.1) 245 (54.1) 1.38 (0.75–2.53)</td>
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<tr>
<td>CT 144 (37.5) 176 (38.9) 1.24 (0.66–2.31)</td>
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<tr>
<td>CC 21 (5.5) 32 (7.1) 1.0 (ref)</td>
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<tr>
<td>n2555622</td>
<td>0.18</td>
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<tr>
<td>AA 158 (42.9) 216 (48.8) 1.0 (ref)</td>
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<tr>
<td>AC 171 (46.5) 186 (42.0) 0.99 (0.59–1.64)</td>
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<tr>
<td>CC 39 (10.6) 84 (19.0) 1.31 (0.79–2.17)</td>
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<tr>
<td>n6444282</td>
<td>&lt;0.0001</td>
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<tr>
<td>CC 132 (34.4) 122 (26.9) 2.38 (1.55–3.67)</td>
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<tr>
<td>CG 198 (51.6) 211 (46.6) 2.31 (1.55–3.45)</td>
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<tr>
<td>GG 54 (14.1) 120 (26.5) 1.0 (ref)</td>
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<tr>
<td>n11724251</td>
<td>0.053</td>
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<tr>
<td>AA 114 (30.0) 171 (37.5) 1.0 (ref)</td>
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<tr>
<td>AG 197 (51.8) 208 (45.6) 1.01 (0.68–1.51)</td>
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<tr>
<td>GG 69 (18.2) 77 (16.9) 1.47 (0.96–2.26)</td>
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<tr>
<td>n10019035</td>
<td>0.065</td>
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<tr>
<td>CC 317 (83.0) 374 (82.2) 1.0 (ref)</td>
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<tr>
<td>CT 64 (16.8) 71 (15.6) 11.9 (1.46–97.5)</td>
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<tr>
<td>TT 1 (0.3) 10 (2.2) 11.9 (1.49–94.6)</td>
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<tr>
<td>n2332897</td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td>CC 181 (47.0) 175 (38.8) 4.59 (2.60–8.11)</td>
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<td></td>
</tr>
<tr>
<td>CA 185 (48.1) 198 (43.9) 4.46 (2.53–7.87)</td>
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<tr>
<td>AA 19 (4.9) 78 (17.3) 1.0 (ref)</td>
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</table>

**Odds ratio (OR) for colon cancer risk and 95% confidence interval (CI) for having one or two risk alleles, compared to having no risk alleles, and the additive model p-value from logistic regression adjusting for age and gender, but not adjusted for multiple testing.**

**Quantitative Real-Time PCR Measurement of 15-PGDH**

Integrity of isolated total RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and concentrations were determined using a ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). All reverse transcription quantitative real-time PCR assays were performed following the MIQE guidelines [19]. cDNA was synthesized from 1 μg of input
of the different tests. To address multiple testing, we then utilized the false discovery rate (FDR) method of Benjamini and Hochberg [21] to the combined p-values.

The top 9 SNPs identified were then evaluated for association with colon cancer risk in the replication set using the same statistical methods as the discovery set. We combined the results from the first and second case-control samples using a random effect model. All statistics except for the meta-analysis were computed using SAS 9.2 and p-values ≤ 0.05 were considered statistically significant.

Results

SNP-Colon Cancer Association Discovery

Cases in Phase 1 were more likely to be male and were, on average, older than the controls (Table 1). Of the 102 SNPs evaluated in the discovery population, 25 were either monomorphic or had a MAF <5% in our population. Of the remaining, 28 SNPs were excluded from further analyses. Among the remaining 75 SNPs, 8 were significantly associated with colon cancer risk in the logistic regression model at the p < 0.05 (unadjusted for multiple testing) level (Table S1): rs1365611, p = 0.0001; rs6844282, p = 0.0001; rs2332897, p = 0.0001; rs10520282, p = 0.0035; rs1426936, p = 0.012; rs34299544, p = 0.024; rs2555639, p = 0.038, and rs5007089, p = 0.046. All results are given in Table S1, and five of these that met criteria for inclusion in the replication set (based on results in both risk association and the colon tissue gene expression experiment, see also below) are detailed in Table 2.

Association with 15-PGDH Expression

The same complete set of 102 SNPs was evaluated for association with tissue expression levels of 15-PGDH in an independent set of 69 patients (complete results in Table S2). Of these patients, 38 (53%) were male and 31 (45%) were female. The

| Table 3. Association to 15-PGDH Colon Expression of Selected SNPs (Phase 2). |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Genotype (N)                  | Mean (SD) PGDH Expression | p*              | p (combined)**  | p (FDR adjusted)† |
| rs1365611 CC (26)             | 76.3 (32.1)      | 1               | 0.0019          | 0.038            |
|                              | CT (26)          | 72.2 (26.1)     |                 |                  |
|                              | TT (5)           | 71.7 (33.2)     |                 |                  |
| rs2253442 GG (30)            | 67.5 (28.5)      | 0.017           | 0.053           | 0.10             |
|                              | AG (30)          | 86.9 (35.0)     |                 |                  |
|                              | AA (2)           | 84.1 (35.0)     |                 |                  |
| rs2555639 TT (28)            | 67.3 (29.4)      | 0.012           | 0.0040          | 0.063            |
|                              | CT (33)          | 80.0 (30.6)     |                 |                  |
|                              | CC (5)           | 99.0 (47.1)     |                 |                  |
| rs2555642 TT (34)            | 67.6 (28.7)      | 0.014           | 0.044           | 0.10             |
|                              | CT (30)          | 86.9 (35.0)     |                 |                  |
|                              | CC (3)           | 84.1 (35.0)     |                 |                  |
| rs2555622 AA (24)            | 84.7 (34.9)      | 0.050           | 0.051           | 0.10             |
|                              | AC (34)          | 74.9 (33.4)     |                 |                  |
|                              | CC (10)          | 65.8 (20.0)     |                 |                  |
| rs6844282 CC (19)            | 79.7 (37.1)      | 1               | 0.0019          | 0.038            |
|                              | GG (35)          | 76.2 (30.0)     |                 |                  |
|                              | GG (14)          | 75.6 (34.8)     |                 |                  |
| rs11724251 AA (19)           | 92.2 (34.8)      | 0.045           | 0.017           | 0.04             |
|                              | AG (30)          | 73.8 (36.1)     |                 |                  |
|                              | GG (15)          | 64.2 (15.3)     |                 |                  |
| rs10019035 CC (19)           | 63.2 (17.7)      | 0.028           | 0.013           | 0.11             |
|                              | CT (6)           | 80.4 (20.0)     |                 |                  |
|                              | TT (0)           |                 |                 |                  |
| rs2332897 CC (31)            | 77.7 (34.9)      | 0.040           | 0.0008          | 0.032            |
|                              | AC (31)          | 74.1 (26.6)     |                 |                  |
|                              | AA (6)           | 88.1 (49.9)     |                 |                  |

*p-value of number of minor alleles (0, 1 or 2; additive model) in linear regression for 15-PGDH expression level in colon mucosa (one-sided).

**Combined p-value of association to 15-PGDH expression and with risk of colon cancer (from logistic regression presented in Table 1) using Fisher’s method for combining p-values, unadjusted for multiple testing.

†p-value adjusted for multiple testing using FDR method.

PLOS ONE | www.plosone.org 4 May 2013 | Volume 8 | Issue 5 | e64122
with expression levels at p {remaining 84 SNPs, four were statistically significantly correlated monomorphic. These were excluded from further analyses. Of the 102 genotyped SNPs, two failed QC and 14 were average age was 70.1 (SD = 13.8), and the age range was 18–94. Of the 102 genotyped SNPs, two failed QC and 14 were monomorphic. These were excluded from further analyses. Of the remaining 84 SNPs, four were statistically significantly correlated with expression levels at p < 0.05 (Table S2). Detailed expression results are provided for the same 9 SNPs selected for validation (see also below) in Table 3.

### Table 4. SNP Association Validation Population.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (N = 525)</th>
<th>Controls (N = 816)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1365611</td>
<td>244 (47.7)</td>
<td>359 (44.6)</td>
<td>1.14 (0.78–1.66)</td>
<td>0.30</td>
</tr>
<tr>
<td>CT</td>
<td>215 (42.0)</td>
<td>358 (44.5)</td>
<td>0.99 (0.67–1.45)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>53 (10.3)</td>
<td>88 (10.9)</td>
<td>1.0 (ref)</td>
<td></td>
</tr>
<tr>
<td>rs2555639</td>
<td>218 (41.8)</td>
<td>303 (37.4)</td>
<td>1.50 (1.05–2.15)</td>
<td>0.026</td>
</tr>
<tr>
<td>CT</td>
<td>244 (46.8)</td>
<td>385 (47.5)</td>
<td>1.29 (0.91–1.94)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>59 (11.3)</td>
<td>122 (15.1)</td>
<td>1.0 (ref)</td>
<td></td>
</tr>
<tr>
<td>rs2555642</td>
<td>285 (54.5)</td>
<td>421 (51.7)</td>
<td>1.42 (0.89–2.28)</td>
<td>0.17</td>
</tr>
<tr>
<td>CT</td>
<td>209 (40.0)</td>
<td>233 (40.9)</td>
<td>1.33 (0.82–2.15)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>29 (5.5)</td>
<td>60 (7.4)</td>
<td>1.0 (ref)</td>
<td></td>
</tr>
<tr>
<td>rs6844282</td>
<td>213 (41.0)</td>
<td>368 (45.3)</td>
<td>1.0 (ref)</td>
<td>0.15</td>
</tr>
<tr>
<td>AA</td>
<td>245 (47.1)</td>
<td>358 (44.0)</td>
<td>1.18 (0.93–1.50)</td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>62 (11.9)</td>
<td>87 (10.7)</td>
<td>1.23 (0.85–1.78)</td>
<td></td>
</tr>
<tr>
<td>rs11724251</td>
<td>178 (33.9)</td>
<td>239 (29.4)</td>
<td>1.07 (0.78–1.47)</td>
<td>0.44</td>
</tr>
<tr>
<td>AA</td>
<td>161 (30.8)</td>
<td>290 (35.9)</td>
<td>1.0 (ref)</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>276 (52.8)</td>
<td>377 (46.6)</td>
<td>1.31 (1.02–1.68)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>107 (20.4)</td>
<td>155 (19.1)</td>
<td>1.10 (0.79–1.53)</td>
<td></td>
</tr>
<tr>
<td>rs10019035</td>
<td>424 (81.2)</td>
<td>644 (80.5)</td>
<td>1.0 (ref)</td>
<td>0.85</td>
</tr>
<tr>
<td>CC</td>
<td>62 (11.9)</td>
<td>87 (10.7)</td>
<td>1.23 (0.85–1.78)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>92 (17.6)</td>
<td>148 (18.5)</td>
<td>0.94 (0.71–1.26)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>6 (1.2)</td>
<td>8 (1.0)</td>
<td>1.22 (0.41–3.58)</td>
<td></td>
</tr>
<tr>
<td>rs2332897</td>
<td>250 (47.7)</td>
<td>360 (44.4)</td>
<td>1.13 (0.78–1.65)</td>
<td>0.27</td>
</tr>
<tr>
<td>CA</td>
<td>219 (41.8)</td>
<td>362 (44.0)</td>
<td>0.97 (0.66–1.42)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>55 (10.5)</td>
<td>89 (11.0)</td>
<td>1.0 (ref)</td>
<td></td>
</tr>
</tbody>
</table>

*Odds ratio (OR) for colon cancer risk, 95% confidence interval (CI) and p-value for trend from logistic regression, with SNP in additive model adjusting for age and gender, but not adjusted for multiple testing.

doi:10.1371/journal.pone.0064122.t004

### Discussion

Here we present evidence of the association between the T allele of the 15-PGDH rs2555639 SNP and risk of colon cancer in a staged study design. This allele was also associated with decreased 15-PGDH expression in colon tissue in an independent patient population. This SNP maps 17.74 Kb upstream of the 5' UTR of the 15-PGDH gene, in the presumed regulatory region of the gene (Fig. 1). Our study thus highlights the importance of considering genetic variation in promoter regions when assessing the association of inherited variation with predisposition to disease.

While rs2555639 was the only SNP that was significantly associated with risk of colon cancer in each of the discovery and validation sets independently, several additional SNPs had highly significant association with risk when combining data from the discovery and validation samples (Table 5), including rs1365611 (OR = 1.73, 95% CI: 1.26–2.37, p = 0.0008), rs6844282 (OR = 1.42, 95% CI: 1.10–1.83, p = 0.0078), and rs2332897 (OR = 1.74, 95% CI: 1.27–2.38, p = 0.0006). The discovery and replication sets however show evidence of heterogeneity (Table 5), and these 3 SNPs were not significant in the validation sample. Further study with a larger sample size will be required before any final conclusions can be reached regarding the association of these three additional SNPs with risk of colon cancer.

SNP rs2555639 falls into an extremely small LD block, with poor correlations with neighboring SNPs. This may explain why no other SNPs in the tagging panel we tested were significantly associated with disease risk (Fig. 2). For this same reason, rs2555639 would be unlikely to have been detected in previous genome-wide association studies (GWAS) that relied on selections of panels of tagging SNPs, and did not find a statistically significant association in the 15-PGDH region [22,23,24].

Upon combining the p-values from the expression and association results using Fisher’s method, the 9 most statistically significant SNPs (Table 2), were selected for validation by testing for association with colon cancer risk in an independent replication set of cases and controls. Of these top 9 most significant SNPs, 3 (rs1365611, rs6844282 and rs2332897) remained significant after adjustment for multiple testing (Table 3) (rs1365611 p = 0.038, rs6844282 p = 0.038 and rs2332897 p = 0.032), and one more had a multiple testing adjusted p-value of just over 0.05 (rs2555639, p = 0.065).

### Validation Association

In the Phase 3 validation sample, cases were more likely to be male and were older, on average, than the controls, as in Phase 1 (Table 1). The top 9 SNPs, based on the combined p-values showing evidence for association with colon cancer risk and/or 15-PGDH expression in the colon, were selected for validation in the independent set of 325 colon cancer patients and 816 controls. Of these SNPs, rs2555639 demonstrated statistically significant evidence for association with colon cancer risk at the p<0.05 level (via a logistic regression analysis) (Table 4).

Combining the data from the case-control discovery and validation populations, our data suggest that having two copies of the T allele of rs2555639 confers an estimated 58% (95% CI: 19%–109%, p = 0.0015) increase in odds of colon cancer compared to individuals with two copies of the C allele (Table 5). Furthermore, the rs2555639 T allele is also associated with decreased expression levels of the 15-PGDH tumor suppressor gene (Table 3, p = 0.012), which is consistent with our expression observations in colon tissue.
Similarly, previous candidate gene studies of the association of the 15-PGDH SNPs with colon cancer risk also failed to detect rs2555639. These earlier studies identified two SNPs in PGDH – rs2612656 and rs8752 – as individually showing significant association with colon cancer risk [25]. However, neither was replicated in a validation study [26]. Both these two previous studies limited the region examined to either the body of the 15-PGDH gene or to only 5 kb of flanking genomic sequence [25,26]. Thus, neither of these studies would have detected the association of rs2555639 with colon cancer risk. Another earlier study evaluated the association of only two non-synonymous coding SNPs in 15-PGDH with colon adenoma risk [27]. We did not evaluate the association of these SNPs with risk of colon cancer in our study because of their low minor allele frequencies (3% and 1%, respectively).

One limitation of our study is that we only evaluated the association of 15-PGDH locus SNPs with colon cancer risk among individuals self-reporting as Caucasian, who are predominantly of European ancestry. Thus we are unable to evaluate whether the association of rs2555639 with both colon cancer risk and 15-PGDH expression holds in other racial groups. In addition, we excluded SNPs with a minor allele frequency less than 5%. This, in combination with our relatively small discovery sample size, may have limited our ability to detect any association to either rare 15-PGDH variants or to more common variants with very low effects.

Another potential limitation is that both our stage 1 and stage 3 samples were drawn from the State of Kentucky population. Validation of our results in other independent, non-Kentuckian populations is thus warranted.

The important role of COX-2 and the arachidonic acid pathway in the development of colon cancer is well established, as is the role of 15-PGDH as a metabolic suppressor of the COX-2 pathway and a colon cancer suppressor gene [4,5]. In this study we have demonstrated evidence for inherited variations in the 15-PGDH gene in potentially regulating 15-PGDH expression levels in the colon as well as conferring susceptibility to colon cancer. We have identified a single SNP, rs2555639, 17.74 kb upstream of the 5' UTR of the 15-PGDH gene, which is associated both with lower colonic 15-PGDH expression and with increased risk of colon cancer. This study illustrates the advantage of combining tests of SNP association with tissue 15-PGDH expression and with disease risk, as this combined approach has allowed us to identify the 15-PGDH rs2555639 T allele as a potentially functional and novel colon cancer susceptibility variant in a 3-stage study despite the modest sample sizes of both the discovery and replication case-control sets. We shall point out that we used $\alpha = 0.05$ as the cut-off to declare replication significance in the validation phase without further adjustment for multiple testing. Although the validation SNPs were selected based on the combined evidence from stages 1 and 2 for their association with both risk of colon cancer and 15-PGDH tissue expression, caution must be taken in interpreting our replication results. Nevertheless, our results should stimulate further studies to validate the rs25556399 variant as predisposing to colon cancer in other independent populations, as well as to investigate other SNP variants in the 15-PGDH locus in the development of colon cancer.

### Table 5. Association of 15-PGDH SNPs with Colon Cancer in Discovery and Validation Populations.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Discovery OR (95% CI)</th>
<th>Validation OR (95% CI)</th>
<th>Combined OR (95% CI)</th>
<th>Combined p</th>
<th>Heterogeneity p</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1365611</td>
<td>4.97 (2.73–9.05)</td>
<td>1.14 (0.78–1.66)</td>
<td>1.73 (1.26–2.37)</td>
<td>0.0008</td>
<td>0.000045</td>
</tr>
<tr>
<td>rs2253442</td>
<td>1.29 (0.71–2.34)</td>
<td>1.44 (0.91–2.27)</td>
<td>1.38 (0.96–1.99)</td>
<td>0.079</td>
<td>0.77</td>
</tr>
<tr>
<td>rs2555639</td>
<td>1.71 (1.09–2.69)</td>
<td>1.50 (1.05–2.15)</td>
<td>1.58 (1.19–2.09)</td>
<td>0.0015</td>
<td>0.66</td>
</tr>
<tr>
<td>rs2555642</td>
<td>1.38 (0.75–2.53)</td>
<td>1.42 (0.89–2.28)</td>
<td>1.40 (0.97–2.04)</td>
<td>0.074</td>
<td>0.94</td>
</tr>
<tr>
<td>rs2555622</td>
<td>1.31 (0.79–2.17)</td>
<td>1.23 (0.85–1.78)</td>
<td>1.26 (0.93–1.69)</td>
<td>0.13</td>
<td>0.84</td>
</tr>
<tr>
<td>rs6844282</td>
<td>2.38 (1.55–3.67)</td>
<td>1.07 (0.78–1.47)</td>
<td>1.42 (1.10–1.83)</td>
<td>0.0078</td>
<td>0.0035</td>
</tr>
<tr>
<td>rs11724251</td>
<td>1.47 (0.96–2.26)</td>
<td>1.10 (0.79–1.53)</td>
<td>1.22 (0.94–1.59)</td>
<td>0.13</td>
<td>0.29</td>
</tr>
<tr>
<td>rs10019035</td>
<td>11.9 (1.49–94.6)</td>
<td>1.22 (0.41–3.58)</td>
<td>1.98 (0.76–5.14)</td>
<td>0.16</td>
<td>0.056</td>
</tr>
<tr>
<td>rs2332897</td>
<td>4.59 (2.60–8.11)</td>
<td>1.13 (0.78–1.65)</td>
<td>1.74 (1.27–2.38)</td>
<td>0.0006</td>
<td>0.000058</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0064122.t005

Figure 1. Schematic of HPGD gene, which encodes 15-PGDH, showing location of rs2555639.

doi:10.1371/journal.pone.0064122.g001
Supporting Information

Table S1 Complete SNP-Colon Cancer Association Results in Discovery Population. Distribution (N(%) of homozygous major allele, heterozygous and homozygous minor allele, and OR of homozygous risk (defined as more common in cases compared to controls) vs. homozygous reference.

Table S2 Complete SNP-Expression Results in Tissue Sample Population.

Author Contributions
Conceived and designed the experiments: LL SDM RCE. Performed the experiments: SPF JDL MLV. Analyzed the data: CLT SPF RCE. Contributed reagents/materials/analysis tools: LL SDM. Wrote the paper: CLT SPF RCE SDM LL.

References

Figure 2. Linkage Disequilibrium (LD) Plot of Genotyped SNPs. LD plot of all SNPs selected for replication in all Caucasian samples. Values within boxes are correlations (R²).
doi:10.1371/journal.pone.0064122.g002

Table S2 Complete SNP-Expression Results in Tissue Sample Population.

(DOCX)

Author Contributions
Conceived and designed the experiments: LL SDM RCE. Performed the experiments: SPF JDL MLV. Analyzed the data: CLT SPF RCE. Contributed reagents/materials/analysis tools: LL SDM. Wrote the paper: CLT SPF RCE SDM LL.


Clear cell papillary renal cell carcinoma is the fourth most common histologic type of renal cell carcinoma in 290 consecutive nephrectomies for renal cell carcinoma

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Received 22 May 2013; revised 1 August 2013; accepted 7 August 2013

Keywords: Clear cell papillary renal cell carcinoma; Incidence; Nephrectomy

Summary Clear cell papillary renal cell carcinoma (CCP-RCC) has recently been recognized as a distinct subtype of renal cell carcinoma (RCC) due to its unique morphologic, immunohistochemical, and genetic features and indolent clinical behavior. However, the incidence of this tumor in a nephrectomy series for renal mass has not been fully investigated. Twelve cases of CCP-RCC were identified from a total of 290 consecutive partial (n = 137) or radical nephrectomies (n = 153) for RCC from 2010 to 2012 in our hospital. In this series, CCP-RCC was the fourth most common (4.1%) kidney tumor following clear cell (conventional) (70%), papillary (16.6%), and chromophobe (5.9%) RCCs. The average age of the CCP-RCC patients was 58.2 years (range, 18-81 years), with an equal sex distribution. Four cases (33.3%) were associated with end-stage renal disease. Of the 12 CCP-RCCs, 9 presented as solitary tumors; 2 coexisted with clear cell RCC; and 1 with papillary RCC. The average size of tumors was 2.5 cm (range, 0.8-6.0 cm). All tumors were pT1 (10 pT1a and 2 pT1b). Two cases were initially misclassified as clear cell RCC. Strong positive cytokeratin 7 stain and negative stains with α-methylacyl-CoA racemase and RCC marker differentiate CCP-RCC from low-grade clear cell RCC with similar histologic features. We conclude that CCP-RCC is a common renal neoplastic entity, representing the fourth most common (4.1%) RCC. It can be easily misclassified due to its overlapping features with low-grade clear cell RCC. In equivocal cases, immunohistochemical stains with a small panel of markers (cytokeratin 7, α-methylacyl-CoA racemase, RCC marker, or CD10) are warranted in making the correct histologic classification. © 2014 Elsevier Inc. All rights reserved.

1. Introduction

Clear cell papillary renal cell carcinoma (CCP-RCC) is a distinctive histologic entity of renal tumor [1-11], which was initially recognized as a benign angioadenomatous tumor [2], and later shown to be associated with end-stage...
renal disease (ESRD) [1]. With the increasing awareness of this entity, published studies of CCP-RCC have demonstrated a better clinical depiction of CCP-RCC. Recent findings indicate that CCP-RCC also occurs in patients without ESRD [3,4,6,7,10,11], often presents as a solitary mass, and may coexist with other renal tumors. Patients with CCP-RCC have very good prognosis, and no tumors of the reported cases have metastasized. It seems that CCP-RCC is relatively common; however, the true incidence of this entity has not been fully investigated.

The histologic features of CCP-RCC [3,5,9] are not exclusive [11], as they may overlap with other renal tumors, especially those that contain clear cells [12]. Therefore, in this study, we report the incidence of CCP-RCC in a consecutive nephrectomy series for renal cell carcinoma (RCC) at our institution, emphasize the overlapping histologic features of CCP-RCC with other clear cell–containing RCCs, and review its immunohistochemical expression for correct classification.

2. Materials and methods

2.1. Cases

We undertook a systematic review of all RCCs with a total of 290 patients who underwent partial (n = 137) or radical (n = 153) nephrectomies from 2010 to 2011 at Houston Methodist Hospital in Houston, TX. All cases with a diagnosis of CCP-RCC as well as those cases with features that resemble CCP-RCC were retrieved. Twelve CCP-RCC cases were identified. Four cases of clear cell RCC (CC-RCC) with features of CCP-RCC were also collected. All slides from each case were evaluated by 2 pathologists (S. Z. and S. S.). Clinicopathologic data of these 12 CCP-RCCs were reviewed.

2.2. Immunohistochemistry

Immunohistochemical studies were conducted with the following panel of markers: cytokeratin 7 (CK7), RCC marker (RCCm), and α-methylacyl-CoA racemase (AMACR). The staining was performed using automatic stainers from Ventana (Ventana Medical Systems, Tucson, AZ) with an enzyme-conjugated polymer complex. The dilution and sources of antibodies are CK7 (1:50; Dako, Carpinteria, CA), CD10 (1:10; Vector, Burlingame, CA), AMACR (1:100; Dako), RCCm (1:10; Novo Castra, Newcastle upon Tyne, UK), and transcription factor E3 (TFE3) (1:100; Cell Marque, Rocklin, CA).

3. Results

3.1. Incidence of CCP-RCC

Among the 290 RCCs, 12 cases (4.1%) of CCP-RCC were identified. Clear cell (conventional), papillary, chromophobe, and unclassified RCC accounted for 70%, 16.6%, 5.9%, and 1.7%, respectively. The remaining 5 tumors (1.7%) were tubulocystic RCC, mucinous tubular and spindle cell RCC, Xp11 translocation RCC, and 2 ESRD-associated RCCs.

3.2. Clinical and pathologic characteristics of the CCP-RCC patients

The clinical and pathologic characteristics of the CCP-RCC patients can be found in Table 1. The patients included 6 men and 6 women with a mean age of 58.2 years (range, 18–81 years). Clinically, 4 cases (33.3%) were associated with ESRD, and 1 patient with von Hippel-Lindau syndrome (VHL) was diagnosed with multiple CC-RCCs. The tumors

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Clinical presentation</th>
<th>Tumor size (cm)</th>
<th>Stage</th>
<th>Follow-up (mo)</th>
<th>Disease progression</th>
<th>Operation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>66</td>
<td>No symptom</td>
<td>0.8</td>
<td>Ta</td>
<td>6</td>
<td>No</td>
<td>RN</td>
<td>Coexist with papillary RCC, Type 1</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>62</td>
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<td>F</td>
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<td>Initial Dx with CC-RCC</td>
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<td>PN</td>
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<tr>
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<td>ESRD</td>
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<td>No</td>
<td>RN</td>
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<tr>
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<td>M</td>
<td>45</td>
<td>ESRD</td>
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<td>Ta</td>
<td>35</td>
<td>No</td>
<td>RN</td>
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</table>

Abbreviations: M, male; F, female; ESRD, end-stage renal disease; VHL, von Hippel-Lindau syndrome; RN, radical nephrectomy; PN, partial nephrectomy; Dx, diagnosis.
in 5 patients were incidentally found, as there were no clinical symptoms. However, 1 patient presented with flank pain, and another had abdominal pain.

The average tumor size was 2.5 cm (range, 0.8-6.0 cm). Thus, the stage was pT1a (≤4 cm) for 10 tumors and pT1b (>4 and 7 cm) for 2 tumors. Of 12 CCP-RCCs, 9 presented as solitary tumors, and 2 coexisted with CC-RCC and 1 with papillary RCC.

Histologically, all of the CCP-RCC tumors were encapsulated, and the architectural patterns identified in most of our cases included solid, papillary, tubular, and cystic. The cytoplasmic clearing and characteristic linear nuclear arrangement away from the basement membrane were often present. The Fuhrman nuclear grade of all tumors was either grade 1 or grade 2. Representative microscopic illustrations of CCP-RCC from the present study are shown in Fig. 1. The mean follow-up duration was 19 months (range, 6-35 months), and there was no case with disease progression or metastasis.

3.3. Overlapping histologic features of CCP-RCC and CC-RCC

Of the 12 CCP-RCC cases, 2 were initially classified as CC-RCC (Fig. 2), as both of these showed a diffuse solid growth pattern predominantly composed of tubules lined by clear cells. Focally, the nuclei of tumor cells were arranged in a linear fashion and away from the basement membrane. However, there were no papillary or significant cystic components. The tumors were reclassified as CCP-RCC after subsequent immunohistochemistry indicated the characteristic CCP-RCC immunophenotype. In addition, during the review process, 4 cases of low Fuhrman grade (grade 2 or less) CC-RCC were found to have areas of solid and tubular growth patterns and clear cells with superficial resemblance to that of CCP-RCC. However, these tumors had a typical staining pattern of conventional CC-RCC (Fig. 3).

3.4. Immunohistochemical features of the CCP-RCC and low Fuhrman grade CC-RCC with cystic or tubular patterns

We assessed the immunohistochemical profile of CK7, AMACR, and RCCm in the 12 CCP-RCC cases and 4 CC-RCC cases with features that were similar to CCP-RCC. All 12 cases (100%) of CCP-RCC showed strong immunoreactivity for CK7, and all were negative for AMACR and RCCm, except for 1 case that showed focal positivity for these 2 markers. All 4 cases of low-grade CC-RCC were negative for CK7 except 1 case was weakly positive. All 4 cases of low-grade CC-RCC were positive for AMACR and RCCm (Figs. 1-3). In addition, all 12 cases of CCP-RCC were negative for TFE3.

4. Discussion

Accumulating data have demonstrated that CCP-RCC is a distinct entity of renal epithelial neoplasm. The tumor is usually well circumscribed with variable architectural patterns, including tubulopapillary, solid, acinar, and cystic...
types. The tumor cells have clear cytoplasm, and the nuclei are characteristically situated away from the basement membrane in a linear fashion, occupying a site within the upper aspect of the cells. Low Fuhrman nuclear grade (1 or 2) is the rule [3,5,9]. Immunohistochemically, the tumor cells express strong positivity for CK7 and are negative or weakly positive for CD10, RCCm, and AMACR; they are also negative for TFE3 [3,4,8,9,11]. Cytogenetic and molecular studies of this tumor indicate no genetic aberration, specifically, loss of chromosome 3p (locus of VHL) and gains of chromosomes 7 or 17, as seen in CC-RCCs or papillary RCCs [6-8,13].

Because of the increasing awareness of CCP-RCC, there have been an increased number of recent case reports and retrospective studies that widen our knowledge of this entity. CCP-RCC is frequently encountered in our routine practice. Very little is known about the incidence of this tumor. Only 1

Fig. 2  Representative photomicrographs of a CCP-RCC case from the present study, which was initially diagnosed as CC-RCC. Hematoxylin and eosin sections show tubular configuration in a lower-power field (A, ×40) and a high-power field (B, ×200). CK7 staining shows diffuse strong positivity (C, ×200). A diagnosis of CCP-RCC is subsequently rendered.

Fig. 3  Representative photomicrographs a CC-RCC case from the present study, which demonstrates the tubular configuration and tumor cell nucleus arrangement characteristically seen in CCP-RCC. Hematoxylin and eosin sections in a lower-power field (A, ×100) and a high-power field (B, ×200) are shown. Negative CK7 staining (C, ×200) and positive RCCm staining with a membranous staining pattern (D, ×200) are seen.
The study has mentioned it [11], although it could be calculated in 2 other studies [4,10] (Table 2). Thus, incorporating the published series [4,10,11], which account for 1795 cases, the average incidence of CCP-RCC is 2.8% (range, 1.9%-4.1%). Our study renders an incidence of 4.1%, which makes it the fourth most common RCC, following CC-RCC, papillary, and chromophobe RCC.

The patients with CCP-RCC typically present at an early stage with small renal tumors and, consequently, have a favorable clinical outcome. By analyzing the published cases, only 3 of 148 cases presented with T2 disease, while the rest of the cases had T1 disease [1,2,4,6,7,9-11]. So far, no patient with CCP-RCC has developed metastasis or died of disease. These reported patients had variable follow-up periods, ranging from 1 to 120 months [1,2,4,6,7,9-11]. Although the relatively “benign” clinical behavior may justify a diagnosis of clear cell “adenoma” or renal clear cell neoplasm of low malignant potential, evaluation of more cases and longer clinical follow-up are warranted before a final conclusion can be made. Therefore, it is crucial to differentiate the CCP-RCC from other RCC counterparts, which are bona fide malignant.

The major differential diagnosis of CCP-RCC is the CC-RCCs with low Fuhrman nuclear grade [14]. Although linear nuclear arrangement is one of the characteristic features of CCP-RCC, it is not always present in all cases. A recent study indicated that the linear nuclear alignment was identified in only 8 (53.3%) of 15 cases of CCP-RCC [11]. Interestingly, a recent study demonstrated rare, biphasic CC-RCC cases presenting as both CCP-RCC and CC-RCC on microscopic morphology [12]. In our study, we showed that 4 cases of low-grade CC-RCC focally have linear nuclear arrangement, which resembles that of CCP-RCC (Fig. 2). Therefore, in those cases of low-grade RCC with clear cells that are not typical for either CC-RCC or CCP-RCC, it is important to use immunohistochemistry as an adjunct to make a distinction as CCP-RCC has unique immunophenotype (ie, diffuse and strongly positive for CK7, negative for AMACR, CD10, or RCCm) [3,4,8-12,15].

Xp11.2 translocation RCC also has papillary and clear cell features, which may be included in the differential diagnosis of CCP-RCC. However, Xp11.2 translocation RCC usually has high nuclear grade and eosinophilic cytoplasm and is typically positive for TFE3 by immunohistochemistry [6,7,11]. Our results showed that all CCP-RCCs are negative for TFE3.

Another clinical feature of CCP-RCC is its coexistence with other renal conditions. Tickoo et al [1] first described a series of CCP-RCC associated with ESRD. Although current data, including ours, show that CCP-RCC often presents with a solitary mass in the kidney without chronic renal disease, association with ESRD is considerably high (24.3%) after evaluating all available data [1,2,4,6,7,9-11]. Although it is reasonable to speculate that the incidence of CCP-RCC in patients with ESRD is much higher than that of the general population, caution should be taken not to misclassify

### Table 2

<table>
<thead>
<tr>
<th>Studies</th>
<th>Mean Age (y)</th>
<th>Sex (M/F)</th>
<th>ESRD</th>
<th>Tumor size (cm), mean (range)</th>
<th>Follow-up (mo)</th>
<th>Immunohistochemistry profile</th>
<th>Incidence among RCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mai et al [4]</td>
<td>58.6 (29-72)</td>
<td>8:2</td>
<td>0/10</td>
<td>4.11 (0.6-4.0)</td>
<td>12-120</td>
<td>CK7 (100%)</td>
<td>100% focal 24% focal 1.9% focal 2.9% focal 3% weak 3%</td>
</tr>
<tr>
<td>Park et al [11]</td>
<td>52 (35-70)</td>
<td>4:11</td>
<td>4/15</td>
<td>1.68 (0.6-4.0)</td>
<td>9-29</td>
<td>CK7 (100%)</td>
<td>100% focal 27% focal 59% focal 100% 5% weak 3%</td>
</tr>
<tr>
<td>Williamson et al [10]</td>
<td>61 (33-87)</td>
<td>19:15</td>
<td>8/15</td>
<td>2.0 (0.2-7.5)</td>
<td>1: pT2</td>
<td>CK7 (100%)</td>
<td>100% focal 2.9%</td>
</tr>
<tr>
<td>Current study</td>
<td>58.2 (17-80)</td>
<td>6:6</td>
<td>4/12</td>
<td>2.5 (0.8-6.0)</td>
<td>6-35</td>
<td>CK7 (100%)</td>
<td>100% focal 4.1%</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.
CCP-RCC as CC-RCC in the setting of ESRD. Coexistence of CCP-RCC with other types of RCCs can also happen. A few cases of CC-RCC, acquired cystic disease-associated RCC, papillary RCC, and chromophobe RCC coexisting with CCP-RCC have been reported [1,6,10,11]. CCP-RCC may also be associated with VHL syndrome. One of our patients and 2 additional patients reported in the literature have had VHL syndrome [6,11].

In summary, our study further suggests that CCP-RCC is a relatively common renal cell neoplasm. Pathologists should be aware of the common incidence of this entity and its overlapping features with other RCCs to avoid the misclassification of CCP-RCC as CC-RCC and vice versa. Coexistence of CCP-RCC with other RCCs is not uncommon. In equivocal cases, immunohistochemical stains with a small panel of markers (CK7, AMACR, RCCm, or CD10) are helpful in determining the correct histologic classification.

Acknowledgments

We would like to thank Philip D. Randall for his critical review and editing of this manuscript. We also like to thank Dr Yong Mee Cho at the Asan Medical Center, Korea, for performing the immunohistochemical staining of TFE3.

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NIH Public Access

Author Manuscript

*Hum Pathol.* Author manuscript; available in PMC 2014 June 01.

Published in final edited form as:


Loss of PTEN Expression Is Associated with Recurrence and Poor Prognosis in Patients with Pancreatic Ductal Adenocarcinoma

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Abstract

Phosphatase and tensin homolog (PTEN) is a tumor suppressor in the AKT/mTOR pathway. Animal model studies have shown that loss of PTEN function is involved in the progression of pancreatic cancer. However, the prognostic significance of loss of PTEN expression in pancreatic cancer is unclear. PTEN expression was evaluated by immunohistochemistry on tissue microarrays consisting of multiple cores of 133 resected stage II pancreatic ductal adenocarcinomas. A PTEN expression score was calculated as the product of the percentage of positive tumor cells and the intensity of PTEN staining. We categorized PTEN expression for each tumor as retained (PTEN score>5) or loss (PTEN score \(\leq\)5). Thirty-four (25.6\%) patients had tumors with loss of PTEN expression, and 99 (74.4\%) had tumors with retained PTEN expression. Recurrence/metastasis was observed in 88.2\% (30/34) of patients whose tumors showed loss of PTEN compared to 68.7\% (68/99) of patients whose tumors showed retained PTEN (p=0.03). Patients whose tumors showed loss of PTEN had a shorter overall survival (median: 19.9±3.6 months) than patients whose tumors had retained PTEN (32.7±5.0 months, p = 0.03). In a multivariate analysis, loss of PTEN expression was an independent prognostic factor for poor overall survival in patients with stage II PDA. No significant correlations between loss of PTEN expression and other clinicopathologic parameters were observed (p>0.05). Assessment of PTEN expression may be used as a prognostic marker for patients with resected pancreatic ductal adenocarcinoma.

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**DISCLOSURE/CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

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INTRODUCTION

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the United States (1, 2). Approximately 15–20% of patients present with localized disease, and the 5-year overall survival rate is less than 5% (3). Small tumor size, absence of lymph node metastases, and negative resection margins (R0) are independent prognostic factors predicting long term survival in patients with pancreatic ductal adenocarcinoma who have undergone pancreatectomy (4–6).

The development of pancreatic ductal adenocarcinoma represents the accumulation of successive mutations in proto-oncogenes and loss of tumor suppressor genes, typically beginning with activating mutations in KRAS followed by inactivation of tumor suppressor genes, including p16/INK4A, p53, and SMAD4/DPC4 (7–11). KRAS mutations are found in greater than ninety-percent of pancreatic ductal adenocarcinoma and occur early in tumorigenesis (9–11). Once activated, KRAS functions through multiple signaling pathways, including the phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway. Coinciding with KRAS activation, Asano and colleagues have demonstrated that activation of the PI3K/AKT pathway and its downstream effectors, NF-κB and MYC, is essential for growth and survival of pancreatic ductal adenocarcinoma (12).

Phosphatase and tensin homolog (PTEN) is an important tumor suppressor encoded on chromosome 10q23.3 (13, 14). Loss of PTEN function has been implicated in the tumorigenesis of many human malignancies, including gliomas, endometrial cancers, thyroid cancers and pancreatic cancers. Loss of PTEN function can be due to multiple mechanisms, including biallelic or monoallelic deletions, mutations, gene silencing by promoter methylation or dysregulation of mRNA by microRNAs. In mouse model studies, pancreatic-specific knockout of Pten leads to ductal metaplasia from the expansion of centroacinar cells and the development of pancreatic ductal adenocarcinoma (15). Single copy deletion of Pten in KrasG12D mice (Pdx1-Cre LSL-KrasG12D PtenLox/Lox mice) results in more extensive acinar-ductal metaplasia, pancreatic intra-epithelial neoplasia, increased epithelial proliferation, and higher frequencies of invasion and metastasis compared to Pdx1-Cre LSL-KrasG12D mice (16). Similar results were observed in the KrasG12D mice with conditional Pten knockout (PtenLox/Lox) (17). Pten also functions synergistically with the tumor suppressor, Smad4, during the development and progression of pancreatic cancer in mouse models (18). These data support the notion that PTEN is one of the major tumor suppressors in pancreatic tumorigenesis. Although deletion or loss of function mutations in PTEN have not been detected with significant frequency in pancreatic cancer (19, 20), low or no PTEN expression by immunohistochemistry has been reported in up to 70% of human pancreatic ductal adenocarcinoma samples (16). Recently, Feng et al. reported that PTEN expression in pancreatic ductal adenocarcinoma patients with liver metastasis is lower than those patients who had no liver metastasis (21). They also showed that high PTEN expression is associated with a better 5-year survival in pancreatic ductal adenocarcinoma patients with liver metastasis (21). However, the prognostic value of PTEN expression and its clinical impact in patients who underwent surgical resection for pancreatic ductal adenocarcinoma is not clear. In this study, we sought to evaluate by immunohistochemistry the frequency of loss of PTEN expression in 133 patients with stage II pancreatic ductal adenocarcinoma. The results of PTEN expression were correlated with clinicopathologic parameters and patient survival.
MATERIALS AND METHODS

Patient population

Our study population consisted of 133 patients (78 male and 55 female) with stage II pancreatic ductal adenocarcinoma who underwent pancreatectomy as initial treatment for pancreatic ductal adenocarcinoma at our institution from 1990 to 2010. Patient age ranged from 24.9 to 84.8 years (median age: 64.6 years). Five patients with other disease stages (one patient each with stage IA, IB, or III disease and two patients with stage IV disease) were excluded because there were insufficient case numbers to be representative. Patients who received any form of neoadjuvant chemotherapy and/or radiation therapy were excluded. One hundred, thirteen patients (85.0%) underwent pancreaticoduodenectomy, 18 (13.5%) underwent distal pancreatectomy, and 2 (1.5%) underwent total pancreatectomy. One hundred eight patients (81.2%) had an R0 resection (all resection margins negative by histology) and 25 (18.8%) patients had an R1 resection (microscopic disease involving one or more margins). No patients had an R2 resection. Twenty-five (18.8%) patients received adjuvant chemotherapy alone, 73 (54.9%) received combined adjuvant chemoradiation therapy and 35 (26.3%) did not receive adjuvant therapy. The study was approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center.

Tissue microarray (TMA) construction

Hematoxylin and eosin (H & E) stained slides and their matched formalin-fixed paraffin embedded tissue blocks were retrieved and reviewed for representative areas of tumor and non-neoplastic pancreatic parenchyma. Two 1.0 mm cores from different areas of each tumor and one 1.0 mm core of the matched non-neoplastic pancreatic tissue were used for the TMA construction. The TMA was constructed as previously described using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI)(22).

Immunohistochemistry for PTEN expression

PTEN immunohistochemistry was performed on 4 μm unstained sections from TMA blocks using a mouse monoclonal antibody against PTEN (DAKO clone 6H2.1, Carpeninteria, CA, 1:100 dilution). The immunohistochemical stained slides were evaluated independently by two pathologists (W.C.F. and H.W.) for percentage of tumor cells with PTEN immunoreactivity and the intensity of PTEN immunoreactivity (0-negative, 1-weak, 2-moderate, and 3-strong). The PTEN expression score was calculated as the product of the percentage of positive tumor cells and the intensity of PTEN staining. We categorized each tumor as either retained PTEN expression (defined as PTEN score >5) or loss of PTEN expression (defined as PTEN score ≤5). For tumors that retained PTEN expression, we further categorized those tumors as either PTEN-low (PTEN score >5, but ≤50) or PTEN-high (PTEN score>50). If there was a discrepancy in PTEN expression between two duplicate cores from the same tumor, the tumor was classified based on the result of the core with positive PTEN staining. All cases with a discrepancy in PTEN expression between pathologists were re-reviewed together, and the consensus results for PTEN expression were used.

Statistical analyses

The immunohistochemical staining results were correlated with clinicopathologic features and survival. Chi-square and Fisher’s exact tests were used to compare categorical data. Overall survival and disease-free survival curves were constructed using a Kaplan-Meier method, and log-rank tests were used to evaluate the statistical significance of differences. The prognostic significance of clinicopathologic parameters and PTEN expression was determined using a Cox-regression multivariate analysis via a backward stepwise procedure.
Statistical calculations were performed using Statistical Package for Social Sciences software (version 12.0, SPSS Inc., Chicago, IL). A p-value of <0.05 were considered statistically significant.

RESULTS

PTEN expression in pancreatic ductal adenocarcinoma

Of the 133 samples, 34 tumors (25.6%) showed loss of PTEN expression (Figure 1A & 1B) and 99 tumors (74.4%) showed retained PTEN expression. In the cases that had retained expression for PTEN, the tumor showed both cytoplasmic and/or nuclear staining (Figure 1C & 1D). Forty-three (32.3%) cases were PTEN-low and 56 (42.1%) cases were PTEN-high. Heterogenous PTEN expression between the two tissue cores of the tumor was observed in 22 (16.5%) cases; in these cases, the tumor was classified based on the core with positive staining. All 22 tumors were classified as retained PTEN expression. The matched non-neoplastic pancreatic tissue cores retained PTEN expression in all cases examined and served as the internal positive controls for our immunohistochemical staining (Figure 1E and F).

Association of PTEN expression and clinicopathologic features

The correlations between PTEN expression and clinicopathologic features are summarized in Table 1. Of 34 patients whose tumor lost PTEN expression, 30 (88.2%) had local recurrence or distant metastasis compared to 68.7% (68/99) of patients whose tumor retained PTEN expression (p=0.03). Lymph node metastasis was present in 88.2% (30/34) in cases that lost PTEN expression, compared to 71.7% (71/99) in the patients whose tumor retained PTEN expression (p=0.05). We did not observe significant correlation between the loss of PTEN expression and patient gender, age, tumor differentiation, tumor size, resection margin status or post operative chemo/radiation therapies (p>0.05, Table 1). Similarly, we did not observe significant difference in any of the clinicopathologic factors between the group with PTEN-low tumors and the group with PTEN-high tumors (data not shown).

Loss of PTEN expression correlated with poor overall survival

The median follow up time was 18.5 months (range: 4.2 to 82.9 months) for 88 patients who died of disease. For 45 patients who did not die from the disease, the follow-up time ranged from 7.1 months to 214.0 months with the median follow-up time of 40.7 months after pancreatectomy. No patients died in the immediate postoperative period, and none were lost to follow up. The median disease free survival and overall survival in this study population were 12.1 ± 1.9 months and 25.2 ± 3.0 months, respectively. Loss of PTEN expression correlated with poor overall survival. For the patients whose tumor retained PTEN expression, the median overall survival was 32.7 ± 5.0 months compared to the median overall survival of 19.9 ± 3.6 months for patients whose tumor lost PTEN expression (p=0.03, log-rank method, Figure 2A). Patients whose tumor retained PTEN expression also had longer disease free survival (median: 15.4 ± 2.0 months) than those whose tumor lost PTEN expression (median: 9.4 ± 1.7 months) but was not statistically significant (p=0.09, log-rank method, Figure 2B). The univariate analyses of overall survival in correlation with the clinicopathologic parameters and PTEN expression are shown in Table 2. In addition to the loss of PTEN protein expression, overall survival was also associated with tumor size, margin status, and lymph node metastasis (p<0.05) but not other clinicopathologic factors (p≥0.05). No significant difference in either disease free survival or overall survival between the group with PTEN-low tumors and the group with PTEN-high tumors was observed (data not shown). In multivariate analysis, loss of PTEN expression was a prognostic factor for overall survival in patients with stage II disease independent of tumor size, margin status, and lymph nodal metastases (Table 3).
DISCUSSION

In this study, we examined the PTEN expression by immunohistochemistry in 133 patients with stage II pancreatic ductal adenocarcinoma. We found that loss of PTEN protein expression correlated with tumor recurrence/distant metastasis after pancreatectomy and shorter overall survival. Loss of PTEN protein expression was an independent prognostic factor in our patient population. Our data supports the notion that PTEN is an important tumor suppressor in the tumorigenesis and progression of pancreatic ductal adenocarcinoma. PTEN has been implicated in the tumorigenesis and progression of pancreatic ductal adenocarcinoma. It has been demonstrated that heterozygous Pten deletion in the pancreas accelerates the development of pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma in Kras\textsuperscript{G12D} pancreatic cancer mouse model\cite{17} and that Pten haploinsufficiency was sufficient to induce development of pancreatic ductal adenocarcinoma in murine models. Moreover, mouse survival was dictated in a Pten dose-dependent fashion\cite{16}. To test this hypothesis in human pancreatic ductal adenocarcinoma samples, we compared the clinicopathologic parameters and survival between a group of patients’ tumors with retained PTEN expression and another with loss of PTEN expression. In this study, low or no PTEN expression was observed in 57.9% of stage II pancreatic ductal adenocarcinoma samples. Our findings were consistent with the previous reports, which identified 38.8% and 70% of pancreatic ductal adenocarcinoma samples have low or no PTEN expression\cite{16,21}. We found loss of PTEN expression in 25.6% of our cohort, which was similar to results reported by others using immunohistochemistry performed on both whole tissue and TMA sections\cite{12,23–25}. Given that our study included tumor samples from 133 patients, our results in conjunction with earlier studies show that loss of PTEN staining is not an infrequent occurrence in pancreatic ductal adenocarcinoma.

PTEN functions as an important tumor suppressor in the tumorigenesis of many human malignancies. Loss of PTEN protein expression is reported to be associated with worse survival in advanced endometrial cancer, lung, breast, and colon cancers\cite{26–28,29,30}. The prognostic significance of loss of PTEN expression in patients with pancreatic ductal adenocarcinoma was unclear. Feng et al reported high levels of PTEN expression in 61.2% pancreatic ductal adenocarcinoma patients without liver metastasis compared to 29.9% in pancreatic ductal adenocarcinoma patients who had liver metastasis\cite{21}. Their data suggest that loss of PTEN plays a role in liver metastasis in patients with pancreatic ductal adenocarcinoma. Consistent with this notion, we found that loss of PTEN expression was associated with higher frequency of recurrence/distant metastasis in patients with stage II pancreatic ductal adenocarcinoma compared to those patients whose tumor retained PTEN expression. In addition, we also found that loss of PTEN expression was associated with poor overall survival and was an independent prognostic factor in our patient population with stage II pancreatic ductal adenocarcinoma by multivariate analysis. Loss of nuclear staining for PTEN has been reported to be associated with poor survival in colon cancer\cite{29,30}. In this study, we did not observe a significant correlation in the loss of nuclear staining for PTEN with either disease free survival or overall survival in our study population. Our data did not support the previous report by Pham and colleagues, who found a higher percentage of stage I and II tumors had low levels of PTEN protein expression than stage III and stage IV tumors\cite{25}. The limitation of our study was the inclusion of only patients with stage II disease as there were only a small number of patients with other stages of disease. However, our findings are corroborated by a recent report demonstrating high levels of PTEN expression associated with a better 5-year survival rate compared to those with no or low PTEN expression in patients with pancreatic ductal adenocarcinoma metastatic to the liver\cite{21}.
The mechanisms of loss PTEN expression in pancreatic ductal adenocarcinoma are unclear. Recently, Ying et al demonstrated the deletion of one or two copies of the PTEN locus on 10q23 in 15% of the 61 pancreatic ductal adenocarcinoma samples examined using high resolution array comparative genomic hybridization (16). This finding suggested that loss of PTEN expression in pancreatic ductal adenocarcinoma may be due to the deletion of PTEN locus, which had been reported in other malignancies. The loss of PTEN expression is accompanied by the gain/amplification in AKT2 locus and activation of AKT. The aberrant activation of PI3K/AKT pathway by loss of PTEN and/or activation of AKT accelerates mutant KRAS driven malignant progression of pancreatic ductal adenocarcinoma (31). Our findings that loss of PTEN expression correlated with recurrence/distant metastasis and poor overall survival in patients with pancreatic ductal adenocarcinoma provides additional support for the functions of PTEN/PI3K pathway in pancreatic cancer progression. Therefore, targeting PI3K/mTOR pathway in combination with other treatment modality may provide more effective treatment for pancreatic ductal adenocarcinoma.

In summary, loss of PTEN is not an infrequent occurrence in pancreatic ductal adenocarcinoma. Loss of PTEN expression correlates with recurrence/distant metastasis and survival in patients with stage II pancreatic ductal adenocarcinoma. Thus, loss of PTEN expression supports the implication that PTEN function plays an important role in progression of pancreatic ductal adenocarcinoma. Evaluation of PTEN expression by immunohistochemistry has prognostic implications in patients with stage II pancreatic cancer and may have therapeutic implications as well. As such, the utility of targeting the PI3K/Akt pathway via PI3K inhibitors and mTOR inhibitors should be evaluated in pancreatic ductal adenocarcinoma.

Acknowledgments

Supported by the National Institutes of Health grant (1R21CA149544-01A1) and G. S. Hogan Gastrointestinal Cancer Research Fund at The University of Texas M.D. Anderson Cancer Center

References


Figure 1.
Representative micrographs show PTEN expression in pancreatic ductal adenocarcinoma samples and benign pancreatic tissue. A and B, a moderately differentiated ductal adenocarcinoma with negative staining for PTEN; C and D, strong cytoplasmic and nuclear staining for PTEN in a moderately differentiated ductal adenocarcinoma; E and F, representative benign pancreatic tissue that is strongly positive for PTEN (Original magnification, 40× for A, C, and E; 400× for B, D, and F).
Figure 2.
Kaplan-Meier curves for overall survival (A) and disease-free survival (B) by PTEN expression in patients with stage II pancreatic ductal adenocarcinoma. Patients whose tumors lost PTEN expression had shorter overall survival than patients whose tumors retained PTEN expression.
Table 1
Clinicopathologic features correlated with PTEN expression in stage II pancreatic ductal adenocarcinomas

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>Retained PTEN (n = 99)</th>
<th>Loss of PTEN (n = 34)</th>
<th>p value</th>
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<td>Age (y)</td>
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<td></td>
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<td>&lt;60</td>
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<td>28</td>
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<td>60–70</td>
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<td>43</td>
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<td>&gt;70</td>
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<tr>
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<td>57</td>
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<tr>
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<td>Recurrence or distant metastasis</td>
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<td>54</td>
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Table 2

Univariate analysis of overall survival in patients with stage II pancreatic ductal adenocarcinomas

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<th>Variables</th>
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<td>&lt;60 years</td>
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<td>60 – 70 years</td>
<td>57</td>
<td>1.07 (0.65 – 1.77)</td>
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<td>&gt;70 years</td>
<td>37</td>
<td>1.45 (0.83 – 2.55)</td>
<td>0.39</td>
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<tr>
<td><strong>Gender</strong></td>
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<tr>
<td>Female</td>
<td>55</td>
<td>1.00</td>
<td>0.14</td>
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<tr>
<td>Male</td>
<td>78</td>
<td>0.73 (0.48 – 1.11)</td>
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<td><strong>Tumor differentiation</strong></td>
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<tr>
<td>Well</td>
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</tr>
<tr>
<td>Moderate</td>
<td>88</td>
<td>2.70 (1.08 – 6.76)</td>
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<tr>
<td>Poor</td>
<td>35</td>
<td>2.62 (0.99 – 6.96)</td>
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<td><strong>Tumor size (cm)</strong></td>
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<td>&lt;= 2.0</td>
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<td>1.00</td>
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<tr>
<td>&gt; 2.0</td>
<td>111</td>
<td>3.25 (1.56 – 6.77)</td>
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<td>Negative (IIA)</td>
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<tr>
<td>Positive (IIB)</td>
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<td>1.00</td>
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<tr>
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<tr>
<td>Loss</td>
<td>34</td>
<td>1.65 (1.06 – 2.58)</td>
<td>0.03</td>
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</table>

a Abbreviations: HR, hazard ratio; CI, confidence interval
Table 3
Multivariate analysis of overall survival in patients with stage II pancreatic ductal adenocarcinomas

<table>
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<tr>
<th>Variables</th>
<th>n</th>
<th>HR^a (95% CI)^a</th>
<th>p value</th>
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<tr>
<td>Tumor size (cm)</td>
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<td></td>
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<tr>
<td>&lt;= 2.0</td>
<td>22</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>&gt; 2.0</td>
<td>111</td>
<td>3.47 (1.66 – 7.25)</td>
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<td>Negative</td>
<td>108</td>
<td>1.00</td>
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<tr>
<td>Positive</td>
<td>25</td>
<td>1.39 (0.82 – 2.36)</td>
<td>0.228</td>
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<td>Lymph node status (stage)</td>
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<td>Negative (IIA)</td>
<td>32</td>
<td>1.00</td>
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<tr>
<td>Positive (IIB)</td>
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<td>1.52 (0.83 – 2.80)</td>
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<td>PTEN expression</td>
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<tr>
<td>Retained</td>
<td>99</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Loss</td>
<td>34</td>
<td>1.79 (1.14 – 2.80)</td>
<td>0.011</td>
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</table>

^a Abbreviations: HR, hazard ratio; CI, confidence interval
Solid pseudopapillary neoplasm of the pancreas with prominent atypical multinucleated giant tumor cells

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Abstract

Aims—Solid pseudopapillary neoplasm of the pancreas (SPN) is a rare low-grade malignant neoplasm. SPN with prominent atypical multinucleated giant tumor cells (MNGTCs) has not been reported.

Methods and results—We identified four cases of SPN with prominent atypical MNGTCs in a cohort of 62 cases of SPN (6.5%). The MNGTCs contained multiple enlarged, hyperchromatic, irregular nuclei with ample eosinophilic cytoplasm, typically present in the solid area of the tumor. The MNGTCs had a typical immunohistochemical profile of the conventional SPN and were positive for vimentin, β-catenin, CD10, and progesterone receptor, but were negative for pan-cytokeratin chromogranin, synaptophysin, trypsin, Ki-67 and CD68 in all four cases. Patients of SPN with prominent MNGTCs were older than those with conventional SPN (p=0.01), tumors were incidentally discovered by imaging studies for an unrelated disease in all four cases, and with a female to male ratio of 1:1. The proliferation index (Ki-67) was <1% in all four cases. None of the three patients, whose follow up information was available, developed recurrence during follow-up of 2.7, 3.8 and 5.0 years.

Conclusions—The presence of MNGTCs in SPN most likely represents degenerative change of the tumor cells and does not seem to affect the prognosis.

Keywords
solid pseudopapillary neoplasm; multinucleated giant cells; prognosis
Introduction

Solid pseudopapillary neoplasm of the pancreas (SPN) is a rare low-grade malignant neoplasm that accounts for about 1% to 2% of all pancreatic tumors. The clinicopathologic features of SPN are distinctive from other pancreatic neoplasms. SPN occurs mainly in young women in their 20s and 30s of age with a median age of 28 years. The tumor sizes are often very large at the time of presentation with a heterogeneous cut surface consisting of solid brown lobulated areas, hemorrhage, and cystic spaces containing necrotic debris. Tumors are confined to the pancreas in 85% of patients. Patients with SPN have excellent prognosis after complete surgical resection with a five-year survival rate over 85%. Even the 10% to 15% of patients who developed recurrent SPN, liver or peritoneal metastases commonly enjoy long-term survival. Although there are no pathological factors that can reliably predict the clinical outcome of patients with SPN, Tang et al recently reported two rare cases of clinically aggressive SPN that were associated with diffuse growth pattern, extensive tumor necrosis, significant nuclear atypia, and high mitotic count. Both of their patients died of disease at 6 and 16 months after presentation.

Despite numerous studies using immunohistochemistry markers, electron microscopy and molecular pathology, the cell origin of SPN remains unclear. Characteristically, SPNs almost always harbor β-catenin mutation and have nuclear localization of β-catenin by immunohistochemistry due to lack of degradation of mutated protein, but lack K-ras mutation that is a hallmark of pancreatic ductal adenocarcinomas. β-catenin immunohistochemistry has been widely used as one of the diagnostic markers for SPN. The typical histologic pattern and cytologic features of SPN are characterized by numerous delicate blood vessels surrounded by variable amount of hyalinized stroma and relative uniform, non-cohesive polygonal cells forming pseudopapillae. Intra-cytoplasmic PAS-positive diastase resistant eosinophilic globules, foamy histiocytes, cholesterol crystals with foreign body giant cell reaction are commonly present in SPN. Rare cases of clear cell variant of SPN had also been reported. The clinical features, gross characteristics, and immunohistochemical staining profile of clear cell variant of SPN had also been reported. The typical histologic pattern and cytologic features of SPN are characterized by numerous delicate blood vessels surrounded by variable amount of hyalinized stroma and relative uniform, non-cohesive polygonal cells forming pseudopapillae. Intra-cytoplasmic PAS-positive diastase resistant eosinophilic globules, foamy histiocytes, cholesterol crystals with foreign body giant cell reaction are commonly present in SPN. Rare cases of clear cell variant of SPN had also been reported. The clinical features, gross characteristics, and immunohistochemical staining profile of clear cell SPN are similar to those patients with conventional SPNs. Recently, we encountered an unusual case of SPN represented by numerous atypical multinucleated giant tumor cells (MNGTCs). This feature, to our knowledge, has not been previously reported. The purpose of this study is to investigate the frequency of SPN with MNGTCs and to describe the clinical and histologic features of four cases of SPN with MNGTCs and compared these to 58 patients with conventional SPNs. The significance of MNGTCs in SPN and the differential diagnoses are discussed.

Materials and Methods

Four patients of SPN with MNGTCs and 58 patients with conventional SPN (control group) identified from the pathology file at the University of Texas MD Anderson Cancer Center from 1994 to 2011 were included in this study. The hematoxylin-eosin (H&E) stained slides from the pancreatectomy specimens of all patients were reviewed and the diagnosis of SPN was confirmed in all cases. Clinical and pathologic information, including patient age, gender, type of specimen, tumor size, tumor location in the pancreas, was collected from computerized patient medical record. The study was approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center.

All four patients of SPN with prominent MNGTCs were evaluated by immunohistochemistry for vimentin (clone V9, 1:900, Dako, Carpinteria, CA), CD10 (clone 56C6, 1:50, Novocastra, Buffalo Grove, IL), β-catenin (clone 14, 1:500, BD Biosciences, San Jose, CA), progesterone receptor (PR, clone 1294, 1:200, Dako), chromogranin (clone LK2H10, 1:4000, Chemicon), synaptophysin (clone 27G12, 1:600, Novocastra), trypsin

Histopathology. Author manuscript; available in PMC 2014 February 01.
(1:130,000, Chemicon, Billerica, MA), Ki-67 (clone MIB-1, 1:100, Dako), and CD68 (clone KP-1, 1:900, Dako). The pancytokeratin cocktail consisted of a mixture of four different antibodies against cytokeratins: AE1/AE3 (1:50), CAM5.2 (1:50), MNF116 (1:50) and 8/18 (1:25). Standard immunohistochemical staining techniques were used as described previously with appropriate positive and negative controls 15.

Patient follow-up information through April of 2012 was extracted from patient medical record and if necessary, by review of the U.S. Social Security Index. The overall survival was calculated as the time from the date of diagnosis to the date of death or the date of last follow-up if death did not occur. The Fisher’s exact tests were used to compare categorical data. Survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences. Statistical analysis was performed using Statistical Package for Social Sciences software (for Windows 12.0, SPSS Inc., Chicago, IL). A two-sided significance level of 0.05 was used for all statistical analyses.

Results

Prevalence and clinicopathologic features of SPN with multinucleated giant tumor cells

We reviewed the H&E slides from 62 pancreatectomy specimens with the diagnosis of SPN in the pathology file at our institution for the presence of multinucleated giant tumor cells (MNGTCs). We found 4 cases (6.5%) of SPN with prominent MNGTCs. In all four cases, many MNGTCs were present either in the solid area of the tumor or in area with conventional SPN histology. The clinicopathologic features of the four patients of SPN with prominent MNGTCs and their comparison with the patients with conventional SPN are shown in Table 1 and Table 2 respectively. The tumor was incidentally identified by computed tomography (CT) during work up for other unrelated diseases in all four cases (Table 1). All four patients had serum CA19–9 levels within the normal range. Fifty percent (2/4) patients of SPN with prominent MNGTCs were male compared to 13.8% (8/58) of male patients with conventional SPN (P=0.06, Table 1). Patients with SPN and MNGTCs had a mean age of 51.3 years (range 36 to 59 years) at the time of diagnosis and were older than those with conventional SPN, whose mean age was 32.1 years (range: 9.4 to 62.2 years, p=0.01).

Gross and morphologic features

SPNs with MNGTCs were located in the head of pancreas in 3 patients and in the tail of the pancreas in one patient. Grossly three tumors were well-circumscribed without capsule and with capsule in one remaining case (case #2). All four tumors showed tan, soft cut surfaces with areas of cystic degeneration and hemorrhage. The tumor size ranged from 1.8 cm to 10.3 cm (mean tumor size: 5.0 ± 3.8 cm), which was not significantly different from the mean tumor size of 6.7 ± 4.2 cm for the conventional SPN (p=0.41).

In all four cases, many atypical MNGTCs were seen in otherwise conventional SPN histology. However, MNGTCs were more prominent in the solid areas of the tumor (Figure 1). Most of the MNGTCs contained multiple enlarged, hyperchromatic, irregular nuclei with ample eosinophilic cytoplasm (Figure 1). While some of the MNGTCs had the nuclear features similar to the mononuclear tumor cells, most MNGTCs showed smudged chromatin, suggestive of degenerative changes. Intra-cytoplasmic eosinophilic hyaline globules were present in the MNGTCs in one case (Figure 1D). Areas of hemorrhage, collections of foamy histiocytes and foreign body multinucleated giant cells with cholesterol crystals were present in three of four cases. Focal calcification was identified in two cases. In all four cases, mitoses were rare and no mitoses were identified in the MNGTCs.

Although the tumors were grossly well-circumscribed, microscopic tumor invasion into the
adjacent normal pancreas (Figure 1C) was present in three cases and peripancreatic soft tissue in two cases. Perineural invasion was present in one case. Lymphovascular invasion, lymph node metastasis or positive resection margin was not present in any of the four cases.

**Immunohistochemistry**

The immunohistochemical profile of the MNGTCs was similar to the mononuclear tumor cells for all four cases. Both mononuclear tumor cells and MNGTCs were diffusely positive for vimentin, beta-catenin (nuclear and cytoplasmic staining), CD10, and progesterone receptor (PR) in all four cases (Figure 2). Immunohistochemical stains for pancytokeratin and synaptophysin were focally positive in one case and were negative in the other three cases. All four cases were negative for chromogranin and trypsin. The MNGTCs were negative for CD68 in all four cases (Figure 2F). Thus the immunohistochemical staining profile suggests that the MNGTCs have similar origin as the mononuclear tumor cells, but not from the histiocytes that are commonly present in SPNs. Immunohistochemical stain for Ki-67 was performed on all four cases and all cases showed less than 1% nuclear staining for Ki-67 in the tumor cells, and no staining of MNGTCs (Figure 2E).

**Clinical Outcome**

The follow up information was available in three patients with the follow up time of 2.7, 3.8 and 5.0 years and one patient was lost to follow up one month after the surgery. During follow-up, one patient died of abdominal abscess at 2.7 years after the distal pancreatectomy. The other two patients were alive with no evidence of recurrence at 3.8 and 5.0 years after the surgery. Although there was no significant difference in overall survival compared to the 58 patients with conventional SPN, whose mean survival was 15.26 ± 0.72 years with a median follow up of 3.41 years (P=0.21), the number of patients with MNGTCs was too small to be meaningful.

**Discussion**

Multinucleated giant cells of histiocytic origin are common findings in SPNs. They represent a reaction to the necrotic and degenerative changes that are commonly seen in these tumors. However, MNGTCs have not been reported in SPNs of the pancreas. In this study, we reported four cases of SPN with prominent MNGTCs in a cohort of 62 patients with SPN. The index case of SPN with prominent MNGTCs was found incidentally during routine histologic evaluation of an otherwise usual SPN. Three additional cases of SPN with prominent MNGTCs were found in 61 pancreatectomy specimens with the diagnosis of SPN after retrospective review. The prevalence of SPN with prominent MNGTCs was 6.5% in our study population. We demonstrated that the MNGTCs in all four cases had the same immunohistochemical profile as the mononuclear tumor cells of SPNs and were immunoreactive to vimentin, beta-catenin, CD10, and PR, but not to histiocytic marker CD68. The MNGTCs are also negative for pan-cytokeratin, chromogranin, synaptophysin and trypsin. These findings suggest that the MNGTCs are originated from the mononuclear tumor cells, instead of histiocytic origin. Application of immunohistochemistry would help to differentiate SPN with prominent MNGTCs from other tumors with cells similar to those seen in our cases, including pleomorphic pancreatic neuroendocrine tumor, pancreatic acinar cell carcinoma, undifferentiated carcinoma of the pancreas, undifferentiated carcinoma of the pancreas with osteoclast-like giant cells, metastatic carcinoma or sarcoma.

The MNGTCs in our cases showed multiple enlarged, irregular or bizarre, hyperchromatic nuclei with significant nuclear atypia. In addition, many MNGTCs were present in the solid areas of SPN in our cases. These findings raised the concern for an
aggressive behavior of this tumor. Rare cases of clinically aggressive SPN of the pancreas that are associated with diffuse growth pattern, extensive tumor necrosis, significant nuclear atypia, and high mitotic count have been reported. However, all four patients of SPN with prominent MNGTCs in this study were identified incidentally by CT scan during work up for other unrelated diseases (three patients during work up for inflammatory conditions and one during follow-up for breast cancer after lumpectomy and cyclophosphamide/epirubicin/5-fluorouracil therapy) and two of the tumors were less than 3.0 cm in size. None of our patients had tumor invasion to the other organs, lymphovascular invasion or lymph node metastasis. In addition, all four cases of SPN with MNGTCs showed very low proliferation index and all giant cells were negative for Ki-67 in this study. No recurrence was detected during the follow up of three patients whose follow up information were available, which was similar to those with conventional SPN. These findings argued against the possibility that the presence of prominent MNGTCs in SPN was associated with more aggressive behavior of SPN. Long term follow-up and more cases are needed to further confirm the indolent behavior of this tumor. The histologic appearance and clinical follow-up data in our study of SPN with prominent MNGTCs bear analogy to the degenerative, pleomorphic atypia seen in pleomorphic pancreatic neuroendocrine tumors, symplastic leiomyomas, symplastic glomus tumor, symplastic hemangioma, and bizarre giant cells of mammary fibroadenomas. The presence of atypical cells in these tumors has also been shown to have no significant effect on the prognosis, but the demographic trend appeared to be similar to their conventional counterparts.

In summary, we identified, for this first time, four cases (6.5%) of SPN with prominent MNGTCs of the pancreas in a cohort of 62 patients with SPN at our institution. The patients of SPN with prominent MNGTCs were older, slightly more frequent in males and located in the head of the pancreas compared to the patients with conventional SPN. The patients of SPN with prominent MNGTCs seem to have a good prognosis similar to those with conventional SPN. We speculate that the MNGTCs in our cases may represent degenerative changes in tumor cells.

Acknowledgments

Supported by the National Institutes of Health grant (1R21CA149544–01A1) and G. S. Hogan Gastrointestinal Cancer Research Fund at The University of Texas M.D. Anderson Cancer Center

References


Figure 1.
Representative micrographs showing many atypical MNGTCs in case #4 present in the solid area of the tumor (A), in area with conventional SPN histology and foamy histiocytes (B), and tumor invasion into the adjacent pancreatic parenchyma (C). D. Representative micrograph from Case #3 show atypical MNGTCs present in area with conventional SPN histology with intracytoplasmic globules (H&E stain, original magnification, 200x).
Figure 2.
Representative micrographs from Case #1 show immunohistochemical staining for pan-cytokeratin (A); β-catenin (B), CD10 (C), PR (D), Ki-67 (E) and CD68 (F). Normal pancreatic acinar cells as internal control were marked with dot line in A, B, and C. The atypical MNGTCs were marked with arrows in B–F. (Original magnification, 100x for A and E; 200x for B, C, D and F).
Table 1

Clinicopathologic features of solid pseudopapillary neoplasm with prominent MNGTCs.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age at diagnosis</th>
<th>Gender</th>
<th>Clinical presentation</th>
<th>CA19.9 (unit/ml)</th>
<th>Type of surgery</th>
<th>Location</th>
<th>Tumor Size</th>
<th>Percentage of tumor with MNGTCs (%)</th>
<th>Follow-up (months)</th>
<th>Vital status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52.7</td>
<td>Female</td>
<td>Incidental finding by CT during follow up for breast cancer</td>
<td>10.7</td>
<td>Pylorus-preserving pancreaticoduodenectomy</td>
<td>Head</td>
<td>1.8 cm</td>
<td>60</td>
<td>45.2</td>
<td>Alive with no evidence of disease</td>
</tr>
<tr>
<td>2</td>
<td>36.0</td>
<td>Female</td>
<td>Pneumonia, incidental finding by CT</td>
<td>15.2</td>
<td>Distal pancreatectomy</td>
<td>Tail</td>
<td>10.3 cm</td>
<td>90</td>
<td>32.4</td>
<td>Dead of abdominal abscess</td>
</tr>
<tr>
<td>3</td>
<td>59.0</td>
<td>Male</td>
<td>Kidney stone and lithotripsy, incidental finding by CT</td>
<td>11.2</td>
<td>Pancreatectoduodenectomy</td>
<td>Head</td>
<td>5.0 cm</td>
<td>30</td>
<td>60.0</td>
<td>Alive with no evidence of disease</td>
</tr>
<tr>
<td>4</td>
<td>57.2</td>
<td>Male</td>
<td>Diverticulitis, incidental finding by CT</td>
<td>16.6</td>
<td>Pancreatectoduodenectomy</td>
<td>Head</td>
<td>2.7 cm</td>
<td>25</td>
<td>1.1</td>
<td>Lost to follow-up</td>
</tr>
</tbody>
</table>
**Table 2**

Comparison of the clinicopathologic features of SPN with prominent MNGTCs with conventional SPN

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>SPN with prominent MNGTCs</th>
<th>Conventional SPN</th>
<th>p value</th>
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<tbody>
<tr>
<td>Number of cases</td>
<td>4</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Female: male ratio</td>
<td>1:1</td>
<td>4:25</td>
<td>0.06</td>
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<tr>
<td>Mean age ± SD (years)</td>
<td>51.3 ± 10.5</td>
<td>32.1 ± 14.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Head</td>
<td>3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Body</td>
<td>0</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Tail</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Mean tumor size ± SD (cm)</td>
<td>5.0 ± 3.8</td>
<td>6.7 ± 4.2</td>
<td>0.41</td>
</tr>
<tr>
<td>Liver metastasis or other organ involvement at the time of presentation</td>
<td>0/4</td>
<td>5/58</td>
<td>1.00</td>
</tr>
<tr>
<td>Recurrence</td>
<td>0/3*</td>
<td>10/57*</td>
<td>0.42</td>
</tr>
</tbody>
</table>

*Number of patients whose follow-up information is available.
The Expression of PTEN Is Associated With Improved Prognosis in Patients With Ampullary Adenocarcinoma After Pancreatectoduodenectomy

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• **Context.**—Phosphatase and tensin homolog (PTEN) is one of the most frequently inactivated tumor suppressor genes in sporadic cancers. Somatic mutations of **PTEN** occur in many tumors including those of the gastrointestinal and hepatobiliary tracts. Loss of **PTEN** expression is associated with poor prognosis in patients with metastatic colonic adenocarcinoma, gastroesophageal junction adenocarcinoma, gastric adenocarcinoma, and pancreatic ductal adenocarcinoma.

**Objective.**—To study the expression of PTEN and its significance in ampullary adenocarcinoma (AA).

**Design.**—We constructed tissue microarrays by using archival tissue from 92 patients (55 males, 37 females; median age, 63 years; age range, 37 to 87 years) with previously untreated AA who underwent pancreatectoduodenectomy at our institution. **PTEN** expression was evaluated by immunohistochemistry, scored semiquantitatively (based on staining intensity and percentage positive tumor cells), and correlated with clinicopathologic features and survival.

**Results.**—Of 92 cases, 23 (25.0%) were **PTEN** negative. Loss of **PTEN** expression correlated with lymph node metastasis (**P** = .004), advanced American Joint Committee on Cancer (AJCC) stage (**P** = .02), and higher frequency of recurrence (**P** = .03). Patients with **PTEN**-negative tumors had shorter disease-free survival (DFS, median: 26 months) and overall survival (OS, median: 92 months) than those with **PTEN**-positive tumors (DFS, mean: 161.4 ± 11.7 months, **P** = .001; OS, mean: 175.4 ± 11.0 months, **P** = .001). In multivariate analyses, **PTEN** expression was a prognostic factor for both DFS and OS, independent of AJCC stage, lymph node status, pathologic tumor (pT) stage, and differentiation.

**Conclusions.**—Loss of **PTEN** expression is associated with poor DFS and OS in patients with AA after curative surgery. **PTEN** expression may be used as a prognostic marker for patients with resected AA.

phosphatidylinositol-3,4,5 triphosphate (PI(3,4,5)P3), the product of phosphatidylinositol 3-kinase (PI3K) function, to form phosphatidylinositol-3,4 bisphosphate. The dephosphorylation of PI(3,4,5)P3 antagonizes the PI3K pathway, thereby abolishing the PI3P-mediated activation of survival kinases, such as phosphoinositide-dependent kinase 1, and the AKT/mammalian target of rapamycin (mTOR) pathway.9,10 PTEN is the only known lipid phosphatase abrogating the PI3K pathway, and therefore, loss of PTEN has a significant impact on multiple aspects of tumorigenesis.10

Loss of PTEN expression has been shown to be associated with poor prognosis in patients with malignancies from gastrointestinal and hepatobiliary tracts.11–16 In patients with metastatic colon adenocarcinoma and wild-type K-ras who received cetuximab-based treatment, loss of PTEN expression by immunohistochemistry has been identified in 20% of these patients and is an independent prognostic factor for poor OS by multivariate analysis.13 Similarly, loss of PTEN expression has also been shown to be associated with poor disease-free survival (DFS) and OS in patients with advanced gastroesophageal junction cancer who received cetuximab with irinotecan, and 5-fluorouracil as first-line treatment. Loss of heterozygosity of PTEN was reported in 17% of patients with gastric cancer and correlated with patient survival.18 Other studies19,20 have shown that loss of nuclear staining for PTEN is associated with poor survival in colon cancer. On other hand, Lee et al14 showed that overexpression of PTEN is an independent prognostic factor associated with better patient survival in patients with intrahepatic cholangiocarcinoma. In AAs, allelic imbalance of the PTEN tumor suppressor gene has been reported to be present in 15% of cases.17 However, the expression and the role of PTEN in AA have not been studied in detail. In this study, we examined 92 previously untreated cases of AA for loss of PTEN expression by immunohistochemistry. Using univariate and multivariate analyses, we determined whether the loss of expression of PTEN was associated with OS and DFS and other clinicopathologic features in patients with AA. Our data showed that loss of PTEN expression was associated with poor prognosis in patients with AAs. Targeting the PI3K/AKT pathway may potentially be an effective treatment option for patients with ampullary adenocarcinoma.

MATERIALS AND METHODS

Case Selection

We retrospectively reviewed medical records and tissue specimens of 92 patients with AA who underwent pancreatoduodenectomy at the University of Texas M. D. Anderson Cancer Center (Houston, Texas) between 1995 and 2009. Patients who received preoperative neoadjuvant chemotheraphy and/or radiation were not included. There were 55 males and 37 females with mean patient age at time of surgery of 63 years (range, 37–87 years). This study was approved by the institutional review board of the University of Texas M. D. Anderson Cancer Center.

Tissue Microarray Construction

To construct the tissue microarray used in this study, formalin-fixed, paraffin-embedded archival tissue blocks and their matching hematoxylin-eosin–stained slides were retrieved, reviewed, and screened for representative tumor regions by 2 pathologists (Huamin W. and D. C.). For each patient, 2 cores of tumor were sampled from representative areas by using a 1.0-mm punch. The tissue microarray was constructed with a tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin) as described previously.18

Immunohistochemical Analysis for PTEN

Immunohistochemical staining for PTEN was performed on 4-μm unstained sections from the tissue microarray blocks by using a mouse monoclonal antibody against PTEN (6H2.1, Dako, Carpinteria, California). After deparaffinization, antigen retrieval was performed on the tissue sections at 100°C in a steamer containing Tris-EDTA buffer (pH 9.0) for 20 minutes. The sections were then immersed in anti-PTEN antibody (1:100 dilution) at 35°C for 15 minutes. Subsequently, they were immersed in 3.0% hydrogen peroxide at 35°C for 5 minutes to block the endogenous peroxidase activity. A primary enhancer solution was then applied to the slides, which were incubated at 35°C for 8 minutes. The sections were then incubated with secondary anti-mouse immunoglobulin at 35°C for 5 minutes. Diaminobenzidine was used as a chromogen and diaminobenzidine enhancer was applied, and hematoxylin was used for counterstaining.

Measurement of PTEN Expression Levels

The immunohistochemically stained slides of AA tissue microarrays were examined by using standard light microscopy (Olympus BX41, Olympus America Inc., Melville, New York). The staining results were independently scored semiquantitatively by staining intensity (0, negative; 1, weak; and 2, moderate to strong) and percentage of positive tumor cells by 2 pathologists (S. S. and Huamin W.), who were blinded to the clinicopathologic data. The background stromal or nonneoplastic epithelial cells were used as an internal positive control. The PTEN expression score was calculated as the product of the staining intensity and the percentage of positive tumor cells. PTEN expression was categorized as loss of PTEN (no positive staining for PTEN, PTEN expression score = 0) and as PTEN positive. To further examine the effect of PTEN expression levels on survival in patients with PTEN-positive AA, we further classified the PTEN-positive cases into PTEN-low (PTEN expression score of 1–100) and PTEN-high (PTEN expression score >100) by using the median score for PTEN expression as the cutoff.

Patient Follow-up and Statistical Analysis

The patients’ follow-up information through May 2011 was extracted from the medical records and, if necessary, updated by review of the US Social Security Death Index. The recurrence information was updated when a patient came to the clinic for a follow-up visit. χ² analysis or Fisher exact tests were used to compare categorical data. The survival curves were constructed by using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences. Disease-free survival and OS were calculated as previously described.18 The prognostic significance of clinicopathologic characteristics were determined by using univariate and multivariate Cox regression analysis. Two multivariate models for survival analyses were used to determine the prognostic significance of loss of PTEN expression for DFS and OS. In the first model, pathologic tumor (pT) stage and lymph node status (pN) were included as 2 independent variables. In the second model, pT and pN were combined as 1 variable (American Joint Committee on Cancer [AJCC] stage). All statistical analyses were performed with Statistical Package for Social Sciences software (for Windows 12.0, SPSS Inc, Chicago, Illinois). We used a 2-sided significance level of .05 for all statistical analyses.

RESULTS

PTEN Expression in Ampullary Adenocarcinoma

Immunohistochemical staining for PTEN showed predominantly cytoplasmic staining with scattered nuclear staining in benign stromal cells, benign amillary/duodenal mucosa (Figure 1, A through F), and AAs that were positive
Figure 1. Representative micrographs show phosphatase and tensin homolog (PTEN) expression in ampullary adenocarcinoma. A and B, A moderately differentiated ampullary adenocarcinoma with complete loss of PTEN expression (negative staining for PTEN). C and D, Weak cytoplasmic and nuclear staining for PTEN in a moderately differentiated ampullary adenocarcinoma (PTEN-low). E and F, A moderately differentiated ampullary adenocarcinoma with strong positive staining for PTEN (PTEN-high). The intervening tumor stromal cells serve as internal positive control (original magnifications, ×40 [A, C, and E] and ×200 [B, D, and F]).
Table 1. Clinicopathologic Characteristics of PTEN Expression

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of Patients</th>
<th>Loss of PTEN</th>
<th>PTEN Positive</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>55</td>
<td>13</td>
<td>42</td>
<td>.81</td>
</tr>
<tr>
<td>Female</td>
<td>37</td>
<td>10</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Mean age, y (range)</td>
<td>92</td>
<td>63.0 (37.0–76.0)</td>
<td>63.1 (56.0–87.0)</td>
<td>.96</td>
</tr>
<tr>
<td>Histology pattern</td>
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<tr>
<td>Intestinal</td>
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<td>5</td>
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<td>.60</td>
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<td>Pancreatobiliary</td>
<td>32</td>
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</tr>
<tr>
<td>Mixed</td>
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<td>8</td>
<td>22</td>
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<tr>
<td>Presence of adenoma</td>
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<tr>
<td>No</td>
<td>52</td>
<td>15</td>
<td>37</td>
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<tr>
<td>Yes</td>
<td>40</td>
<td>8</td>
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<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Well to moderate</td>
<td>59</td>
<td>14</td>
<td>45</td>
<td>.80</td>
</tr>
<tr>
<td>Poor</td>
<td>33</td>
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<td>CDX2 expression</td>
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<tr>
<td>Negative</td>
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<td>17</td>
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<td>Lymph node positivity</td>
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<td>4</td>
<td>37</td>
<td>.004</td>
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<td>&gt;3 positive nodes (1)</td>
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<td>AJCC pathologic stage</td>
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<td>.03</td>
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<tr>
<td>Yes</td>
<td>31</td>
<td>12</td>
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</tbody>
</table>

Abbreviations: AJCC, American Joint Committee on Cancer; PTEN, phosphatase and tensin homolog.

for PTEN. Among the 92 cases, 69 (75%) were PTEN positive and 23 (25%) had complete loss of PTEN expression (PTEN negative). The lowest PTEN expression score in the PTEN-positive AAs was 30. Fifty-seven AA samples (62%) showed low levels of PTEN expression (PTEN-low) and 12 (13%) were PTEN-high.

Correlation of Loss of PTEN Expression With Clinicopathologic Features and Other Molecular Markers

The clinicopathologic characteristics of the study population are correlated with the loss of PTEN expression (Table 1). The patients whose tumor had loss of PTEN expression had more frequent lymph node metastasis, higher tumor stage, and developed subsequent recurrence or metastasis as compared to patients with PTEN-positive tumors. Lymph node metastasis was present in 19 of 23 AAs (83%) with loss of PTEN expression, compared to 32 of 69 AAs (46%) that were PTEN positive (P < .001). At the time of surgical resection, the tumor was stage I, II, and III in 2 of 23 (9%), 16 of 23 (70%), and 5 of 23 (22%) patients whose tumor had loss of PTEN expression, respectively, as compared to 28 of 69 (41%), 32 of 69 (46%), and 9 of 69 (13%) patients whose tumors were PTEN positive, respectively (P = .02). No patient had stage IV disease. During follow-up, 52% of patients whose tumor had loss of PTEN expression had recurrence/metastasis as compared to 27% for patients whose tumors were PTEN positive (P = .03). There were no correlations between loss of PTEN expression and the histologic subtype, tumor differentiation, presence of adenomatous component, resection margin status, pT stages, adjuvant therapy status, or expression of cytokeratin (CK) 7, CK20, and CDX2.

Survival Analysis

After pancreaticoduodenectomy, the median follow-up was 48 months, ranging from 8 to 222 months. No patients were lost to follow-up. Loss of PTEN expression for patients with AA correlated with poor DFS and OS. Patients whose tumor had loss of PTEN expression had a shorter mean DFS (89.0 ± 20.8 months) than those whose tumors were PTEN positive (mean DFS: 164.1 ± 11.7 months, P = .01; Figure 2A). The mean OS was 93.1 ± 19.1 months for patients whose tumor had loss of PTEN expression, compared to 175.4 ± 11.0 months for patients whose tumor was PTEN positive (P = .001, Figure 2B). To examine the effect of PTEN expression levels on survival, we further classified the cases that were PTEN positive into PTEN-low and PTEN-high. We found that patients with PTEN-low tumors had a better OS (mean survival: 164.5 ± 12.8 months) than the patients whose tumors had loss of PTEN expression (mean survival: 93.1 ± 19.1 months, P = .007). Patients with PTEN-low tumors had a shorter DFS and OS than those with PTEN-high tumors (Figure 3, A and B); however, the difference in either DFS or OS between these 2 groups was not statistically significant.

In univariate analysis, both DFS and OS correlated with pT stage, lymph node status, AJCC stage, and loss of PTEN expression. However, no significant correlation of either DFS or OS with CDX2 expression, adjuvant therapy, or other clinicopathologic parameters was observed (Table 2). Two multivariate models for survival analyses were used to determine the prognostic significance of loss of PTEN expression for DFS and OS, and the results are shown in Table 3. In the first model, lymph node status was an independent prognostic factor for DFS (P = .002) and pT stage was an independent prognostic factor for OS (P = .03). In the second model, AJCC (pathology) stage was an independent prognostic factor for both DFS (P = .003) and OS (P = .046). The loss of PTEN expression was a prognostic factor for OS in both models, independent of tumor differentiation, pT stage, lymph node status, and AJCC stage (P < .05, Table 3). However, the correlation between the loss of PTEN expression and DFS was not statistically significant in either model (P > .05, Table 3).

COMMENT

In this study, we examined the protein expression of PTEN tumor suppressor gene by immunohistochemistry in 92 previously untreated AAs. We found loss of PTEN
expression in 25% of AAAs. Loss of PTEN was associated with lymph node metastasis, AJCC stage, and recurrence in our study population. In addition, we showed that loss of PTEN expression in AAAs correlated with poor DFS and OS and was an independent prognostic factor for OS in multivariate analysis. Our data suggest that loss of PTEN expression plays a role in AAAs and may be used as a prognostic factor in patients with resected AA.

PTEN is an important tumor suppressor gene involved in many types of human malignancies. Loss of function of PTEN leads to constitutive activation of the PI3K/AKT pathway, which, in turn, activates various downstream target genes and results in cellular growth, proliferation, and survival. In this study, we found that PTEN expression was lost in one-fourth of the resected AA samples. However, the mechanism(s) of the loss of PTEN expression in AAAs is (are) unclear. Multiple possible mechanisms may be involved in regulating the expression and the functions of PTEN, which include mutations of PTEN, heterozygous and homozygous deletions, transcriptional silencing by promoter methylation, microRNA targeting, posttranslational regulation, and stability of the PTEN protein.6,20–23 Transcriptional silencing by PTEN promoter methylation has been reported in endometrial, thyroid, gastric, lung, breast, and ovarian carcinomas, as well as glioblastomas.24–30

Figure 2. Kaplan-Meier curves for disease-free survival (A) and overall survival (B) by loss of phosphatase and tensin homolog (PTEN) expression in patients with ampullary adenocarcinoma after curative surgery. Patients whose tumors had loss of PTEN expression had shorter disease-free survival and overall survival than patients whose tumors retained PTEN expression.

Figure 3. Kaplan-Meier curves for disease-free survival (A) and overall survival (B) by phosphatase and tensin homolog (PTEN) expression levels in patients with ampullary adenocarcinoma after curative surgery.
### Table 2. Univariate Analysis of Survival

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of Patients</th>
<th>Disease-Free Survival HR (95% CI)</th>
<th>P Value</th>
<th>Overall HR (95% CI)</th>
<th>Survival P Value</th>
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Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio; PTEN, phosphatase and tensin homolog.

### Table 3. Multivariate Analysis of Survival

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<th>Characteristics</th>
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<th>P Value</th>
<th>Overall Survival HR (95% CI)</th>
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Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence interval; HR, hazard ratio; PTEN, phosphatase and tensin homolog.
microRNA targeting has also been reported in gliomas and prostate and lung carcinomas.\textsuperscript{23,31,32} Given the previous report that allelic imbalance of PTEN tumor suppressor gene is present in 13% of AA cases,\textsuperscript{37} it is possible that heterozygous and homozygous deletions may be involved in the loss of PTEN expression in ampullary carcinoma. Further studies are needed to elucidate the mechanism of loss of PTEN expression in these patients.

Loss of PTEN has been shown to be a poor prognostic factor in several cancers, including carcinomas of the gastrointestinal and hepatobiliary tracts.\textsuperscript{11–16,33–36} Intact PTEN expression has been shown to be associated with lower stage and negative lymph nodal status in endometrial carcinomas.\textsuperscript{37} Consistent with previous reports for other cancers, we found that loss of PTEN expression in our study population was associated with lymph node metastasis and higher AJCC stage at the time of surgery and was associated with higher frequency of recurrence and poor DFS and OS after curative surgery. Similar to our results, loss of PTEN expression has been reported in 25.6% of pancreatic ductal adenocarcinomas, and it was associated with tumor recurrence/metastasis and poor prognosis in a study of 135 cases of surgically resected stage II pancreatic ductal adenocarcinoma by Foo et al.\textsuperscript{38} It is interesting to note that patients whose tumors were PTEN-low had a DFS and OS intermediate to the DFS and OS of patients in this study whose tumors were PTEN-high and of those whose tumor had loss of PTEN expression; although the difference did not reach statistical significance. It is therefore possible that PTEN may affect the prognosis in patients with AA in a dose-dependent manner. In contrast to the previous studies that showed that loss of nuclear staining for PTEN is associated with poor survival in colon cancer,\textsuperscript{11,12} we did not observe significant correlation between nuclear PTEN expression and either survival or other clinicopathologic parameters (data not shown). This may reflect the difference in the antibodies used for immunohistochemistry in these studies or the difference in molecular alterations between cases of surgically resected stage II pancreatic ductal adenocarcinoma and AAs.

In addition to loss of PTEN expression as an independent prognostic factor for DFS and OS, our data also showed that advanced tumor (pT) stage, lymph node metastasis, and AJCC stage were correlated with both DFS and OS by AJCC stage were correlated with both DFS and OS. Our findings are corroborated by several previous studies that demonstrated prognostic significance of pathologic stage in patients with AA after curative surgery.\textsuperscript{39–41} Consistent with these previous studies,\textsuperscript{39–41} patients with AAs of intestinal subtype also had better prognosis than those with pancreaticobiliary subtype in our patient population (data not shown). However, we did not observe significant correlation of either DFS or OS with commonly used intestinal markers such as CK20 or CDX2.

In summary, our study showed that loss of PTEN expression is an independent prognostic factor for poor disease-free and overall survival in patients with ampullary adenocarcinoma after curative surgery. Immunohistochemical analysis of PTEN expression can be useful as a prognostic factor and have therapeutic implications in this patient population. Targeting the PI3K/AKT pathway via PI3K inhibitors and mTOR inhibitors may prove to be an effective treatment for patients with ampullary adenocarcinoma.
Original Article

Parametric transfer function analysis and modeling of blood flow autoregulation in the optic nerve head

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1Discoveries in Sight Research Laboratories, Devers Eye Institute, Legacy Research Institute, Portland, Oregon;
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Received December 10, 2013; Accepted December 25, 2014; Epub March 13, 2014; Published March 30, 2014

Abstract: The aim of the study was to establish a parametric transfer function to describe the relationship between ocular perfusion pressure (OPP) and blood flow (BF) in the optic nerve head (ONH). A third-order parametric theoretical model was proposed to describe the ONH OPP-BF relationship within the lower OPP range of the autoregulation curve (< 80 mmHg) based on experimentally induced BF response to a rapid intraocular pressure (IOP) increase in 6 rhesus monkeys. The theoretical and actual data fitted well and suggest that this parametric third-order transfer function can effectively describe both the linear and nonlinear feature in dynamic and static autoregulation in the ONH within the OPP range studied. It shows that the BF autoregulation fully functions when the OPP was > 40 mmHg and becomes incomplete when the OPP was < 40 mmHg. This model may be used to help investigating the features of autoregulation in the ONH under different experimental conditions.

Keywords: Optic nerve head, autoregulation, intraocular pressure, transfer function

Introduction

Blood flow (BF) autoregulation (AR) is an intrinsic ability of the retina and optic nerve head (ONH), among many other bodily tissues, to maintain a relatively constant level of blood in response to variations in perfusion pressure and metabolic demand [1-3]. This process involves multiple mechanisms that may fail as a part of pathogenic processes e.g. glaucoma [2, 4-7]. Thus, assessment of autoregulation capacity in ocular tissues is important to understand the mechanisms of the disease and to establish potential therapeutic targets.

The classic method to assess autoregulation capacity within ocular tissue is to compare BF changes before and after ocular perfusion pressure (OPP) is altered, by manipulation of either arterial blood pressure (BP) or intraocular pressure (IOP) [6, 8-11]. After this induced OPP change, autoregulation proceeds chronologically in two phases: dynamic (dAR) and static (sAR). During the dAR phase, the pressure change evokes a rapid BF response (increase or decrease), which lasts on average 10-20 sec. When the vascular resistance and BF stabilizes to either its original or a new level, the sAR phase is then reached [12]. A series of BF changes measured during the sAR phase in response to varying levels of perfusion pressure constitute a classic AR curve, or P-F relationship [1] as demonstrated in Figure 1.

Whilst sAR analysis utilizes the BF change in amplitude before and after the OPP change, analysis of the dAR phase includes both amplitude and time latencies [13]. Thus, dAR analysis has been considered as a more effective clinical method at revealing potential autoregulation dysfunction, as demonstrated in ischemic and traumatic brain injuries [14-18]. Yet, previous studies relating to ocular autoregulation have focused on sAR, and overlooked the dAR component.

The inherent complexities of BF AR encourage the use of mathematical modeling. Utilizing the mechanic features of autoregulation, discrepancies between theoretical systems behaviors and actual behaviors measured in vivo can point to hitherto unknown components that are missing, thereby assisting in developing a more comprehensive picture of this biological pro-
Mathematic modeling of blood flow autoregulation

cess. However, there are limited BF AR mathematical models [19, 20] and few studies specifically focusing on the eye [3, 21]. One of the difficulties applying a mathematic model to describe autoregulation capacity is the nonlinearity of the relationship between BF response and OPP [22].

Previously, first-order linear time-invariant transfer functions have been used for analysis of dAR. However, most of these first-order linear models are focused specifically upon the plateau region of the autoregulation curve [22, 23]. Consequently, grading the autoregulatory response is limited to either, the lower or upper critical points of the autoregulation curve or the shifts of the curve by altering the physiological parameters.

Second-order transfer functions, designed within the scenario of spontaneous BP fluctuations have been used to represent the properties of BF autoregulation in which the perfusion pressure changes do not extend to the nonlinear range of the autoregulation curve, though it is adequate to describe the descending phase and the steady state of the BF response, the model is too rigid to depict the gradually ascending property during recovery so that it describes only the linear property of autoregulation but not the full course of autoregulation from dAR phase to sAR.

As such, a higher order of differential model may be more appropriate to represent the autoregulation pattern since it is capable of providing more flexibility to describe both the linear and nonlinear features of autoregulation during the BF response. In our previous study [24], it has shown that the magnitude of both maximal dAR change and the steady state BF are closely related to the IOP and BP and for both dAR and sAR, the OPP is a determining factor to mediate the interaction between sAR and dAR. To incorporate sAR and dAR into a single model, BF response to variations of different OPP need to be assessed.

To accomplish the previously stated goals a theoretical third-order parametric model was established to describe the OPP-BF relationship incorporating both dAR and sAR. This model was then validated utilizing in vivo data, where the ONH dAR and sAR responses were induced by rapidly increasing the IOP. The resultant parametric transfer function may serve as a tool to predict and gauge the impairment of autoregulation in disease conditions and may have a potential application to assess the autoregulation capacity in humans.

Materials and methods

Quantification of rapid IOP increase induced BF autoregulation in ONH

Six male rhesus (Macaca mulatta) monkeys without observable eye diseases, ranging from 9 to 12 (10.7 ± 1.4, mean ± SD) years old and weighing 5.6 to 13 kg (10.1 ± 3.4 kg), were used. All experimental protocols and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Legacy Research Institutional Animal Care and Use Committee.

The animal was sedated initially by an intramuscular injection of ketamine/xylazine (15 mg.kg⁻¹ and 0.8 mg.kg⁻¹); anesthesia was maintained thereafter by continuous administration of pentobarbital (6-9 mg.kg⁻¹.hour⁻¹, IV) using an infusion pump (Aladdin, World Science Instruments Inc., Sarasota, FL). Pentobarbital was selected because this anesthetic, unlike the volatile gas anesthetics [18, 25], has minimal impact on autoregulation capacity [26-28]. Animals were intubated and breathed with room air.

The animal was placed on a table in prone position. Body temperature was maintained with a 37°C circulating warm-water heating pad. Pulse rate and oxygen saturation were monitored continuously (Propaq Encore model 206EL; Protocol Systems, Inc., Beaverton, OR) and maintained between 85-125 per minute and O₂ above 95%, respectively. One of the superficial arteries in a leg was cannulated with a 27G needle, which was connected to a pressure transducer (BLPR2, WPI, NH) and a four-channel amplifier system (Lab-Trax-4/24T, WPI, NH). BP in the artery was recorded continuously and stored into a computer.

A laser speckle flowgraphy (LSFG. Softcare, Japan) device was used to estimate the BF in the ONH. In brief, a fundus camera equipped within the LSFG device was focused on an area centered on the ONH. The area is approximate-
output power, 1.2 mW), a speckle pattern is generated due to random interference of the scattered light from the illuminated tissue area. The speckle pattern is continuously imaged by a charge coupled device (700 x 480 pixels) at a frequency of 30 frames per second for 4 seconds at a time. Offline analysis software (LSFG Analysis, Softcare, Iizuka, Japan) computed a mean blur rate (MBR) of the speckle images. MBR is a squared ratio of mean intensity to the standard deviation of light intensity of the image, which varies temporally and spatially according to the velocity of blood cells movement and correlates well with capillary BF within the ONH validated by the microsphere [29] and the hydrogen clearance methods [30]. Thus, the MBR has been used as a BF index. A composite MBR map representing BF distribution within the ONH disc was generated from the images of each 4-second series. After eliminating the area corresponding to large blood vessels within the images, capillary BF within the remaining ONH disc area was averaged and reported in arbitrary units (A.U.) of MBR.

Manometrical IOP control and recording

Two 27G needles were inserted into the anterior chamber of the eye via the temporal corneoscleral limbus. One of the two needles was connected to a pressure transducer to register the IOP; the other needle was connected to either one of two sterile saline reservoirs, each set at a different height. The connection of the reservoirs to the anterior chamber was controlled by a solenoid valve (Valcor Engineering, Springfield, NJ), which allows one of the reservoirs to be opened and the other closed so that the IOP can be changed from one level to the other nearly instantaneously. A computer mouse synchronized the valve control with the BF measurement program. The OPP was computed using the equation OPP = BP - IOP - 5 (mmHg), where 5 mmHg is the height difference from eye to heart when the animal was in a prone position.

IOP step increase induced sAR and dAR responses

Under a range of BP between 80 and 95 mmHg and IOP set manometrically at 10 mmHg, baseline ONH BF images was acquired by the LSFG. Ten seconds after the completion of baseline imaging, the electronic valve connected to a saline reservoir at a height equivalent to either 30 or 40 or 50 mmHg (IOP_130, IOP_140, IOP_150 respectively) was switched open by the synchronized computer mouse to induce a rapid IOP change. The BF recording continued for 60 seconds and, thereafter, for 10 seconds every minute for a total of 5 minutes. The above tests were repeated three times in both eyes of the 6 animals (12 eyes) to obtain the empirical BF response at varied OPPs. The percentage difference of BF between the baseline value and that at the end of 5 min of IOP increase was calculated as sAR. All the sAR measured over different OPPs was used for the construction of the autoregulation curve and the subsequent mathematical modeling.

Mathematical modeling of autoregulation system

As the schematically illustrated OPP-BF relationship, or sAR autoregulation curve shown in
Mathematic modeling of blood flow autoregulation

Figure 1. a plateau covers a range of OPP where the autoregulation remains effective. When the OPP exceeds the critical range defined by this plateau, i.e. beyond the upper and below lower limits of the AR range, BF will passively become either greater or lower following the OPP changes.

Because BF autoregulation is not a simple response to a mechanical stimulus, but also to different chemical and biological stimuli through a variety of scales and pathways, thus complicates efforts to represent it. In order to simplify the mathematical analysis of the system, all procedures could be described as an input-output model (black-box) between OPP and BF (Figure 2). Subsequently, a linear or nonlinear transfer function, in terms of spatial or temporal frequency, can be derived to depict the relationship between OPP and BF. The transfer function then can be estimated using a system identification method or curve fitting from a series of experimental measurements.

The transfer function in Figure 2 can be expressed as a quotient of polynomials

\[ G(s) = \frac{p(s)}{q(s)} = \frac{b_m s^m + \ldots + b_1 s + b_0}{a_n s^n + \ldots + a_1 s + a_0}, n \geq m \]  

where \( s \) is the Laplace operator; \( n \geq m \) according to the initial value theorem and final value theorem [31].

Considering the model is an approximation of a physiology procedure, the degree of activation of the vascular smooth muscle arising through
Mathematical modeling of blood flow autoregulation

BF autoregulation is clearly a nonlinear phenomenon, thus, a linear approximation of a nonlinear model can be justified in a small range of OPP around a point of equilibrium by utilizing the segmental linear property of the autoregulation curve without altering the features of the curve. Therefore, a piecewise linear transfer function, i.e., a parametric transfer function, was used to describe the static and dynamic properties of autoregulation under different OPP. Since BF is a nonlinear function of OPP, the OPP level can be used as a parametric input of the piecewise transfer function, that is, each parameter in Equation 4 could be described as a function of the OPP level. Defining \( x \) as an independent variable representing the OPP level, it is possible to find the functions \( K_P = f_1(x) \), \( T_P = f_2(x) \), \( T_w = f_3(x) \), and \( \zeta = f_4(x) \), from experimental data to complete the parametric transfer function over the full range of OPP. All values of \( K_P \) at a full range of OPP, is composed of the static autoregulation curve from the final value theory of linear system step response. In the scenario of this study, the OPP was limited to the lower half of the autoregulation curve (OPP \(< 80 \) mmHg) since BP was from 80 mmHg to 100 mmHg in all experiments.

Results

Quantification of rapid IOP increase induced BF autoregulation in ONH

Static BF autoregulation curve of ONH: Due to varied BP in animals during each test, the IOP increase from 10 mmHg to either 30, 40 or 50 mmHg resulted in a range of OPP decrease between 15 and 67 mmHg. The percentage BF change after IOP elevation normalized to their baseline values are plotted against each corresponding OPP to construct portion of a complete autoregulation curve as shown in Figure 5.

The BF and OPP were fitted to a polynomial function to describe the static relationship between OPP \((x)\) and BF \((y_{BF})\) at the lower half \((\text{OPP} < 70 \text{ mmHg})\) of the static autoregulation curve:

\[
y_{BF} = 7.6 \times 10^{-6}x^3 - 1.1 \times 10^{-3}x^2 + 5.6 \times 10^{2}x
\]

\(P<0.0001, r=0.8\)  

The shape of the fitted curve resembles the theoretical autoregulation curve in the lower half of OPP shown in Figure 1. In the curve, BF maintained at a constant level when the OPP

---

**Figure 5.** The sAR measured at a range of OPP. Each data point (averaged from three repeated measures) represents the sAR at a given level of OPP. The data is best fit with a polynomial function. Since the highest OPP in these anesthetized animals can only be reached to a certain level below 70 mmHg, the curve represents only a portion of the autoregulation curve within the lower OPP range.

the myogenic mechanism is given by a first order transfer function \([32, 33]\):

\[
G_1(s) = \frac{k_1}{T_s + 1}
\]

where \( k_1 \) is the regulation gain and \( T_s \) the time constant.

Based on published models on transfer function analysis, the degree of activation arising through the metabolic and other mechanisms was hypothesized to fit by a second order transfer function of the form \([34, 35]\):

\[
G_2(s) = \frac{k_2}{1 + 2\zeta T_s s + T_s^2 s^2}
\]

where \( k_2 \) is regulation gain, \( T_s \) is a time constant, and \( \zeta \) is the damping ratio.

Combining the two procedures, the autoregulation procedure of BF could be expressed by a third-order transfer function of the form:

\[
\frac{BF}{OPP} = G(s) = \frac{K_P}{1 + 2\zeta T_s s + T_s^2 s^2} / (1 + T_s s)
\]

where \( K_P = k_1 k_2 \).

To verify the validity of the third order model, we analyzed the experimental data from six monkeys. This third order system conformed to the model prepared from in vivo measurements as shown in Figure 3. When applying the same input to the theoretical model with parameters \( K_P = 0.03, T_s = 14.46, T_w = 2.54, \zeta = 1.20 \) in Equation 4, the output of the transfer function model (simulated BF) correlates with the in vivo experimental output (measured BF) as illustrated in Figure 4.
was higher than 40 mmHg, reflecting BF recovering to baseline completely and a fully functional autoregulation. When the OPP was below 40 mmHg, BF decreased with the decrease of OPP, indicating an incomplete recovery.

Parameters of transfer function

Figure 6 shows the four parameters ($K_p$, $T_p$, $T_w$ and $\zeta$) in Equation 4 estimated from the experiments measured at different OPP levels. The data points of each parameter in Figure 6 were derived from the measured BF curves at a different range of OPP (see Figure 4). Each parameter was fitted to a polynomial function of the OPP ($x$) at steady state:

$$K_p = 1.31 \times 10^{-5} x^2 - 1.5 \times 10^{-3} x + 6.15 \times 10^{-2}$$

($P<0.0001$, $r=0.94$) (6)

$$T_p = 9.9 \times 10^{-3} x^2 - 0.347 x + 12.50$$

($P<0.0001$, $r=0.85$) (7)

$$T_w = 6.9 \times 10^{-4} x^2 - 0.0852 x + 4.84$$

($P<0.0001$, $r=0.69$) (8)

$$\zeta = 2.41 \times 10^{-2} x^2 - 0.047 x + 2.69$$

($P<0.0001$, $r=0.92$) (9)

In Figure 6A, the gain of the transfer function ($K_p$) has a nonlinear relationship with OPP, which decreases when OPP increases and reaches a constant when OPP is above a certain value. Panel B and Panel C illustrate the relationship of the two time constants ($T_p$ and $T_w$) with the OPP. $T_p$ increases monotonically with OPP and approaches to a constant of 10 sec when OPP becomes lower than 25 mmHg. $T_p$ is a monotonically descending function of OPP and converges to a value of 2.5 seconds when OPP is above 50 mmHg. $T_p$ is relative stable and varies in a range of 1.7-3.8 seconds. The damping ratio $\zeta$ is a quasi-linear function of

<table>
<thead>
<tr>
<th>IOP</th>
<th>BF deviation at 45 sec</th>
<th>BF deviation at 55 sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-30 mmHg</td>
<td>2 ± 3%</td>
<td>2 ± 3%</td>
</tr>
<tr>
<td>10-40 mmHg</td>
<td>3 ± 1%</td>
<td>4 ± 1%</td>
</tr>
</tbody>
</table>

IOP: intraocular pressure; BF: blood flow.

Table 1. Percentage change of BF at 45 sec and 55 sec compared to 5 min under IOP$^{1030}$ and IOP$^{1040}$

$K_p$, $T_p$, $T_w$, and $\zeta$ from experimental data. Each parameter is a nonlinear function of OPP to gain (A), the time constants $T_p$ (B) and $T_w$ (C), and a damping ratio $\zeta$ (D).
OPP and monotonically decreasing with OPP; the lower the OPP, the lower the damping ratio.

**Dynamic autoregulation time duration**

In order to find out the dynamic duration of the BF autoregulation, BF measured at 45 seconds and 55 seconds were compared with averaged BF measured from 4-5 minutes, as shown in Table 1. Since BF stabilizes at approximately a constant after 5 minutes, averaged BF measured from 4-5 minute can be readily assumed as final value. At 45 seconds, the BF reached a state which was less than 5% variation from the steady state (2 ± 3% and 4 ± 1% for IOP$_{1030}$ and IOP$_{1040}$, respectively). If a percentage variation of ± 5% from the final value was considered a new steady state, it is reasonable to conclude that the dynamic autoregulation happened in duration of about 1 min; thus, a continuous time course measurement of BF of 1 min provides enough spatial resolution for dynamic analysis.

The transient changes of OPP induced by different IOP elevations, the experimentally predicted BF changes are presented in Figure 7. The OPP was considered as the input of transfer function and the parameters of the transfer functions were calculated from Equations 6 to 9 using the steady state OPP of each mean. The simulated BF followed the mean in vivo BF, suggesting that the transient BF change under different OPPs can be modeled by a parametric transfer function even though autoregulation is nonlinear to OPP.

**Discussion**

In this study, a mathematical model was developed that utilized a parametric transfer function to analyze the relationship between BF and OPP following acute IOP elevations. This transfer function consists of 4 parameters: gain $K_p$, time constant $T_p$, time constant $T_q$ and a damping ratio, $\zeta$. The real parts of all poles of the denominator within Equation 4 are negative, which requires that $\zeta > 0$. This criterion is satisfied as long as OPP < 80 mmHg, as shown in Figure 6D.

The gain $K_p$ is a monotonically decreasing function of the OPP and is directly related to the recovery rate of the BF in the steady state. After the transient phase of the BF response following IOP elevation, $K_p$ is the only functional parameter in this model; and it resembles the capability of static autoregulation. The higher the value of $K_p$, the less the BF recovers to the baseline.

The first-order model represents the response time of the vascular muscle after acute OPP drop [33, 36]. $T_p$ is the only parameter in this...
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The challenge of modeling a physiological system mathematically is to balance its predictive capability and its simplicity to enable interpretation of the physiology dynamics. There must also be a balance between the scope of questions posed and comprehensiveness of the model. The first limitation of the model is that the parameters of this model were limited to an OPP less than 80 mmHg since the regression of all curves were performed under OPP 80 mmHg. Clearly, the model applies to dynamic autoregulation caused by an acute IOP elevation or an acute BP decrease, but is not applicable to spontaneous fluctuations of OPP. The second limitation is that this model does not specify each parameter with the underlying physiological processes. The first-order differential equation likely represents the action of vascular smooth muscle [33], but the second-order differential equation may better correspond with the processes [34, 37]; more than metabolic mechanism, which remains to be determined in future work. The other limitation is the use of multiple parameters to simulate spatially distributed hemodynamics, i.e., the parametric linear approximation of the autoregulation curve. Clearly the predicted BF autoregulation is just an approximation around small equilibrium points, but it enables valuable data to be extrapolated from the experimental data.

As has been noted above, the ocular BF autoregulation system is more complex than the parametric transfer function presented in this study. However, one of the advantages of the parametric transfer function is that it describes the relationship between OPP and BF not only within the linear range but also in the nonlinear range of autoregulation. In addition, it predicts the quantitative BF change over a range of OPP that represents the systematic property of BF autoregulation. Though this estimated parametric transfer function cannot be phrased as a theorem, the model gives reasonable behavior despite many uncertainty and inaccuracies in parameter estimation. Presumably it reflects the fact that the biological system is evolved to be robust.

Conclusions

The simulated BF response within the transfer function model corroborated the in vivo measured BF response in not only static autoregulation but also within the dynamic autoregulation.
Thus, a parametric third-order transfer function can effectively describe the ONH BF autoregulation following IOP elevation, because it illustrates the linear and nonlinear features of autoregulation and predicts quantitative BF change over a wide range of OPPs. Future studies may extend the application of the model to investigate the effect of physiological variation such as aging effects and blood pressure induced OPP changes in BF autoregulation.

Acknowledgements

The study was supported by NIH EY019939 (LW); non restriction fund from Translational Medicine, Pfizer Inc. (LW); Good Samaritan Foundation (LW); Reserve Talents of Universities Overseas Research Program of Heilongjiang by Heilongjiang Education Department, P R China (JY).

Disclosure of conflict of interest

None.

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References

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Glaucoma

Static Blood Flow Autoregulation in the Optic Nerve Head in Normal and Experimental Glaucoma

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Submitted: December 5, 2013
Accepted: January 2, 2014

Purpose. To characterize the static blood flow autoregulation in the optic nerve head (ONH), and to investigate its role in hemodynamic changes in experimental glaucoma (EG).

Methods. Unilateral elevation of intraocular pressure (IOP) was induced in 15 adult rhesus macaques by laser treatment to the trabecular meshwork. Prior to and after laser treatment, retinal nerve fiber layer thickness (RNFLT) was assessed, biweekly, by spectral-domain optical coherence tomography. Optic nerve head static autoregulation was assessed by determining the percentage blood flow (BF) change after the IOP was acutely increased from 10 to 30, 40, or 50 mm Hg manometrically, utilizing a laser speckle flowgraphy device.

Results. Postlaser IOP (measured during average 7.7 ± 2.6 months) was 20.2 ± 5.9 mm Hg in EG eyes and 12.3 ± 2.6 mm Hg in control eyes (P < 0.0001). Retinal nerve fiber layer thickness was reduced by 35 ± 22% of the baseline values (P < 0.001) on average in EG eyes and by 0.4 ± 2.3% in control eyes (P > 0.05). The ONH BF remained at a constant level within a range of ocular perfusion pressure (OPP), 41 mm Hg and above. The autoregulation curves, created by all 723 tests in control and 352 tests in EG, were not significantly different (P = 0.07).

Conclusions. Optic nerve head BF in normal nonhuman primate (NHP) eyes is effectively regulated within a range of OPP approximately 41 mm Hg and above. Chronic IOP elevation causes no remarkable change to the static autoregulation within the ONH of EG eyes.

Keywords: autoregulation, intraocular pressure, ocular perfusion pressure, experimental glaucoma

Open angle glaucoma is an ocular disease characterized by progressive retinal ganglion cell death and axon loss, ultimately leading to irreversible visual field loss. Though elevated intraocular pressure (IOP) is still the only treatable risk factor for glaucoma, accumulating evidence suggests that blood flow (BF) in the optic nerve head (ONH) is compromised.1–12 This hemodynamic alteration, including the observation in our recent studies in experimental glaucoma (EG),13,14 has been proposed to play a role in the pathological processes of glaucomatous optic neuropathy, either directly or indirectly by increasing the susceptibility of the ONH to IOP.15–18 One possible mechanism underlying this compromised BF in the ONH is autoregulation dysfunction.16–23

Autoregulation is an intrinsic ability of vascular beds, including ocular tissues, to maintain a constant level of BF in the face of perfusion pressure fluctuation and varied metabolic demand. The capacity of autoregulation may become less potent, or may completely fail, in glaucoma leading to the tissue’s being underperfused24 while greater diurnal IOP variations25,26 and more pronounced reductions in nocturnal blood pressure occur.27,28 Based upon the concept of autoregulation, a line of studies has examined the autoregulation capacity in glaucoma by determining the relative BF change in retina,29,30 choroid,31,32 and retrobulbar arteries33–36 after either BP29,37 or IOP30,32 or blood carbon dioxide concentration35,38 is artificially altered from an ambient level to another level. Although these studies suggest that BF autoregulation is altered in those tissues, to date, no clear evidence of autoregulation dysfunction has been demonstrated in the ONH.32,35 the primary site of glaucomatous damage to the retinal ganglion cell axons.39–41 These incomplete results hinder our understanding of the vascular role in glaucoma pathophysiology and are due, at least partially, to the methodological limits of how autoregulation is examined.

The common methodology used in the clinic to quantify autoregulation capacity is measurement of BF differences before and after the ocular perfusion pressure (OPP) is artificially increased or decreased. The normalized BF change thus represents the autoregulation capacity at a given OPP level tested. A series of such BF changes in response to OPP changes, over a wide range of OPP, constitutes a classic autoregulation curve or pressure–flow (P-F) relationship (see Fig. 1).42 This curve includes a plateau across the range of OPP where the BF is fully compensated by autoregulation. When the OPP fluctuations exceed the critical range defined by this plateau, that is, beyond the lower and upper limits of the autoregulation range (LLA and ULA, respectively), vasomotor adjustments are incomplete and BF will gradually decrease or increase passively as OPP changes. In this study, the P-F relationships within these ranges of OPP are referred to as “lower slope” and “upper slope” (Fig. 1).

When autoregulation capacity is assessed in glaucoma, BF is often measured at a given level of OPP chosen depending on the manner in which the OPP is challenged, for example, by increasing the blood pressure or by altering IOP. It is assumed that any BF abnormality at a measured OPP represents the
autoregulation status in glaucoma. However, according to studies in cerebral circulation, autoregulation may change differently depending upon the disease. For example, the autoregulatory curve may shift toward the higher OPP in chronic hypertension, in ischemic cerebrovascular diseases, a loss of autoregulation could be simply attributed to an alteration within the lower slope of OPP, while the normal plateau can be absent in diabetes. Because the autoregulation curve changes within glaucoma are still unknown, the BF change measured at a given OPP may not represent the exact autoregulation status. This is a likely source of discrepancy between the studies in which autoregulation capacity is examined at different OPPs. However, to construct a complete autoregulation curve covering a full range of OPP is difficult, if not impossible, in a clinical setting.

The nonhuman primate (NHP) EG model with chronic IOP elevation has been used to study ONH BF. In our previous studies using this model, ONH BF in the EG eye has been found to be significantly lower than in the control eye when the BF was measured at a reduced OPP by increasing the IOP to 40 mm Hg. In these eyes, the basal ONH BF (i.e., under manometric IOP control at 10 mm Hg) changes in a biphasic manner over the course of longitudinal follow-up through stages of EG: ONH BF is slightly increased during the early stage and then undergoes a continuous decline through later stages of EG. This altered ONH BF observed under manometric IOP control at both normal and raised IOP suggests that ONH autoregulation is likely interrupted after a period of chronic IOP elevation in EG and progresses secondary to the continuous optic neuronal degeneration.

In the current study on this cohort of NHP EG, we tested an assumption that chronic IOP elevation results in autoregulation impairment, which is associated with the reduced ONH BF demonstrated in our previous studies. This was achieved by comparing the autoregulation curve between EG and normal control eyes. The autoregulation curve was created based on a series of relative ONH BF changes, each measured in response to an acute OPP decrease induced by instantaneous IOP elevation monitored across stages of EG. In addition to the comparison of the whole autoregulation curve between the study eye groups, parameters that characterize the autoregulation curve, including the lower slope, LLA, and plateau (see Fig. 1), were compared for individual animals (pairs of eyes) as well. Although the experimental model precludes a scenario whereby vascular dysregulation could serve as a primary insult in glaucomatous damage, the results for the first time reveal a general pattern for an autoregulation curve within the ONH after chronic IOP elevation. Since the BF was measured after it reached a steady state post-OPP challenge, these measurements represent so-called static autoregulation, in contrast to “dynamic autoregulation” that describes the rapid, continuous course of BF response during an OPP challenge, which will be reported separately.

**Methods**

**Animals and Anesthesia**

Fifteen adult rhesus monkeys (Macaca mulatta) were included in the study, 14 females and 1 male. Their average age at the beginning of the study (±SD) was 9.0 ± 2.6 years (range, 5–14 years), and average weight was 6.4 ± 1.7 kg. In all cases, anesthesia was induced with intramuscular ketamine (15 mg/kg; Henry Schein Animal Health, Dublin, OH) and xylazine (1.5 mg/kg; Akorn, Inc., Decatur, IL), along with a single subcutaneous injection of atropine sulfate (0.05 mg/kg; Butler Schein Animal Health, Dublin, OH). Animals were then intubated and breathed air plus 10% oxygen spontaneously. Heart rate, end tidal CO2, and arterial oxygenation saturation were monitored continuously. Body temperature was maintained with a heating pad at 37°C. Pupils were fully dilated with 1.0% tropicamide (Alcon Laboratories, Inc., Fort Worth, TX). One of the superficial branches of a tibial artery was cannulated with a 27-gauge needle, which was connected to a pressure transducer (BLPR2; World Precision Instruments, Sarasota, FL) and a four-channel amplifier system (Lab-Trax-4/24T; World Precision Instruments) for continuous arterial blood pressure (BP) recording. Anesthesia was maintained by continuous administration of pentobarbital (8–12 mg/kg/h, intravenous) using an infusion pump (Aladdin; World Science Instruments, Inc., Sarasota, FL) for all procedures except during trabecular meshwork laser sessions. All procedures were performed with the animals under general anesthesia, adhering to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee at Legacy Research Institute.

**Retinal Nerve Fiber Layer Thickness Measurement**

The progression of structural damage during the development of EG was monitored by longitudinal measurements of peripapillary retinal nerve fiber layer thickness (RNFLT) in
both eyes of each animal using a spectral-domain optical coherence tomography (SD-OCT) instrument (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany). For each measurement, a single circular B-scan (12° diameter) was performed; 9 to 16 individual sweeps were averaged to comprise a final B-scan at each session. The automated layer segmentations generated by the instrument were manually corrected when the algorithm had obviously erred during delineation of the RNFL inner and outer borders. Spectral-domain OCT data were exported for extraction of RNFLT values by custom software.49

Induction of Chronic Unilateral Experimental IOP Elevation

Laser treatment to one eye of each animal was performed under ketamine and xylazine anesthesia. One hundred eighty degrees of the trabecular meshwork (50-µm spot size, 1.0-second duration, 600–750 mW power) was treated in each of two separate sessions at least 2 weeks apart. After each treatment, a sub-Tenon’s injection of 0.5 mL dexamethasone (10 mg/mL; APP Pharmaceuticals LLC, Schaumberg, IL) was given in the inferior fornix of the treated eye. Laser treatments were repeated (but limited to a 45° or 90° sector) on subsequent occasions as necessary to achieve sustained IOP elevation.

IOP Measurement

Intraocular pressure was measured at each test session by rebound tonometry (Tonopen XL; Reichert, Inc., Depew, NY) within both eyes of each animal (a mean of three measurements per eye) within 30 minutes of general anesthesia induction. After the initial IOP measurements, IOP was set in both eyes manometrically to 10 mm Hg. Two 27-gauge needles were inserted into the anterior chamber of each eye. One needle was connected to a manometer set at 10 mm Hg; the other was connected to a pressure transducer to record actual eye pressure as previously described.13

Blood Flow Measurement With Laser Speckle Flowgraphy

The laser speckle flowgraphy (LSFG) technique (Softcare; lizuka, Japan) was used to measure BF in the NHP ONH and has been described in detail within previous publications.14,50,51 In brief, a fundus camera equipped within the LSFG device was focused on an area centered on the ONH. The area is approximately 3.8 × 3 mm (width × height). After the laser is switched on (λ = 830 nm, maximum output power, 1.2 mW), a speckle pattern is generated due to random interference of the scattered light from the illuminated tissue area. The speckle pattern is continuously imaged by a charge-coupled device (700 × 480 pixels) at a frequency of 30 frames per second for 4 seconds at a time.

Offline analysis software (LSFG Analysis; Softcare) computed mean blur rate (MBR) of the speckle images. Mean blur rate is a squared ratio of mean intensity to the standard deviation (SD) of the image, which varies temporally and spatially according to the velocity of blood cell movement and correlates well with capillary BF within the ONH validated by the microsphere and the hydrogen clearance methods.52 Thus, the MBR has been used as a BF index. A composite MBR map representing BF distribution within the ONH disc was generated from the images of each 4-second series. After eliminating the area corresponding to large blood vessels within the images, capillary BF within the remaining ONH disc area was averaged and reported in arbitrary units (AU) of MBR.

Quantification of Static Blood Flow Autoregulation

For each measurement of static BF autoregulation, the IOP in the test eye was manometrically set at 10 mm Hg and left to equilibrate for at least 5 minutes. With all vital signs (oxygen saturation, heart rate, and end tidal CO₂ stabilized, baseline BF (MBR) was measured with the LSFG for 4 seconds. The saline reservoir was then switched to a height calibrated to be equivalent to 30, 40, or 50 mm Hg and kept at the higher level for at least 5 minutes. After the BF was measured again for another 4 seconds, the reservoir was returned to the previous level (10 mm Hg). The percentage BF change measured at each acutely elevated IOP level relative to that measured at an IOP of 10 mm Hg was calculated. During each test session, the above measurement protocol was repeated for all three levels of acute IOP elevation. A series of the relative ONH BF changes measured at different OPP levels during multiple test sessions was used to create autoregulation curves for both EG and control eyes.

The OPP was estimated by subtracting the IOP from the recorded mean arterial BP (diastolic pressure + one-third of pulse pressure) and an additional 5 mm Hg to account for the height difference between the eye and the artery where BP was recorded.

Experimental Design

For each animal, three to five prelaser baseline sessions were included to establish baseline values of IOP, RNFLT, and ONH autoregulation in each eye. Then chronic IOP elevation was initiated by laser treatment in one eye of each animal. Thereafter, the same measurements were repeated once every 2 weeks for the duration of the postlaser follow-up. Most animals (9 of 15) were followed until a relatively advanced stage, when RNFLT in the EG eye was reduced by more than 35% of its prelaser baseline value. Six of the 15 animals were followed only during an earlier stage of EG, then killed in order to evaluate histological and molecular changes in early EG eyes. In these animals, the average RNFLT was reduced by less than 10%, on average. At the end of each experiment, the animals were killed humanely under deep anesthesia for histopathological studies.

Data Analysis and Statistics

The relative ONH BF changes obtained at different OPPs were fitted with segmental linear regression to determine three parameters: the lower slope, LLA, and plateau (Prism 6.03; GraphPad Software, Inc., La Jolla, CA). The difference between autoregulation curves of the EG eye and control eye was evaluated by Student’s t-test after the data were confirmed to have normal distribution.

RESULTS

Intraocular Pressure and RNFLT

Within the baseline testing sessions, there was no difference between the two eyes for mean IOP (14.1 ± 2.1 vs. 13.8 ± 2.3, P = 0.28). Postlaser IOP in the EG eyes was significantly
linear regression was fitted for all the data (Y = 0.01 × X + 10.94, R² = 0.003; red dotted lines: standard error).

increased compared with that in the control eyes (20.2 ± 5.9 vs. 12.3 ± 2.6 mm Hg, respectively (P < 0.0001). Peak IOP was 42.2 ± 10.2 mm Hg for EG eyes and 18.9 ± 2.8 mm Hg for control eyes. The average duration of postlaser treatment was 7.7 ± 2.6 months (ranging from 3.6 to 12.5 months). The average values for RNFLT in the control eyes at the end of longitudinal follow-up were −0.4% ± 2.3% relative to baseline (ranging from −6.2% to +4.3%). The average RNFLT in the EG eyes at the end of the experiment was −33 ± 22% relative to baseline values (P < 0.001). In the six animals followed only to an earlier stage of EG, the average RNFL loss was 9 ± 8% relative to baseline (ranging from 3.5% above to 17.1% below baseline) in the EG eyes. The nine advanced EG eyes had RNFLT loss of 46 ± 11%, ranging from 36% to 62%.

**ONH BF Autoregulation in Control Eyes**

To describe the P-F relationship within the range of ambient OPP or ONH autoregulation curve, all the tests (n = 723) from eyes without laser treatment (contralateral control, n = 15 eyes) and the designated EG eyes before any laser treatment (“prelaser,” n = 15) were included for the analysis. This analysis aims to determine the range of OPP within which the ONH BF remains relatively stable. As shown in Figure 2, the ONH BF (AU) was measured at OPPs from 48 to 115 mm Hg. Since the IOPs during the BF measurements were all set at 10 mm Hg, the varied OPP resulted from the difference of BP between animals and between longitudinal tests. Analysis of a linear regression showed that the ONH BF within the range of OPP did not correlate (R² = 0.003).

Figure 3 shows the percentage ONH BF change from each of the test dates in Figure 2 after the IOP was acutely increased from 10 to 30, 40, or 50 mm Hg. This analysis aims to establish an additional portion of the autoregulation curve, specifically to determine the LLA (OPP from where BF starts to decline) and to characterize the lower slope. As shown, a segmental linear regression generated two distinguishable regression lines. The LLA was defined as the intersection of the two regression lines, which was approximately 40.7 mm Hg OPP (95% confidence limit (CL): 38.81–42.52). The ONH BF changed little (plateau) if the resultant OPP was above the LLA, but the BF started to decline (lower slope) once the OPP was below the LLA. The intercept of the lower slope was −39%.

In Figure 4, the same BF data in Figure 3 are plotted against percentage OPP change; that is, the relative decrease of OPP (%) resulted from the acute IOP increase from 10 to 30, 40, and 50 mm Hg. Segmental linear regression analysis showed that the BF started to decline when the OPP decreased more than 43%.

**ONH BF Autoregulation in EG Eyes**

**Comparison of Autoregulation Curves Between EG and Control Eyes.** In this analysis, the ONH BF changes (%) in response to the acute OPP decrease in all postlaser tests (n = 352) in EG eyes were pooled together to construct an autoregulation curve. This curve was then compared with that derived from control eyes (n = 723 tests) in Figure 3. The reason for pooling all the tests together was to enhance the statistical power. However, since RNFLT loss progressed more slowly in some animals, the results may be biased toward those animals with a greater number of tests. This random effect was controlled by the Satterthwaite method while a mixed model of ANCOVA was used to test the difference between EG and control eyes. The result showed no difference between the two groups of eyes (P = 0.71), although the lower slope and the plateau showed a tendency toward a slightly greater BF...
A decrease in EG eyes. A further comparison specifically for the lower slope, LLA, and the plateau showed no remarkable difference between the two groups of eyes as well (P = 0.50, P = 0.72, and P = 0.85, respectively; see Fig. 5).

Since the BF changes in EG eyes were collected throughout the experiment after laser treatment, pooling all the data, including the early stage, could potentially mask a possible ONH autoregulation change. Therefore, the above analysis was repeated in two subdivided groups, (1) earlier-stage EG (n = 15 eyes), in which RNFLT loss was less than 20%, and (2) advanced EG eyes (n = 9 eyes), in which RNFLT loss was more than 20%. Therefore, the autoregulation curve representing each subgroup was compared with the control eyes for the whole curve, the slope, and the plateau. The results showed that none of the parameters or LLA in the EG eye was significantly different compared with normal control eyes (Fig. 6, Table).

**Comparison of the Autoregulation Curve Between Individual EG Eye and Pooled Controls or the Contra-lateral Control Eye.** In the previous analyses, the BF measurements in the EG and control eyes were pooled together to generate the autoregulation curves. Although the approach enhanced the overall statistical power, it might have overlooked the individual eyes with possible autoregulation changes. Therefore, in this analysis the autoregulation curve from each EG eye was compared with either pooled controls or the contralateral control eye. Three animals were excluded due to too few data points on the lower slope (0–2) to perform the analysis. The results demonstrate no significant difference between each EG eye and pooled control eyes or their own contralateral control eye (data not shown).

**DISCUSSION**

To test the prediction that chronic IOP elevation impairs ONH static autoregulation capacity in this NHP glaucoma model, the ONH BF autoregulation curve was created from BF responses across a wide range of OPP and compared between EG eyes and control eyes. The results showed that within the ONH of the control eyes, BF was effectively regulated within the OPP range from 41 to 115 mm Hg. Conversely, when the OPP was below 41 mm Hg, the BF declined linearly with OPP. In the EG eyes, the autoregulation curve and the corresponding parameters showed no significant difference from the control eyes, regardless of whether the comparison was made between pooled data or individual animals.

An important parameter measuring the autoregulation efficacy is the critical OPP point (LLA), from where and below the BF starts to decline. Previous studies investigating the ONH autoregulation showed that LLA varied in a wide range, within and between species, from 20 to 48 mm Hg53,54 in cats and 30 mm Hg in monkeys.55 In humans, the LLA was estimated to be between 10 and 26,56 22,57 or 39 mm Hg58 based on the BP and IOP provided in the papers. In these studies the critical point at which BF started to decline was expressed by percentage OPP change; this corresponding critical point was 20%,59 40%,60 or 60%.57,61 OPP decrease from an ambient level.57,61 In the current study in NHP ONH, the BF started to decline from OPP 41 mm Hg (Fig. 3), or 43% if the OPP change was expressed by a percentage decrease (Fig. 4).

Besides the difference in species, BF measurement methodology, speed of OPP change,73 and individual variation,58 the approach to determining the LLA may account for the differences of the measured LLA. To define a LLA often requires identifying an OPP level below which the BF decreases significantly. In studies with a smaller sample size and larger measurement variation, this BF level is often found at a much lower OPP compared to that in a study with a larger sample size and smaller measurement variation. In the current study, the LLA was estimated by determining the intersection of two regression lines generated by a segmental linear regression model, which defines the LLA as an OPP where the BF just begins to decline.

A previous study on this same cohort of NHP glaucomatous animals62 showed that beyond approximately 10% of RNFLT loss, the ONH BF in EG eyes progressively declined to more than 20% below the baseline value, and the decrease was...
correlated closely with the loss of RNFLT. It was proposed in that study that this reduced BF was at least partially a result of reduced metabolic demand after retinal ganglion axon loss and/or possibly a result of autoregulation dysfunction. In the current study, when the P-F relationship was described based upon static BF autoregulation and compared between EG and control eyes, it failed to show any significant difference in static autoregulation. Thus, it seems that at least in these chronic IOP-induced EG eyes, the static autoregulation is not a predominant factor accounting for the reduced ONH BF observed in the previous study.62

There are additional factors that should be considered regarding this insignificant autoregulation difference. First, in this NHP EG model, the ONH BF in the prelamina and lamina region during the early stage was in fact increased.62 In a subgroup of these experiments, the increased BF was also observed during the later stages within the retrolaminar region.14 These findings agree with earlier studies in the same NHP EG model, which showed both increased and decreased ONH BF59 and upregulated retinal BF in the early stages of human glaucoma.63 On the one hand, this strongly suggests autoregulation dysfunction occurring in glaucoma; on the other hand, the up- and downregulated BF change may obscure the observation of a potential autoregulation change in the ONH. More importantly, the different sensitivities of autoregulation components should also be taken into account. As mentioned previously, static and dynamic aspects are two related but different components during the process of autoregulation. Studies in the brain have shown that each autoregulation component should also be taken into account. As mentioned previously, static and dynamic aspects are two related but different components during the process of autoregulation. Studies in the brain have shown that each component may represent different mechanisms.64 Under certain diseased conditions, such as acute ischemic stroke and brain injuries, the impairment to these two components has been demonstrated to be disassociated.55,66 One additional factor that likely contributes to the insignificant change observed here for static autoregulation is the wide variation in the ONH BF response to the acute IOP increase in both control and EG eyes (see Fig. 3). This large variation has been observed in the human ONH as well. In those studies, after the IOP was acutely increased by a negative pressure suction cup on the sclera, the ONH BF reduction varied between approximately 15%59 and 50%57 (estimates based on the data provided in these previous reports). Thus, this variation is likely one of the sources of variability in the detection of autoregulation changes in both human glaucoma and this EG model.

Several limitations are associated with this study. As has been detailed in previous reports, the LSFG, like most other optically based techniques, has limited tissue penetration in addition to the inherent calibration error between in vitro and in vivo measurements.14,51,62 The BF measured by the LSFG derives largely from the anterior portion of the optic nerve, and to a certain degree underestimates true BF. This is also part of the reason for the higher intercept in normal eyes (−39%) at OPP = 0, although a nonzero BF intercept is possible at “zero” OPP because the systolic arterial pressure can be ~30 mm Hg above mean arterial pressure67 used for the calculation of OPP. It should also be noted that the present study was conducted in anesthetized animals. Even though the anesthetics were carefully selected to minimize the inhibitory effect upon autoregulation during the tests, caution is warranted in comparing the results with human subjects. In addition, chronic IOP elevation and the course of EG in NHP eyes are still relatively rapid compared to observations in human glaucoma because peak IOP in the EG eyes was probably higher than most treated human glaucoma and damage developed in mere months. It is possible that the ONH autoregulation system is still functionally intact despite loss of neuronal tissue (i.e., RNFLT loss). In summary, the current study demonstrated, first, that the normal ONH in NHP has a strong autoregulation capability to maintain a constant BF within the OPP range from 41 mm Hg and above. Secondly, it showed that chronic IOP elevation causes no remarkable autoregulation change in the ONH of EG eyes when examined by the static component. Thirdly, although the results may support our previous premise that reduced ONH BF in the EG eye is largely a result of reduced metabolic demand after neuronal tissue loss, continuing investigation utilizing different approaches, specifically examining the dynamic autoregulation component within the ONH of these same EG animals, is necessary. A parallel report on this series of studies will describe the details of dynamic autoregulation in the ONH of this EG model.

Acknowledgments

The authors thank Chelsea Piper for her technical assistance, Manoj Pathak for consultation on the statistical analysis, and Leo Schmetterer for valuable comments. Supported by National Institutes of Health Grant R01-EY019939 and Legacy Good Samaritan Foundation, Portland, Oregon.

Disclosure: L. Wang; None. C.F. Burgoyne; None. G. Cull; None. S. Thompson; None. B. Fortune; None

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Longitudinal Hemodynamic Changes Within the Optic Nerve Head in Experimental Glaucoma

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Submitted: March 11, 2013
Accepted: May 23, 2013
Citation: Cull G, Burgoyne CF, Fortune C, and Wang L. Longitudinal hemodynamic changes within the optic nerve head in experimental glaucoma. Invest Ophthalmol Vis Sci. 2013;54:4271–4277. DOI:10.1167/iovs.13-12013

Purpose. To characterize longitudinal changes in basal blood flow (BF) of the optic nerve head (ONH) during progression of structural damage in experimental glaucoma (EG).

Methods. Unilateral elevation of IOP was induced in 15 adult rhesus macaques by laser treatment to the trabecular meshwork. Prior to and after laser, retinal nerve fiber layer thickness (RNFLT) and ONH BF were measured biweekly by spectral-domain optical coherence tomography and a laser speckle flowgraphy device (LSFG), respectively.

Results. Average postlaser IOP was 20.2 ± 5.9 mm Hg in EG eyes and 12.3 ± 2.6 mm Hg in control eyes (P < 0.0001). Longitudinal changes in basal ONH BF were strongly associated with changes in RNFLT as EG progressed from early through moderately advanced stages of damage, with Pearson correlation coefficients ranging from 0.64 to 0.97 (average = 0.81) and an average slope of 1.0. During early stage (RNFLT loss < 10%), basal ONH BF was mildly increased (9% ± 10%, P = 0.004) relative to baseline and compared with fellow controls (P = 0.02). Basal ONH BF declined continuously throughout subsequent stages in EG eyes reaching 25.0% ± 9.6% (P < 0.0001) below baseline at the final stage studied (RNFLT loss > 40%). In fellow control eyes, there was no significant change in basal ONH BF over time (P = 0.27).

Conclusions. In EG based on chronic mild-to-moderate IOP elevation, a two-phase pattern of ONH BF alteration was observed, ONH BF increased during the earliest stage (while RNFLT was within 10% of baseline) followed by a linear decline that was strongly correlated with loss of RNFLT.

Keywords: intraocular pressure, blood flow, optic nerve head, experimental glaucoma
neural loss manifest as RNFLT changes. We postulated a priori that the BF requirement of the ONH ultimately diminishes with neural tissue loss or damage in EG.

METHODS

Animals and Anesthesia

Fifteen adult rhesus monkeys (Macaca mulatta) were included in the study. 14 females and 1 male: their average age at the beginning of the study ($\pm$SD) was 9.0 ± 2.6 years (range, 5–14). All procedures were performed with the animals under general anesthesia, adhered to the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee (IACUC) at Legacy Research Institute. In all cases, anesthesia was induced with ketamine (10 mg/kg) and xylazine (1.5 mg/kg), along with a single subcutaneous injection of atropine (0.05 mg/kg). Animals were intubated and breathed air plus 10% oxygen spontaneously. Heart rate, end tidal CO$_2$, and arterial oxygen saturation were monitored continuously.

Body temperature was maintained with a heating pad at 35°C. Puppies were fully dilated with 1.0% tropicamide (Alcon Laboratories, Inc., Fort Worth, TX). One of the superficial branches of a tibial artery was cannulated with a 27-gauge needle, which was connected to a pressure transducer (BLPR2; World Precision Instruments, Sarasota, FL) and a four-channel amplifier system (Lab-Trax-4/24T; World Precision Instruments) for continuous arterial blood pressure (BP) recording. Anesthesia was maintained by continuous administration of pentobarbital (8-12 mg/kg/h, IV) using an infusion pump (Aladdin; World Precision Instruments) for all procedures except during trabecular meshwork laser sessions.

IOP Measurement and RNFLT Imaging Protocol

IOP was measured at each session by rebound tonometry (Tonopen XL; Reichert, Inc., Depew, NY) in both eyes of each animal (mean of three measurements per eye) within 30 minutes of general anesthesia induction. After the initial IOP measurements, IOP was set in both eyes manometrically to 10 mm Hg. Two 27-gauge needles were inserted into the anterior chamber of each eye. One needle was connected to a custom-made manometer set at 10 mm Hg, the other needle was connected to a pressure transducer (BLPR2; World Precision Instruments) to record actual eye pressure, as previously described. Peripapillary RNFLT was measured in both eyes of each animal using a commercial spectral-domain optical coherence tomography (SDOCT) instrument (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany). For this study, the average peripapillary RNFLT is reported from a single circular B-scan (12° diameter) consisting of 1536 A-scans. Nine to 16 individual sweeps were averaged in real time to comprise the final stored B-scan at each session. At the initial imaging session, the operator centered the position of the scan on the ONH and all subsequent scans were pinned (identical) to this location using the instrument’s eye-tracking software. A trained technician manually corrected the accuracy of the instrument’s native automated layer segmentations when the algorithm had obviously erred from the inner and outer borders of the RNFL to an adjacent layer (such as a refractive element in the vitreous instead of the internal limiting membrane, or to the inner plexiform layer instead of the outer border of the retinal nerve fiber layer). SDOCT data, including segmentations, were then exported for extraction of RNFLT values by custom software.

Induction of Chronic Unilateral Experimental IOP Elevation

Laser treatment to one eye of each animal was performed under ketamine and xylazine anesthesia. One hundred eighty degrees of the trabecular meshwork (50-μm spot size, 1.0-second duration, 600- to 750-mW power) were treated in each of two separate sessions at least 2 weeks apart. After each treatment, a sub-Tenon’s injection of 0.5 mL dexamethasone (10 mg/mL) was given in the inferior fornix of the treated eye. Laser treatments were repeated (but limited to a 45° or 90° sector) on subsequent occasions as necessary to achieve sustained IOP elevation.

BF Measurement With Laser Speckle Flowgraphy

The principles of the laser speckle flowgraphy (LSFG) technique (Softcare, Iizuka, Japan) and its application to measure ONH BF in nonhuman primates have been described in detail within previous publications. In brief, a frame camera equipped within the LSFG device was used to define an area centered on the ONH, with dimensions of approximately $3.8 \times 3.0$ mm ($width \times height$). After switching on the laser ($\lambda = 830$ nm, maximum output power, 1.2 mW), a speckle pattern appears due to random interference of the scattered light from the illuminated tissue area, which is continuously imaged by a charge-coupled device ($700 \times 480$ pixels) at a frequency of 30 frames per second for 4 seconds at a time.

Offline analysis software (LSFG Analysis; Softcare) computed mean blur rate (MBR) of the speckle images. MBR is a squared ratio of mean intensity to the SD of light intensity, which varies temporally and spatially according to the velocity of blood cell movement and correlates well with capillary BF within the ONH validated by the microsphere method. Thus, the MBR has been used as a BF index. A composite MBR map representing BF distribution within the ONH disc was generated from the images of each 4-second series. After eliminating the area within the images corresponding to large blood vessels, capillary BF within the remaining ONH disc area was averaged and recorded in arbitrary units (AU) of Mean BF (MBF).

Basal ONH BF could be measured either at the ambient IOP or under manometric IOP control. Measurement under ambient IOP in EG eyes can be complicated by potential failures of autoregulation when ambient IOP is high. Moreover, IOP is inevitably and progressively reduced from its initial level at the start of each recording session due to IOP-lowering effects of anesthesia. Therefore, ONH BF was measured under uniform conditions whereby IOP was manometrically set to 10 mm Hg in all eyes at all sessions. This IOP level is close to that measured under general anesthesia in normal NHP eyes (11.2 ± 3.1 mm Hg). ONH BF was measured at least 5 minutes after the IOP was set at 10 mm Hg.

For each animal, three to five prelaser baseline sessions were included to establish baseline values of IOP, RNFLT, and ONH BF in each eye. Then chronic IOP elevation was initiated by laser treatment in one eye of each animal. The contralateral eye served as control. Thereafter, IOP, RNFLT, and ONH BF measurements were repeated once every 2 weeks for the duration of the postlaser follow-up (Table 1, column 5). Most animals (9 of 15) were followed until a relatively advanced stage when RNFLT in the EG eye was reduced by more than 5% of its prelaser baseline value (Table 1, rightmost column). However, 6 of the 15 animals were followed only during an earlier stage of EG to evaluate histological and molecular changes in early EG eyes (Table 1, bottom six rows). At the end of each experiment animals were killed humanely under deep anesthesia for tissue preservation and histopathological studies.
**Optic Nerve Blood Flow in Experimental Glaucoma**

**Table 1. Demographics, IOP Information, and Final RNFLT of Each Animal**

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<th>ID</th>
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<th>Mean IOP EG, mm Hg</th>
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**Data Analysis and Statistics**

All data were reported as average ± SD unless specified. A commercial software package (Prism 5; GraphPad Software, Inc., La Jolla, CA) was used to perform ordinary least squares linear regression, ANOVA, and one-sample t-tests to evaluate longitudinal changes in ONH BF over time. The statistical test applied to analyze each experimental result is specifically identified within the text of the results section.

**RESULTS**

**Demographics and IOP**

Table 1 lists the demographics of all experimental animals as well as the IOP measured during the postlaser follow-up period in both eyes and the stage of EG at termination (i.e., the final RNFLT measurement relative to the baseline average of each EG eye).

There was no difference at baseline between experimental and control eyes for mean IOP (14.1 ± 2.1 vs. 13.8 ± 2.3, P = 0.28). Over the total duration of postlaser follow-up, the average IOP in EG eyes was 20.2 ± 5.9 mm Hg compared with 12.3 ± 2.6 mm Hg in control eyes (P < 0.0001, Table 1). Over this same period, peak IOP ranged from 18 to 58 in EG eyes and from 14 to 23 in control eyes (Table 1).

**RNFLT**

The extent of glaucomatous damage varied across the group of EG eyes as measured by RNFLT (Table 1). Two of the 15 animals were killed before significant loss of RNFLT was observed (28,675 and 23,583; note, significant loss is defined as 7% below baseline consistent with previously published measurement variability). The rate of progressive RNFLT loss also varied across animals, as shown in Figure 1.

The rate of progressive RNFLT loss was only modestly associated with mean IOP (R = 0.45, P = 0.20) or peak IOP (R = 0.53, P = 0.08). In fellow control eyes, RNFLT was consistent across all follow-up time points after laser had been initiated in the EG eyes. The average (±SD) of all follow-up values for relative RNFLT in control eyes was −0.4% ± 2.5% with a range from −6.2% to +4.3% relative to the baseline average for each control eye, consistent with our previously published range of 7% measurement noise for RNFLT.

**Basal ONH BF**

Longitudinal changes in basal ONH BF were closely associated with stage of EG as measured by RNFLT. Figure 2 shows a scatter plot of the change in basal ONH BF from baseline against the change in RNFLT from baseline for each eye at each longitudinal measurement session. For clarity, the nine animals that progressed to a relatively more advanced stage (35% or more loss of RNFLT) are shown in panel A and the other six animals are shown in panel B. Although the data for most animals in Figure 2 appear to share a similar relationship, formal statistical testing indicates that not all 15 EG eyes can be described by a single function (F(28,84) = 5.7, P < 0.0001 and Akaike’s Information Criterion difference = 41.7, P < 0.0001).

Therefore, linear regression was applied to the data for each of the EG eyes independently (Table 2). The results listed in Table 2 demonstrate that longitudinal changes in basal ONH BF were strongly associated with changes in RNFLT as EG progressed from early through moderately advanced stages of damage, with Pearson correlation coefficients ranging from 0.64 to 0.97 (average = 0.81) and an average slope of 1.0. Importantly, the Y-intercept was positive in 8 of the 12 EG eyes, ranging from 2% below to 33% above (average = 10.4% above) the basal ONH BF at baseline. This result suggests that basal ONH BF is actually higher than baseline during the earliest stage of EG.

To further test whether basal ONH BF was associated with the stage of disease, the data were binned into EG severity stages according to relative loss of RNFLT as shown in Figure 3. There was a significant effect of EG severity stage on basal ONH BF (R² = 0.70, F = 20.5, P < 0.0001, ANOVA). There was no significant effect of severity stage in EG eyes on the relative basal ONH BF in control eyes measured at the corresponding time points (R² = 0.14, F = 1.4, P = 0.27, ANOVA). During the earliest EG stage when RNFLT loss was less than 10% from baseline values, basal ONH was significantly higher than baseline (P = 0.004, one-sample t-test). During the subsequent stage when RNFLT loss was between 10% and 20% below baseline, there was no significant difference in basal ONH BF as
compared to baseline ($P = 0.74$). Basal ONH progressively declined through subsequent stages to reach levels of $8.7\% \pm 8.3\%$ below baseline ($P = 0.03$), $21.4\% \pm 11.8\%$ below baseline ($P = 0.003$), and $25.3\% \pm 9.3\%$ below baseline ($P < 0.0001$), respectively. Basal ONH BF in EG eyes was significantly elevated above fellow control eye levels during the earliest stage (RNFLT loss < 10%; $P = 0.02$) and significantly reduced below the level in fellow control eyes during the latter stages (RNFLT loss 30%–40% and greater than 40%, $P = 0.03$ and $P = 0.002$, respectively), but was not significantly different from fellow control eye levels during the two intermediate stages (RNFLT loss 10%–20% and 20%–30%, $P = 0.38$ and $P = 0.74$, respectively).

**BP at the Time of BF Measurement During Baseline and EG Stages**

The average arterial BP at the time corresponding to baseline ONH BF measurement was $89.3\% \pm 4.6$ mm Hg in control eyes and $89.6\% \pm 4.2$ mm Hg in EG eyes ($P = 0.49$). During the progression of EG, BP showed no change over time ($F$-test, $P = 0.59$). Table 3 shows BP measured during baseline sessions and across the five different stages of EG. The corresponding ocular perfusion pressure (OPP) can be estimated by subtracting $10$ mm Hg for IOP and additional $5$ mm Hg for the height difference between the eye and the femoral artery where BP was recorded. Note that the OPP levels during all stages were above the lower limit of autoregulation in the ONH of monkeys previously reported (Wang L, et al. IOVS 2012: ARVO E-Abstract 6842).

**DISCUSSION**

The results of this study show that basal ONH BF was strongly associated with the stage of EG severity as measured by loss of RNFLT and underwent a two-phase pattern of change: during the earliest stage, when RNFLT was within $10\%$ of baseline, ONH BF exhibited mild increase, after which it progressively declined with increasing degree of EG severity. At the most severe stage evaluated in this study, when more than $40\%$ of the RNFLT was lost, ONH BF was reduced by more than $25\%$ below baseline. The decline of ONH BF was strongly correlated with RNFLT thinning over the range of EG studied.

In this longitudinal observation in EG, RNFLT, rather than IOP, has been used to define different stages of progression.

**FIGURE 1.** The extent and rate of progressive loss of RNFLT varied across animals. For clarity, the group of 15 animals is split into $n = 9$ that were followed to moderate stage of damage (i.e., beyond 35% loss, [A]) and $n = 6$ that were killed at a relatively early stage of damage (B).

**FIGURE 2.** Longitudinal change in basal ONH BF (% from baseline) versus loss of RNFLT (% from baseline); for clarity, the group of 15 animals is split into $n = 9$ that were followed to a moderate stage of damage (A) and $n = 6$ that were killed at a relatively early stage (B). Basal ONH BF in EG eyes is greater than prelaser baseline during early-stage EG when RNFLT loss is minimal.
This is because the magnitude of IOP elevation varies substantially between animals and during the course of EG development that it does not correlate with optic nerve axon loss, the most reliable landmark to define the stages of EG. In contrast, RNFLT is a more definitive marker than IOP for the stage of progression in this model with a close correlation to orbital optic nerve axon loss.39

The relationship between chronically increased IOP and ONH BF has been investigated in a similar EG model in cynomolgus monkeys.29 In that study, which had a cross-sectional design, the EG eyes had a slightly higher average level of IOP elevation (34 ± 8 mm Hg) and a much longer duration (more than 8 months) than in our study; however, no significant ONH BF changes were demonstrated even at very late stages of EG. Possible reasons for this discrepancy may include the limited time window observed by a single “snapshot” and/or the lower sensitivity of the iodoantypyrine tracer method. Caprioli and Miller50 demonstrated that iodoantypyrine tracer measurements of ONH BF were largely influenced by tracer diffusion from the nearby choroid, which has high BF. In the current study, a significant two-phase pattern of ONH BF change was demonstrated following chronic IOP elevation and the BF changes were also closely correlated with RNFLT loss. Although the initial, “early-stage” increase of ONH BF was not strongly correlated with IOP summary parameters, it was specific to EG eyes (i.e., did not occur in fellow control eyes), which suggests this initial increase of ONH BF is most likely a direct effect of chronic IOP elevation in the EG eye. Thereafter, the continuous decrease of ONH BF in correlation with RNFLT loss suggests that diminished metabolic demand in ONH due to progressive axon loss is likely the cause of reduced ONH BF. The significance of these changes and their potential mechanisms are discussed further in the following sections.

The observation of increased BF during early-stage EG is interesting. However, potential confounding factors that might result in a similar apparent increase need to be ruled out before further consideration of its importance. Basal ONH BF in this study was always measured at a fixed IOP of 10 mm Hg by inserting a needle into the anterior chamber for manometric IOP control. According to previous studies, this procedure may result in a temporary hyperperfusion because of sudden increase of perfusion pressure associated with decreasing IOP from a level that was otherwise chronically elevated.40,41 It is possible that the magnitude of this transient hyperperfusion was higher in EG eyes than in control eyes because the IOP in EG eyes was generally higher than normal and/or because BF autoregulation might be abnormal in EG eyes. To rule out such a possibility, we compared ONH BF measured before the needle insertion with that measured 5 minutes after the needle insertion in the same eyes. In total, 226 pairs of such measurements from 24 eyes (12 EG eyes and 12 control eyes) were available and show that ONH BF in both the EG and control eyes was increased after manometric IOP lowering to 10 mm Hg. However, the magnitude of transient ONH hyperperfusion was not significantly different between EG and control eyes, nor was there any significant difference in the experimental eyes at baseline versus after induction of EG. Thus, it is unlikely that the ONH hyperperfusion observed during early-stage EG was due to a higher transient hyperemic response following manometric IOP lowering in EG eyes as compared with control eyes. It is rather more likely to reflect a chronic difference in basal ONH BF during this early EG stage.

The mechanisms underlying ONH BF increase during early EG are not clear. One possible reason is that the initial IOP elevation might cause a functional disturbance of ONH BF autoregulation by affecting either vascular smooth muscles directly or via a signal released from cells that modulate the BF autoregulatory system, such as perivascular astrocytes.42 Speculatively, these changes might have reset both the basal vascular tone that determines the basic BF level (to be higher) and/or the sensitivity of BF autoregulatory mechanisms to respond to altered perfusion pressure. Alternatively, regional metabolism might be increased during early-stage EG due to increased energy demands of ONH gliotic changes or lamina cribrosa remodeling.43-45 In contrast, during the later stages of EG, basal ONH BF was reduced, which may reflect reduced metabolic demand associated with neuronal loss and connective tissue changes and/or a possible disruption of the BF autoregulatory system. Thus, there exists a temporal
transition from increased ONH BF to a decrease in EG eyes: during the early stage, mild increase was initially observed within the anterior ONH; subsequently ONH BF declined gradually below the normal level in association with the progression of structural damage measured by thinning of the peripapillary RNFL. In a parallel study in which a microsphere technique was used to measure ONH BF at various depths through the ONH and anterior orbital ON, we noted that some eyes with early EG exhibited decreased BF in the anterior ONH but an increased BF in the posterior ONH, relative to their contralateral control eyes.\(^{31}\)

Our observation of a high correlation between ONH BF and RNFLT during the progression of EG agrees with a number of prior clinical studies, which have shown similar correlation between ONH BF and RNFLT.\(^{4}\) or between ONH BF and visual field deficit.\(^ {17-21}\) In a separate study on NHPs with idiopathic bilateral optic atrophy, a disease in monkeys that causes nonglaucomatous optic nerve degeneration without apparent IOP elevation, also found that decreased ONH BF was highly correlated with RNFLT.\(^ {35}\) Together, the results from this and previous studies suggest that the compromised ONH BF in human glaucoma is at least in part secondary to structural damage and reduced neuronal activities. This compromised ONH BF in turn, may increase the vulnerability of the ONH to increase to eventual significant decrease, with the latter phase showing a high correlation to the extent of RNFL loss. These findings provide strong experimental evidence suggesting that compromised ONH BF observed in glaucomatous patients with high IOP (or without) is at least in part the result of neural loss (and presumed reduced metabolic demand).

**Acknowledgments**

The authors thank Chelsea Piper and Yao Zhou for technical assistance and Leo Schmetterer for valuable comments.

Supported by National Eye Institute Grant R01-EY019939 (LW); Legacy Good Samaritan Foundation, Portland, Oregon (LW); and unrestricted financial support from Translational Medicine, Pfizer, Inc. (LW). Equipment support was from Heidelberg Engineering GmbH (CFB, BF).

Disclosure: G. Cull, None; C.F. Burgoyne, Heidelberg Engineering GmbH (F); B. Fortune, Heidelberg Engineering GmbH (F); L. Wang, None.

**References**


Basal Blood Flow and Autoregulation Changes in the Optic Nerve of Rhesus Monkeys with Idiopathic Bilateral Optic Atrophy

Chelsea Piper, Brad Fortune, Grant Cull, George A. Cioffi, and Lin Wang

**Purpose.** To characterize the hemodynamic features and the association with structural damage in the optic nerve head (ONH) of idiopathic bilateral optic atrophy (BOA) in rhesus macaque monkeys.

**Methods.** In five animals with BOA and nine healthy animals under general anesthesia (pentobarbital), intraocular pressure (IOP) was manometrically controlled. ONH blood flow was measured with a laser speckle flow graph device. Basal blood flow in global and quadrantial sectors was measured with IOP set at 10 mm Hg; autoregulation capacity was assessed by comparing blood flow changes before and after IOP was increased from 10 to 30 mm Hg. Spectral-domain optic coherence tomography was used to measure retinal nerve fiber layer thickness (RNFLT) by peripapillary circular scans.

**Results.** Compared with control eyes, RNFLT in BOA eyes was significantly less in all sectors (P < 0.001) except the nasal (P = 0.25); the average global and sectoral blood flow in all quadrants was significantly lower (P < 0.001). These blood flow changes were significantly correlated with corresponding sectoral RNFLT (P < 0.01) except the nasal (P = 0.25). After IOP was increased to 30 mm Hg, global blood flow was significantly reduced (P < 0.001), but with no regional preferences despite prominent temporal RNFLT loss; no significant blood flow change was observed in control eyes (P = 0.24).

**Conclusions.** Basal blood flow and autoregulation capacity in the ONH of BOA were significantly compromised, with a close correlation to structural changes. The hemodynamic changes showed no regional preference across the ONH, which was consistent with postmortem histological observations. (Invest Ophthalmol Vis Sci. 2013;54:714–721) DOI:10.1167/iovs.12-97773

Idiopathic bilateral optic atrophy (BOA) is a newly identified disease observed in rhesus macaque monkeys. A previous study has characterized the structural and functional abnormalities found in nine animals with the signs of this disease. Anatomical characteristics include loss of macular retinal ganglion cells, thinning of the retinal nerve fiber layer papillomacular bundles, temporal optic disc pallor, and temporal sector atrophy of the retrobulbar optic nerve. Results of electroretinography (ERG) and visually evoked cortical potential studies reveal loss of macular ganglion cell function consistent with the anatomical findings. It is not yet known, however, whether hemodynamic changes are associated with the affected tissues in BOA of the macaque.

In general, the basal blood flow to a tissue is maintained at, or slightly higher than, a tissue’s minimal metabolic requirement. The basal blood flow in neural tissues, including ocular tissue such as the retina and optic nerve head (ONH), is intrinsically regulated under normal physiological conditions. This regulation maintains a close correlation between local neural activities and blood flow (known as neurovascular coupling) to allow appropriate oxygen and glucose delivery under fluctuating metabolic demand. Autoregulation mechanisms also maintain blood flow at a relatively constant level as perfusion pressure fluctuates. Thus, the efficiency of basal blood flow regulation is essential to maintain normal tissue perfusion and functions. Under pathological conditions, blood flow regulation may be disrupted. Studies in cerebral circulation have demonstrated that autoregulation may be modified or disturbed in several disease conditions resulting in hemodynamic changes and tissue damage. Impaired autoregulation in the ONH associated with altered blood flow was also demonstrated in experimental diabetes and hypercholesterolemia. In BOA eyes, although the specific cause of the disease remains unknown, the profound structural and functional damage in the retina and ONH may be associated with significantly reduced basal blood flow and perhaps also deteriorated autoregulation capacity.

Investigating these potential hemodynamic changes in the ONH of monkeys with BOA may help further our understanding of vascular pathology in diseases associated with optic nerve degeneration, such as glaucoma. For decades, autoregulation dysfunction has been proposed as one of the mechanisms of insufficient blood flow perfusion in glaucoma. Although a line of studies supports the existence of systemic vascular dysregulation, the evidence is mixed about whether local blood flow dysregulation in the ONH occurs as a preexisting condition or as a consequence of glaucomatous optic neuropathy. The current study was a cross-sectional comparison of blood flow in the ONH between five monkeys with BOA and a cohort of healthy controls using a laser speckle flowgraphy (LSFG) device. The LSFG measurement for ONH blood flow has been validated in a recent study. Autoregulation capacity was also investigated through comparison of blood flow changes in the ONH before and after the ocular perfusion pressure (OPP) was challenged by a controlled step increase of intraocular pressure (IOP). The results demonstrate significantly reduced basal blood flow and autoregulation capacity in the ONH of BOA monkeys.

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Supported by NIH Grant R01-019939 (LW), NIH Grant R01-EY019327 (BF), Good Samaritan Foundation, and unrestricted research funds from Pfizer, Inc.

Submitted for publication February 28, 2012; revised June 11 and November 28, 2012; accepted December 17, 2012.

Disclosure: C. Piper, None; B. Fortune, None; G. Cull, None; G.A. Cioffi, None; L. Wang, None

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Methods

Animals

All experimental methods adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local Institutional Animal Care and Use Committee. In total, 28 eyes of 14 adult rhesus monkeys (Macaca mulatta) were studied. Nine animals (seven female and two male, 9.4 ± 3.1 years) served as controls for this study. It was determined that the other five animals (three female and two male, 9.2 ± 5.5 years) had BOA on the basis of initial screening by direct ophtalmoscopy at Oregon National Primate Research Center; this was subsequently confirmed by more extensive ophthalmoscopy and ERG studies in our laboratory. The clinical findings included bilateral, symmetric thinning of the retinal nerve fiber layer thickness (RNFLT), predominantly within the papillomacular bundle; corresponding temporal pallor of the optic disc; and reduced ERG components corresponding to macular retinal ganglion cell function. Three of the five BOA monkeys were among those reported in our previous communication for structural and functional characterization, whereas two others have not been reported previously.

Anesthesia

All procedures were performed with animals under general anesthesia. In all cases, anesthesia was induced with 15 mg/kg intramuscular ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 1.5 mg/kg intramuscular xylazine (Phoenix Scientific Inc., St Joseph, MO), along with a single subcutaneous injection of atropine sulfate (0.05 mg/kg; Phoenix Scientific Inc.). Animals were intubated and breathed air. Heart rate and arterial oxygen saturation were monitored continuously (Propaq Encore model 206EL; Protocol Systems, Inc., Beaverton, OR). Body temperature was maintained with a warm-water heating pad at 37°C. Pupils were fully dilated with 1.0% tropicamide (Alcon Laboratories Inc., Fort Worth, TX). One of the superficial branches of a tibial artery was cannulated with a 27-gauge needle, which was connected to a pressure transducer (BLPR2; World Precision Instruments, Manchester, NH) and a four-channel amplifier system (Lab-Trax-4/24T; World Precision Instruments). Systolic, diastolic, and mean blood pressure (BP) in the artery were recorded continuously. If the arterial cannulation could not be obtained, the BP was intermittently measured with an automated Propaq system sphygmomanometer. Anesthesia was maintained by continuous administration of pentobarbital (6–9 mg/kg, intravenous) using an infusion pump (Aladdin; World Science Instruments Inc., Sarasota, FL). Pentobarbital was selected because unlike the volatile gas anesthetics, it has minimal impact on autoregulation capacity.17–21

Measurement of RNFLT

A spectral-domain optical coherence tomography (SD-OCT) instrument (Spectralis HRA-OCT; Heidelberg Engineering, GmbH, Heidelberg, Germany) was used to measure the RNFLT. Standard RNFLT scans consisted of a peripapillary circular B-scan with 1536 A-scans and a standardized diameter of 12°. Nine individual B-scan sweeps were averaged in real time using the instrument’s eye-tracking software to reduce speckle noise in the final recorded scan. Layer segmentations were evaluated and corrected manually if necessary (wherever the RNFL borders were incorrectly identified by the instrument’s native segmentation algorithm).

Blood Flow Measurement with Laser Speckle Flowgraphy

A laser speckle flowgraphy device (LSFG; Softcare, Iizuka, Japan) was used to measure the blood flow in the ONH. The principles of the laser speckle technique and its application in our lab to measure ONH blood flow in nonhuman primates have been described in previous publications.22–25

First, a fundus camera within the device was used to define an area centered on the ONH, which has dimensions of approximately 3.8 mm × 3 mm (width × height). After switching on the laser (λ = 850 nm, maximum output power 1.2 mW), a speckle pattern appeared due to random interference of the scattered light from the illuminated area, which was continuously imaged by a charge-coupled device (700 × 480 pixels) at a frequency of 30 frames per second for 4 seconds at a time. A first analysis software computed mean blue rate (MBR) of the speckle images. MBR is a squared ratio of mean intensity to the standard deviation of light intensity, which varies in time according to the velocity of blood cell movement and correlates well with capillary blood flow within the ONH.22–25 A composite MBR map representing blood flow arbitrarily distributed within the ONH was generated from each of the images within each 4-second series. After the area within the images corresponding to large blood vessels was eliminated, the capillary blood flow (AU) in the ONH was averaged. In addition, sectoral blood flow was determined for temporal, nasal, superior, and inferior quadrants of the ONH.25

Assessment of ONH Basal Blood Flow and Autoregulation by Stepped Increase in IOP

The animal was placed on a table in the prone position. Head position was fixed with an adjustable headrest and a bite bar to keep the face forward. Proparacaine HCl (0.5%) was administered topically, and a speculum was used to keep the eye open. The pupil was fully dilated with 1.0% tropicamide (Alcon Laboratories Inc., Fort Worth, TX). The IOP was then elevated from 10 to 30 mm Hg, by switching the solenoid valve, and maintained at the new level for at least 3 minutes. A second blood flow measurement was performed in the same manner as the first. Based on one of our previous studies,24 blood flow stabilizes at 5 minutes after IOP elevation. This measurement pair therefore represents a static phase of autoregulation, in contrast to dynamic-phase autoregulation as reported in previous studies.25 The blood flow difference between before and after the IOP elevation was calculated as a measure of autoregulation capacity in the ONH.

Histology

In one eye of four BOA and five normal control animals, retrobulbar optic nerves were sampled approximately 3 mm posterior to the globe (tissues from the other eyes were used for other histopathological studies). Transverse sections (0.5 mm thick, ∼2 mm behind the globe) were cut with a vibratome for each nerve and processed for immunohistochemical labeling of capillaries and astrocytes. The sections were first permeabilized with 3% Triton X-100 in 0.01 M PBS for 1 hour and then incubated with a mixture of 10% serum corresponding to the host species of secondary antibodies and 2% bovine serum albumin for 1 hour. Primary polyclonal rabbit anti-human glial fibrillary acidic protein (GFAP) (Z0334, 1:400; DakoCytomation, Glostrup, Denmark) and monoclonal mouse anti-human CD31 (1:50, kDa, 1:400, Dako North America, Carpinteria, CA) were applied and incubated at 4°C for 4 days. After three 1-hour rinses in 0.01 M PBS, corresponding secondary antibodies (Alexa Fluor 555 goat anti-rabbit, 1:400, and Alexa Fluor 488 goat anti-mouse, 1:100; Invitrogen Corporation, Carlsbad, CA) were applied overnight at 4°C. The
sections were washed in PBS (0.01 M) and mounted for microscopy. Negative controls for immunohistochemical stains were performed with omission of corresponding primary antibody from the solution.

Statistics

All blood flow data are reported as mean ± SD. Statistical analysis was performed using commercially available software (Statistica; StatSoft Inc., Tulsa, OK). The average ONH blood flow differences between BOA and control eyes were evaluated using analysis of variance (ANOVA). Bonferroni correction was employed to post hoc tests of group differences. The Mann-Whitney test was used to compare the median RNFLT thickness in each retinal region between BOA and normal eyes. Correlation between the RNFLT and blood flow in the ONH in each quadrant and globally in each eye was evaluated using Deming regression. A probability <0.05, unless otherwise specified, was considered the critical level for rejecting the null hypothesis.

RESULTS

Global and Regional RNFLT, BP, and IOP

The global average RNFLT was 69.9 ± 7.6 μm in the BOA eyes, which was significantly thinner than the value of 98.1 ± 6.1 μm observed in the normal eyes (P < 0.001). A typical example is shown in Figure 1. The Table lists the RNFLT for each of the four quadrants. As expected, the most severe RNFL thinning was found in the temporal quadrant, but RNFL loss was somewhat surprisingly found in the other quadrants as well. The average mean arterial BP during blood flow measurements was 75.8 ± 11.2 mm Hg (ranging from 60 to 91 mm Hg) in monkeys with BOA and 79.4 ± 6.36 (ranging from 63 to 90 mm Hg) in normal controls (P = 0.30 between the two groups). The corresponding OPP was 15 mm Hg less than the BP at IOP of 10 mm Hg and was 35 mm Hg less at IOP of 30 mm Hg. The additional 5 mm Hg was to correct the height difference between the tested eye and the level at which BP was measured.

IOP measured with a handheld tonometer (TonoLab; Oculab, Inc., Glendale, CA) with monkeys under ketamine/xylazine anesthesia was 13.9 ± 2.1 and 13.6 ± 2.1 mm Hg for right and left eyes in normal monkeys. The IOP in BOA eyes was 15.8 ± 4.8 and 15.8 ± 5.6 mm Hg for right and left eyes, respectively. The mean difference between the normal and BOA eyes was not statistically significant (P = 0.12).

Mean Blood Flow and Autoregulation Capacity in the ONH of BOA

The average basal ONH blood flow measured at an IOP of 10 mm Hg was 8.44 ± 1.17 (AU) in BOA eyes, which was significantly lower than the value of 10.9 ± 1.28 (P < 0.001) measured in control eyes (Fig. 2). The corresponding mean OPP during basal blood flow measurements in BOA and normal eyes, respectively, was approximately 60 and 63.5 mm Hg. Figure 2 shows the ONH blood flows measured at IOP 10 and 30 mm Hg in both normal and BOA eyes. Three minutes after the IOP was increased to 30 mm Hg, the average blood flow was reduced from 8.44 ± 1.17 to 6.65 ± 1.36 in BOA eyes (P < 0.001, ANOVA); however, there was little change in normal control eyes (10.9 ± 1.28 vs. 11.08 ± 1.36, P = 0.24). On average, the ONH blood flows were reduced by 21% in BOA eyes; they actually increased slightly, by 2%, in normal eyes after IOP was increased to 30 mm Hg. The corresponding OPP level during the IOP 30 mm Hg step challenge in BOA and

<table>
<thead>
<tr>
<th>Peripapillary RNFLT (μm, Mean ± SD)</th>
<th>BOA (n = 10)</th>
<th>Normal (n = 18)</th>
<th>P Value</th>
<th>BOA/Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>69.9 ± 7.6</td>
<td>98.1 ± 6.1</td>
<td>&lt;0.001</td>
<td>-29%</td>
</tr>
<tr>
<td>Superior</td>
<td>90.8 ± 12.8</td>
<td>118.5 ± 7.2</td>
<td>&lt;0.001</td>
<td>-23%</td>
</tr>
<tr>
<td>Temporal</td>
<td>54.3 ± 15.2</td>
<td>71.0 ± 6.2</td>
<td>&lt;0.001</td>
<td>-52%</td>
</tr>
<tr>
<td>Inferior</td>
<td>99.5 ± 18.2</td>
<td>139.8 ± 12.7</td>
<td>&lt;0.001</td>
<td>-29%</td>
</tr>
<tr>
<td>Nasal</td>
<td>53.9 ± 11.1</td>
<td>62.6 ± 8.5</td>
<td>≈ 0.25</td>
<td>-14%</td>
</tr>
</tbody>
</table>
normal eyes, respectively, was approximately 40 and 43.5 mm Hg.

Regional Blood Flow

Regional ONH blood flow difference was also evaluated in four individual ONH sectors (superior, temporal, inferior, and nasal quadrants) to determine if the temporal sector in the BOA eyes had significantly greater blood flow decrease than the other ONH sectors due to significantly more RNFLT loss as shown in the Table (Fig. 3).

This same pattern of regional blood flow variation was observed in BOA eyes for both IOP levels as well, although blood flow in all four quadrants was significantly lower than in control eyes at IOP 30 mm Hg (P < 0.05 for all quadrants with Bonferroni correction). Two-way ANOVA showed that there was no significant interaction between IOP and sector (P = 0.70); thus it was concluded that the relative degree of autoregulation dysfunction is equivalent for all sectors/ quadrants. Further, the results indicate that quadrant blood flow of BOA eyes was evenly reduced across ONH sectors, regardless of the actual IOP level (10 to 30 mm Hg).

Correlation between the RNFLT and Blood Flow

The correlation between the basal blood flow at IOP 10 mm Hg and RNFLT was analyzed for global average values as well as individual quadrants. The global average ONH blood flow for each eye was closely correlated with global average RNFLT (P = 0.0003, Fig. 4, left panel). Similarly, ONH blood flow correlated significantly with corresponding sectoral RNFLT for three of the four quadrants (Fig. 4, right panels), with only the nasal sector showing no significant association. The P values of the correlation for the superior, temporal, inferior, and nasal quadrants were, respectively, 0.01, 0.001, 0.0002, and 0.25.

ONH Astrocytes and Capillaries in BOA and Normal Eyes

In all eyes for which histological studies were available (four BOA and five normal control animals), astrocytes labeled with GFAP and capillaries labeled with CD31 immunohistochemically could be observed across the transversely sectioned optic nerve (see Fig. 5). As expected, GFAP immunoreactivity in BOA eyes was significantly increased in areas with tissue damage compared with normal eyes, as demonstrated in our previous study.1 Surprisingly, the capillaries in the damaged regions of BOA eyes appeared to have a much higher density compared with normal eyes. This pattern of increased capillary density was observed in all four BOA eyes examined; Figure 5 shows one example.
DISCUSSION

The coupling between neural activity and blood flow in neural tissues has been demonstrated in studies mostly through enhancement of the neuronal activities. That is, a provoked neural activity leads to blood flow increase as demonstrated in human ONH by experimental visual stimuli. Following the same concept based on acute activity, one could suppose that chronically reduced neural activity may cause basal blood flow to decrease over the long term. In the normal ONH of nonhuman primates, metabolic rate and blood flow are both relatively high. In BOA eyes, RNFLT was 20% to 50% thinner compared to normal controls (see Table), with fewer axons passing through the ONH; these anatomical findings are associated with significant functional loss assessed by in vivo electrophysiology. Thus, neuronal activities in the ONH of BOA eyes were expected to be low; so were the energy demands and blood supply. In agreement with this premise, blood flow in the ONH of BOA eyes was on average 24% lower and closely correlated with the extent of RNFL thinning of the ONH. Thus, the blood flow in the ONH is coupled not only with increasing but also with decreasing neuronal activities. This notion may have some clinical importance with regard to

FIGURE 4. The relationship between RNFLT and blood flow was assessed for global average (left panel) and each of four sectors (right panels). The open circles represent the values in normal control eyes (n = 18); the solid circles represent values in BOA eyes (n = 10). The correlations were all statistically significant except for the nasal quadrant.

FIGURE 5. Micrographs show histological observations in one representative BOA eye (bottom panels) compared with a representative control eye (top panels). Axons (A, E) were stained with toluidine blue. Capillaries (B, F) and astrocytes (C, G) were immunohistochemically stained with endothelium markers CD31 and GFAP, respectively, in 50 µm thick sections. Compared with those in the normal eye (A), axons in the damaged area of the BOA eye (E, lower-left area) were so scarce that the corresponding fascicles were shrunken and collapsed (red dotted lines encircle one of the collapsed fascicles). The connective tissues in the septa became thickened (marked with asterisks). These changes in the BOA eye led to an appearance of higher capillary density (F) compared with normal eyes (B). In the same area, the GFAP immunoreactivity in the BOA eye was significantly increased (G) compared with the normal eye (C). Overlay of capillaries (D) and astrocytes (H) in the normal and BOA eyes, respectively. Scale bars: (A, E) 50 µm; other photographs 100 µm.
the interpretation of blood flow changes observed under chronic disease conditions such as glaucoma.

Previous studies have shown a close relationship between ONH blood flow and RNFLT in human glaucomatous eyes. However, it is difficult to determine whether the reduced blood flow was a preexisting pathological change or a consequence of glaucomatous optic atrophy—a "chicken or egg" question. More disturbingly, a large portion of axons are likely to have already become degenerated even at an "early" stage of glaucoma diagnosis. The result showing reduced ONH blood flow in BOA suggests that there is likely a similar situation in glaucoma. At least in part, reduced blood flow in the glaucomatous ONH could be a result of optic nerve degeneration after chronic axonal loss and reduced energy demands, though this does not preclude the possibility that it might act as preexisting condition to further progression of damage.

Interestingly, although ONH blood flow was closely correlated with RNFLT overall and for all but the nasal sector, the reduction of blood flow observed in the temporal quadrant in BOA eyes was not significantly worse than the reduction within the other quadrants, even though the RNFLT was an additional 30% thinner compared with relative loss in the other quadrants (Table). Instead, blood flow was even reduced across sectors of the ONH. One possible reason for this uniform blood flow reduction follows from results shown in Figure 5: All capillaries in the optic nerve reside within septa surrounding axonal fascicles. However, in regions of ONH with significant axonal loss in BOA eyes, the axon fascicles were significantly smaller, and the capillaries within the corresponding regions appeared to be more densely distributed compared to those in regions with less axonal loss in BOA or normal eyes. Thus, since the LSFG blood flow estimate represents the average flow density within the scattering volume of the tissue, the histological observations of capillaries were made approximately 5 mm behind the globe whereas the LSFG blood flow measurement was derived from approximately 1 mm of the anterior optic nerve. An additional possible reason for the uniform blood flow decrease is the rich capillary anastomosis within the ONH of BOA eyes. Although the specific cause of BOA remains unknown, a strong suspicion is that it is an inherited disease analogous to dominant optic atrophy or Leber's hereditary optic neuropathy in humans. Thus, the current observations suggest that a similar autoregulation change may occur in glaucoma. However, further study in a more controlled experimental optic atrophy condition, such as optic nerve transection, may be necessary to validate the findings.

The mechanism underlying autoregulation dysfunction in the ONH of BOA are unclear. Several cell types are thought to be involved in normal blood flow autoregulation, including pericytes, smooth muscle cells, vascular endothelial cells, and increasingly also astrocytes. It is possible that the gliotic ONH changes observed here in BOA (e.g., Fig. 5) exacerbate cell signaling and communication and thus disrupt autoregulation. For example, in a recent study by Shibata et al., failure of ONH autoregulation was demonstrated in experimental diabetes in rabbits—similar to our current observation in BOA monkeys—using the same LSFG technique. Interestingly, also in that study, intravitreal injection of a gap junction blocker to interrupt direct intracellular coupling in normal rabbits induced similar autoregulation changes. Since gap junctions are distributed largely within the endfeet of astrocytes in the ONH, which tightly ensheath the blood vessels, this suggests that astrocytes were likely involved in autoregulation dysfunctions in the pathological ONH. Indeed, it has been demonstrated that astrocytes are associated with at least normal blood flow autoregulation in coupling to neuronal activities. Though no studies have specifically documented the functional communication between astrocytes and blood vessels in the ONH, close structural relationships have been demonstrated. In BOA eyes, evidence of massive "activation" of astrocytes with enhanced expression of glial fibrillary acidic protein (GFAP) was shown in this and our previous studies. Together, these
findings suggest that the roles of astrocytes in neurovascular coupling and in the autoregulation system within the ONH in both normal and diseased conditions warrant further investigation.

The LSFG technique used in this study has certain limitations when applied in order to compare blood flow differences in tissues with different light absorbance/scattering properties. This is the case because two key components of the blur rate calibration underlying the LSFG measurement, a constant and the zero offset, are unknown in vivo and may vary between tissues with different absorbance/scattering properties such as in the pathological condition of BOA. It is possible that the absorption and/or scattering properties of ONH differ enough in BOA eyes to become a confounding factor in LSFG measurement (comparison of basal blood flow between BOA and controls). However, in a very recent study in our lab, blood flow changes measured with LSFG in the ONH of monkeys with experimental glaucoma, which exhibit pathological changes similar to those in BOA, were highly correlated with ONH blood flow measured simultaneously by the “gold standard” microsphere technique. This suggests that the basal ONH blood flow differences between BOA and healthy control eyes observed in this study using LSFG reflect true basal blood flow differences and are not due to potential artifact or confounding effects of altered tissue optical properties in the atrophied ONH.

In summary, the present study characterized hemodynamic features in the ONH of eyes with BOA and demonstrated that the basal blood flow and autoregulation capacity in the ONH of BOA were significantly reduced with no regional preferences despite prominent temporal sector neural damage. It also showed that the reduction of basal blood flow was closely correlated with structural changes in the ONH. These findings suggest that similar hemodynamic changes may develop in other diseases with optic nerve atrophy, such as glaucoma.

Acknowledgments

The authors thank Claude F Burgoyne for identifying two BOA animals used in the study and Yi Liang for technical assistance.

References


Anterior and Posterior Optic Nerve Head Blood Flow in Nonhuman Primate Experimental Glaucoma Model Measured by Laser Speckle Imaging Technique and Microsphere Method

Lin Wang, Grant A. Cull, Chelsea Piper, Claude F. Burgoyn, and Brad Fortune

**Purpose.** To characterize optic nerve head (ONH) blood flow (BF) changes in nonhuman primate experimental glaucoma (EG) using laser speckle flowgraphy (LSFG) and the microsphere method and to evaluate the correlation between the two methods.

**Methods.** EG was induced in one eye each of 9 rhesus macaques by laser treatment to the trabecular meshwork. Prior to lasering and following onset of intraocular pressure (IOP) elevation, retinal nerve fiber layer thickness (RNFLT) and ONH BF were measured biweekly by spectral-domain optical coherence tomography and LSFG, respectively, until RNFLT loss was approximately 40% in the EG eye. Final BF was measured by LSFG and by the microsphere method in the anterior ONH (MS-BFANT), posterior ONH (MS-BFPST), and peripapillary retina (MS-BFRP).

**Results.** Baseline RNFLT and LSFG-BF showed no difference between the two eyes (P = 0.69 and P = 0.43, respectively, paired t-test). Mean (± SD) IOP was 30 ± 6 mm Hg in EG eyes and 13 ± 2 mm Hg in control eyes (P < 0.001). EG eye RNFLT and LSFG-BF were reduced by 42 ± 16% (P < 0.0001) and 22 ± 13% (P = 0.005), respectively, at the final time point. EG eye MS-BFANT, MS-BFPST, and MS-BFRP were reduced by 41 ± 17% (P < 0.001), 22 ± 34% (P = 0.06), and 30 ± 12% (P = 0.001), respectively, compared with the control eyes. Interocular ONH LSFG-BF differences significantly correlated to that measured by the microsphere method (R² = 0.87, P < 0.001).

**Conclusions.** Chronic IOP elevation causes significant ONH BF decreases in the EG model. The high correlation between the BF reduction measured by LSFG and the microsphere method provides evidence that the LSFG is capable of assaying BF for a critical deep ONH region. (Invest Ophthalmol Vis Sci. 2012;53:8303–8309) DOI:10.1167/iovs.12-10911

Glaucoma remains a major cause of blindness around the world, yet the sequence of pathophysiologic events defining its etiology remains unknown. Although elevated intraocular pressure (IOP) is one of the major risk factors, impaired microcirculation of the ONH (OH), may contribute to initiation and progression of glaucoma. However, despite a number of studies that have demonstrated reduced blood flow (BF) in the glaucomatous ONH,1–4 the exact role of hemodynamic changes associated with the pathologic mechanisms in glaucoma remains obscure due partially to the complexity of the ONH blood supply, its unique regulatory mechanisms, and limitations of the technologies used for ONH BF measurement.5–7 BF in the ONH is supplied by two arterial systems.8,9 The superficial nerve fiber layer (NFL) is supplied principally by the central retinal artery (CRA); the prelaminar, laminar, and retrolaminar layers are supplied by ciliary arteries (CAS). Physiologically, BF regulatory mechanisms may also differ across these regions supplied by these two vascular systems due to differences in their autonomic innervations.10,11 It has also been proposed that it is the deep ONH region supplied by ciliary arteries that is associated with pathologic changes in glaucoma.6,12 However, the most current techniques available for BF measurement, particularly the optically based techniques, such as laser Doppler flowmetry, are thought to measure principally the most superficial tissue supplied by CRA (i.e., the NFL in the ONH surface).9 Contributions to the measurement from deeper layers supplied by the CAS may vary depending on the techniques and tissue properties.13–16 It becomes even more complicated when BF is measured in patients with significant NFL thinning, such as glaucoma. In these patients, BF from tissues that are otherwise normally too deep to reach using such measures may begin to contribute to the measured BF.17 Thus, application of optically based, in vivo modalities for ONH BF measurement in glaucoma is limited by lack of knowledge about the BF in deep tissues of the ONH, its relative contribution to measurement made by various modalities, and its possible alteration in glaucoma.

The microsphere method is an invasive terminal method for BF measurement that can be applied to virtually all tissues of the body if the tissue volume is sufficiently large to accommodate adequate numbers of microspheres. Because the microsphere method measures actual BF, it has often been used to validate BF measurements made using other techniques.18–20 One particular advantage of the microsphere technique is the capability it provides to measure ONH BF within specific layers.11 In the current study, ONH BF in a nonhuman primate model of experimental glaucoma (EG) based on chronic unilateral IOP elevation was determined by both a laser speckle flowgraphy (LSFG) device and the microsphere method, which measures...
BS within specifically defined depth regions of the ONH. Although the LSFG has been used in clinical settings and in experimental animals to assess ocular hemodynamics, there are concerns that variations in the optical properties of different ocular tissues and variations in those properties due to the transition from normal to a pathologic state, may affect the absorbance and scattering of the laser source, potentially confounding BF estimates made by LSFG (and other optical techniques). Comparing the BF measurement between the two methods may provide insight into how ONH BF changes in EG and validate ONH BF measurements made by LSFG, an emerging in vivo optical technique.

The purpose of the current study was therefore twofold. First, was to characterize ONH blood flow change at approximately 40% retinal nerve fiber layer thickness (RNFLT) loss in the EG model. Second, was to compare and correlate the BF measurements by LSFG and the microsphere method.

**Methods**

**Animals and Anesthesia**

Nine adult rhesus monkeys (*Macaca mulatta*) were included in the study (7 females, 2 males; 9.7 ± 3.1 years old). All procedures were performed with the animals under general anesthesia, adhered to the Association for Research in Vision and Ophthalmology’s Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee at Legacy Research Institute. In all cases, anesthesia was induced with intramuscular ketamine (15 mg/kg) and xylazine (1.5 mg/kg), along with a single subcutaneous injection of atropine sulfate (0.05 mg/kg). Animals were intubated and breathed air spontaneously. Heart rate, blood pressure (BP) was recorded continuously with a single subcutaneous injection of atropine sulfate (0.05 mg/kg). Animals were intubated and breathed air spontaneously. Heart rate, end tidal CO₂, and arterial oxygen saturation were monitored continuously (Propaq Encore model 206EL; Protocol Systems, Inc., Beaverton, OR). Body temperature was maintained with a heating pad at 37°C. Pupils were fully dilated with 1.0% tropicamide (Alcon Laboratories, Inc., Fort Worth, TX). One of the superficial branches of a tibial artery was cannulated with a 27-gauge needle, which was connected to a pressure transducer (BLPR2, World Precision Instruments [WPI], Inc., Sarasota, FL) and a four-channel amplifier system (Lab-Trax-4/24T; WPI). Arterial blood pressure (BP) was recorded continuously. Anesthesia was maintained with continuous administration of pentobarbital (6–9 mg/kg/h, IV) using an infusion pump (Aladdin; World Science Instruments, Inc., Sarasota, FL) in all procedures except during trabecular meshwork lasering sessions (see the following text).

**IOP Measurement and RNFLT Imaging Protocol**

IOP was measured by a handheld tonometer (Tono-Pen XL, Reichert, Inc., Depew, NY) in both eyes of each animal (mean of 3 measures per eye) within 30 minutes of general anesthesia induction. IOP in both eyes was manometrically controlled as described earlier. Peripapillary RNFLT was measured in both eyes of each animal using a commercial spectral-domain optical coherence tomography (SD-OCT) instrument (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany). For this study, the average peripapillary RNFLT was measured from a single circular B-scan consisting of 1536 A-scans. Nine to 16 individual sweeps were averaged in real time to comprise the final stored B-scan at each session. At the initial imaging session, the operator centered the position of the scan on the ONH (based on the view of the optic disc margin in the corresponding infrared reflectance image) and all subsequent scans were pinned (identical) to this location. A trained technician manually corrected the accuracy of the instrument’s native automated layer segmentations when the algorithm had obviously erred from the inner and outer borders of the RNFL to an adjacent layer (such as a refractive element in the vitreous instead of the internal limiting membrane, or to the inner plexiform layer instead of the outer border of the retinal nerve fiber layer). All segmentations were then exported for extraction of RNFLT values by custom software.

**Induction of Chronic Unilateral Experimental IOP Elevation**

Laser treatment to one eye of each animal was performed under ketamine and xylazine anesthesia. In all, 180° of the trabecular meshwork (50-μm spot size, 1.0-second duration, 600- to 750-mW power) were treated in each of two separate sessions at least 2 weeks apart. Laser burns were calibrated to achieve blanching and bubbling of the meshwork without evident “pops” or bleeding. A sub-Tenon’s injection of 0.5 mL of dexamethasone (10 mg/mL) was given in the inferior fornix of the treated eye. Laser treatments were repeated (but limited to a 90° sector) on multiple subsequent occasions as necessary to achieve sustained IOP elevation.

**Blood Flow Measurement with LSFG**

The principles of the LSFG technique (Softcare Ltd., Iizuka, Japan) and its application to measure ONH BF in nonhuman primates have been described in detail within previous publications. In brief, a fundus camera equipped within the LSFG device was used to define an area centered on the ONH, with dimensions of approximately 5.8 × 3 mm (width × height). After switching on the laser (l = 830 nm; maximum output power; 1.2 mW), a speckle pattern appears due to random interference of the scattered light from the illuminated tissue area, which is continuously imaged by a charge-coupled device (700 × 480 pixels) at a frequency of 30 frames per second for 4 seconds at a time. Offline analysis software computed the mean blur rate (MBR) of the speckle images. MBR is a squared ratio of mean intensity to the SD of light intensity, which varies temporally and spatially according to the velocity of blood cells’ movement and correlates well with capillary BF within the ONH. A composite MBR map representing BF distribution within the ONH disc was generated from the images of each 4-second series. After eliminating the area within the images corresponding to large blood vessels, capillary BF within the remaining ONH disc area was averaged and recorded in arbitrary units (AU) of MBR.

**BF Measured with the Microsphere Method**

Under general anesthesia, both femoral arteries and a vein were cannulated with polyethylene tubes (PE50) for BP monitoring, reference blood sample collection (see the following text), and administration of drugs, respectively. The left ventricle was cannulated via the right brachial artery with the same type of polyethylene tubing. Once arterial BP and end-tidal PCO₂ were stabilized within normal ranges, 60 million fluorescent microspheres (Polysciences, Inc., Warrington, PA) with a 10-μm diameter, suspended in a solution of 5 mL of 0.15 M NaCl and 0.05% Tween 20, were injected into the left ventricle over a period of 25 to 45 seconds after heparinizing the blood (500 IU/kg, IV). Note that in two male monkeys with larger body weight, 100 million microspheres were injected. A reference blood sample was drawn from one of the cannulated arteries, starting from the onset of microsphere injection, for 1 or 2 minutes. Animals were then euthanized by overdose of intravenous pentobarbital (Euthasol; Delmarva Laboratories, Inc., Midlothian, VA), then both eyes were enucleated and postfixed in 4% paraformaldehyde for 48 hours, after which they were embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek O.C.T. Compound; Sakura Fine Tek USA, Inc., Torrance, CA).

For each embedded eye, consecutive serial longitudinal frozen sections were obtained by a cryostat with 25 μm thickness. These sections were collected on a series of numbered glass slides and air dried. The microsphere concentration in the reference blood was determined in a hemacytometer (Fuchs–Rosenthal Counting Chamber,
Electron Microscopy Sciences, Hatfield, PA). Each ONH section was photographed with a 10× lens under a fluorescent microscope equipped with an automated imaging system. Montages of the photographs for each ONH section were created digitally. The total microspheres within a given region of ONH (see the following text) were counted. With the microsphere counts in both reference blood and ONH, total BF in a given defined region (microspheres within a given region of ONH) was calculated as:

\[ \text{BF} = \frac{N_{\text{microspheres}}}{C_{\text{ref}}} \]

where \( N_{\text{microspheres}} \) is the microsphere count in the tissue of interest and \( C_{\text{ref}} \) is the microsphere number per \( \mu L \) reference blood per minute.

The ONH BF was determined for two layers (depth regions) divided by the posterior border of the lamina cribrosa: the first layer included the superficial NFL, prelaminar tissue, and lamina cribrosa (anterior ONH); the second layer included the first 1 mm of the retrolaminar optic nerve (posterior ONH). BF in the peripapillary retina was also measured in seven pairs of the eyes from an annulus of tissue obtained by two trephine cuts (6 and 2.5 mm) centered on the optic disc. The number of microspheres within this 23.4 mm² peripapillary annulus of retina was counted and BF was calculated using the same method as described earlier for ONH BF.

### Experiment Protocol

Three to five baselines of IOP, RNFLT, and LSFG-BF were acquired from both eyes of each animal. EG was then induced by laser treatment to one eye of each animal to induce chronic IOP elevation. The IOP, RNFLT, and LSFG-BF measurements were then repeated biweekly following the onset of laser treatment to induce IOP elevation until RNFLT in the EG eye had declined by approximately 50% of the baseline average. A final BF measurement was then performed by LSFG and the microsphere method on the day when the animal was euthanized. In six of the nine animals, the last LSFG BF measurement was followed immediately by the microsphere BF measurement. In two animals, the LSFG-BF was measured 4 and 7 days prior to the microsphere method, respectively. In one animal (ID 28506), the last LSFG-BF measurement was measured 45 days earlier than that measured by the microsphere method due to development of cataract in the control eye, thus preventing clear in vivo imaging. All BF measurements by both LSFG and the microsphere method were performed at least 5 minutes after IOP had been manometrically set at 40 mm Hg.

### Statistics

All data were reported as mean ± SD. Statistical analysis was performed using commercially available software (Prism V 5.0.4; SoftPad Software, Inc., La Jolla, CA). Paired Student’s \( t \)-tests were used to evaluate the mean BF difference between the eyes and between baseline and final measurements within eyes. Linear regression was used to correlate the interocular BF differences measured by LSFG and the microsphere method. Probability <5% was considered as the critical level for rejecting the null hypothesis.

### RESULTS

#### IOP and RNFLT

Average postlaser IOP in the EG eyes was 30 ± 6 vs. 13 ± 2 mm Hg in the contralateral controls (\( P < 0.001 \)). The highest IOP recorded in the EG eyes averaged 46 ± 7 mm Hg, ranging from 35 to 57 mm Hg and the total duration of elevated IOP was 4.8 ± 2.6 months, ranging from 1 to 10 months. Baseline RNFLT showed no difference between the two eyes of each animal (\( P = 0.69 \)). RNFLT loss in the EG eye relative to prelaser baseline values was 42 ± 16% on average (\( P = 0.0001 \)), ranging from 6% to 59% at the end of last data collection, with one animal being terminated before the endpoint due to development of a cataract in the control eye.

#### LSFG BF Measurement

Mean baseline LSFG-BF was 11.1 ± 1.4 (AU) in the EG eyes versus 10.8 ± 1.4 in the fellow control eyes (\( P = 0.45 \)). Mean EG eye LSFG-BF at the last imaging session was 9.2 ± 1.7 (AU) versus 11.7 ± 1.9 in the contralateral control eyes (\( P = 0.005 \)), corresponding to a 22 ± 13% reduction (Table). In one of the EG eyes, the LSFG-BF was 9% higher than that in the contralateral control eye. This EG eye was also one of the two EG eyes with increased BF in the posterior ONH (see the following text and Table).

Longitudinal comparison of LSFG-BF between baseline and the endpoint showed no significant difference in the control eyes (\( P = 0.20 \)), but the LSFG-BF was significantly reduced at the endpoint in EG eyes (\( P = 0.01 \)).

Figure 1 illustrates the baseline and the final time point of optic disc photographs, peripapillary RNFLT measured by SD-OCT, and LSFG MBR composite maps in both eyes of a representative experimental animal (ID 28849).

### BF Measurement with the Microsphere Method

**BP during the BF Measurement.** Average mean BP was 93.2 ± 9.9 mm Hg immediately prior to microsphere injection. During injection (duration of 1 or 2 minutes), mean BP either did not change (\( n = 5 \)) or decreased transiently by <10 mm Hg.
BF in the Anterior and Posterior ONH (Figs. 2B–D). In control eyes, the average MS-BFANT was 1.37 ± 0.56 μL/min and in EG eyes it was 0.88 ± 0.54 μL/min (P < 0.0001, paired Student’s t-test; Fig. 2B). The reduction in MS-BFANT corresponded to a 41 ± 17% in the EG eyes relative to their fellow control eyes (Table). Average MS-BFPPOST was 4.60 ± 1.1 μL/min in control eyes and 3.45 ± 1.4 μL/min in EG eyes (P = 0.064), representing a 22 ± 34% reduction in EG eyes (Fig. 2C, Table). Although the overall trend was similar to MS-BFANT, the EG MS-BFPPOST reduction was complicated by the fact that two EG eyes demonstrated an increase rather than a decrease (Table). The combined BF of both anterior and posterior ONH (MS-BFANT, P) was 5.98 ± 1.5 μL/min in control eyes and 4.32 ± 1.4 in EG eyes (P = 0.016, Fig. 2D), representing a 25 ± 25% reduction in the EG eyes relative to their fellow control eyes (Table).

BF in the Peripapillary Retina (Fig. 1E). Total BF in the 23.4 mm² peripapillary annulus of retina (MS-BFPP) was 3.11 ± 0.85 μL/min in control eyes and 2.17 ± 0.63 μL/min in EG eyes (P = 0.001, n = 7). This difference represented a 30 ± 12% reduction in EG eyes relative to fellow controls (Table).

The Table lists the RNFLT changes measured by SD-OCT (column 2) and interocular BF difference (%) in each pair of the eyes measured by the LSFG (column 3) and by the microsphere method (columns 4–7). Note that the MS-BFANT values in all EG eyes were reduced (column 4). The average MS-BFPPOST (column 5) was also reduced in EG eyes except in two of the nine eyes, in which the MS-BFPPOST was 30% and 35% higher than that in their fellow control eyes (see numbers in bold in Table). The combined BF (BFANT, column 6) showed similar change to the MS-BFPPOST.

In the two EG eyes (ID 139 and ID 28506) with increased MS-BFPPOST as measured by the microsphere method, one of them (ID 139) showed 9% higher LSFG-BF as well. The other EG eye with higher MS-BFPPOST (ID 28506), had lower LSFG-BF.
measured 42 days earlier than the microsphere method.

**Correlation of Interocular BF Difference Measured by LSFG and the Microsphere Method**

LSFG and Microsphere BF data for eight of the nine animals were included in this analysis. Animal 28506 was excluded because the last available LSFG BF measurement occurred 42 days prior to euthanization (and its associated microsphere measurement). As shown in Figure 3, interocular BF differences measured by LSFG correlated significantly to microsphere MS-BFPOST ($R^2 = 0.88; P < 0.001$; Figs. 3B, 3C, respectively), but not with MS-BFANT ($R^2 = 0.051, P > 0.05$; Fig. 3A) in these eight eyes.

**DISCUSSION**

In the current study, compromised BF in the anterior ONH of EG eyes was demonstrated in nonhuman primates’ EG measured by both LSFG and the microsphere method. Compromised BF in the retrolaminar region was also demonstrated by the microsphere method, which enables evaluation of BF within specific depths of the ONH. The high correlation between the BF reduction in the posterior ONH but not anterior ONH measured by LSFG and that measured by the microsphere method provides evidence that the LSFG technique is capable of assaying BF for a critical region of the ONH, which served primarily by the short posterior ciliary circulation (and recurrent pial branches).

Increased IOP is one of the major risk factors associated with glaucomatous optic neuropathy including structural damage and likely also compromised microcirculation within the ONH. Biomechanical alterations of glaucoma within the ONH tissues is a topic of extensive investigation. The effect of glaucoma on ONH microcirculation, however, remains inconclusive. One of the longstanding questions about glaucomatous optic neuropathy is whether reduced BF is a preexisting pathologic change or a consequence of glaucomatous optic atrophy. This has been difficult to determine in part because a substantial portion of retinal ganglion cells/axons may have already become degenerated even at an ‘‘early’’ clinical stage of glaucoma. Previous studies in experimental models of glaucoma based on chronic IOP elevation failed to show significant BF changes probably because of either low sensitivity of the methods used to measure blood flow or insufficient statistical power. In the current study, the EG eyes had undergone approximately 5 months of chronic IOP elevation and achieved 42% RNFLT loss on average at the time of the microsphere BF measurement. The percentage of retinal ganglion cell/axon loss in the ONH was likely even higher, approaching approximately 60%, suggesting that the reduced BF detected in the glaucomatous ONH could be, at least in part, a result of ONH degeneration and consequently diminished metabolic demand.

Interestingly, the ONH BF was not reduced uniformly in depth as demonstrated by the microsphere method. Although all EG eyes had reduced MS-BFANT and the majority of the EG eyes had reduced MS-BFPOST, two of the nine EG eyes in fact had higher MS-BFPOST (see Table). This hyperemic response was also observed during earlier stages measured by LSFG in additional EG eyes (results of “staging” analyses; i.e., longitudinal LSFG versus RNFLT will be the subject of a separate communication). Previous studies by Quigley et al. showed similarly mixed BF changes between individual animals and

![Figure 2](image-url)  
**FIGURE 2.** BF in EG (black columns) and contralateral control eyes (white columns) measured by LSFG (A) and by the microsphere method (B–E). (B) Anterior ONH. (C) Posterior ONH. (D) Total BF in combined anterior and posterior ONH. (E) Peripapillary retina. Error bars = ±SD.

![Figure 3](image-url)  
**FIGURE 3.** Linear regression analysis shows the correlation of interocular BF differences in the anterior ONH (A), posterior ONH (B), and the combined anterior and posterior ONH (C) measured by LSFG ($y$) and microsphere method ($x$). Note the outlier in (A) (top left corner) was one of the two eyes with high MS-BFPOST (ID 139 in Table).
ONH depths, with an overall slight decrease of BF in the anterior ONH and a slight increase of BF in the retrolubar (posterior) ONH in nonhuman primate EG models, suggesting that during any given stage of EG, the vasculature in the posterior ONH may develop temporary hyperemia. This may explain, at least in part, the insignificant BF change in the two previous studies37,39 and perhaps also contradictory results of BF studies in human glaucoma.41

In the current study, the ONH BF was measured at IOP set at 40 mm Hg in both eyes. At this IOP, no BF change was expected in control eyes because the corresponding OPP was close to, but above, the lower limit of autoregulation in the ONH (Wang L, et al. IOVS 2012;53:ARVO E-Abstract 6851). In the EG eyes, since the OPP was just above the margin of the lower limit of autoregulation, the BF was more likely to be compromised if the autoregulation had failed. Thus, in addition to the structural damage, impaired autoregulation may also be one of the possible mechanisms contributing to the reduced BF observed in EG eyes. Although compromised ONH BF was demonstrated in EG eyes both by the microsphere method and by LSFG, each of these methods has certain limitations. When BF is measured by the microsphere method in small volume tissues, such as the ONH, the number of microspheres entrapped within the tissue is small, which may cause larger measurement variation. For this reason, we counted all three superficial ONH layers together (anterior ONH) to maximize the total microsphere numbers. Nevertheless, these numbers were still below the number typically recommended for tissue BF measurement.42 However, the relatively large effect size (i.e., the 25% BF difference between EG and control eyes) strengthened the statistical power and overcame the relatively small tissue volume, as has been observed in previous studies.43–45 Another limitation of the microsphere method in this study was the potential that the volume of tissue was reduced by the significant degree of neurodegeneration, which could result in fewer microspheres counted, irrespective of BF density within the tissue volume. The LSFG technique measures BF for the capillaries within an approximately fixed volume of tissue (i.e., effectively the capillary blood flow “density”), so the lower LSFG-BF and the strong correlation between LSFG-BF and BF measured by the microsphere technique would suggest that the capillary BF reduction in EG eyes was the dominant factor over potential reduction of tissue volume.

The laser speckle imaging technique also has certain limitations. It is thought that since the parameters of the calibration function (i.e., its constant and zero offset) used to calculate the blur rate of speckle images are derived from in vitro tests, the assumption that they may be appropriate for all tissues with varying light absorbance/scattering properties may be flawed.27 For example, large differences in the absorbance/scattering properties exist between myelinated and nonmyelinated regions of retina such that the LSFG measures in these areas might not be comparable.28 For the same reason, tissue properties may also differ under pathologic conditions, although this has never been verified. Thus, it has been recommended that BF comparisons between tissues with different properties should be avoided.27 In the current study, the BF comparison was restricted to the same tissue type (i.e., ONH). Under such a condition, the LSFG-BF between the pairs of eyes showed no difference prior to lasering. It was significantly decreased in EG eyes after chronic IOP elevation. Because the ONH in EG eyes at the endpoint had undergone significant tissue degeneration, it might be the tissue property change that contributed to the blur rate change, at least in part, rather than being strictly reflective of BF changes. However, this is not likely because, as the results of the current study demonstrated, the BF change measured by LSFG was strongly correlated with that measured by the microsphere method. Moreover, if the decreased blur rate in EG eyes was predominantly caused by tissue property changes, the blur rate would be expected to change uniformly in all EG eyes (either all increasing or all decreasing) in conjunction with the relatively uniform degree of neurodegeneration (e.g., as measured by RNFLT), regardless of whether the BF increased or decreased in these eyes. On the contrary, in several of the EG eyes with significantly reduced RNFLT, increased BF was observed for both LSFG and for the posterior ONH measured by the microsphere method. Together, the results of the current study leave little doubt that the potential confound on calculated blur rate caused by tissue degeneration, if any, is much smaller compared with the dominant signals derived from actual BF changes.

The results of this study also suggest that the MBR scale for LSFG (in AU) is compressed relative to BF measured by the microsphere technique. For example, the interocular LSFG-BF differences were smaller compared with that measured by the microsphere method as shown in Figure 3. This smaller interocular LSFG-BF difference is likely a result of error in the MBR calibration parameters having been derived from in vitro studies as mentioned earlier. Ongoing studies are being conducted to address at least the offset parameter for the LSFG calibration in vivo.

Notwithstanding these technical limitations, the present study demonstrated two important observations: the first is significantly reduced BF in the ONH after chronic IOP elevation measured by two independent techniques; the second is a strong correlation of BF measurement between the LSFG and the microsphere method. These findings provide experimental evidence of direct association between chronic IOP elevation and impaired ONH microcirculation, and validate the BF measurement by LSFG in ONH with experimentally glaucomatous neural degeneration.

References


Metformin Ameliorates Hepatic Steatosis and Inflammation without Altering Adipose Phenotype in Diet-Induced Obesity

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is closely associated with obesity and insulin resistance. To better understand the pathophysiology of obesity-associated NAFLD, the present study examined the involvement of liver and adipose tissues in metformin actions on reducing hepatic steatosis and inflammation during obesity. C57BL/6J mice were fed a high-fat diet (HFD) for 12 weeks to induce obesity-associated NAFLD and treated with metformin (150 mg/kg/d) orally for the last four weeks of HFD feeding. Compared with HFD-fed control mice, metformin-treated mice showed improved glucose tolerance and insulin sensitivity. Additionally, metformin treatment caused a significant decrease in liver weight, but not adiposity. As indicated by histological changes, metformin treatment decreased hepatic steatosis, but not the size of adipocytes. In addition, metformin treatment caused an increase in the phosphorylation of liver AMP-activated protein kinase (AMPK), which was accompanied by an increase in the phosphorylation of liver acetyl-CoA carboxylase and decreases in the mRNA levels of lipogenic enzymes and proinflammatory cytokines. However, metformin treatment did not significantly alter adipose tissue AMPK phosphorylation and inflammatory responses. In cultured hepatocytes, metformin treatment increased AMPK phosphorylation and decreased fat deposition and inflammatory responses. Additionally, in bone marrow-derived macrophages, metformin treatment partially blunted the effects of lipopolysaccharide on inducing the phosphorylation of JNK1 and nuclear factor kappa B (NF-kB) p65 and on increasing the mRNA levels of proinflammatory cytokines. Taken together, these results suggest that metformin protects against obesity-associated NAFLD largely through direct effects on decreasing hepatocyte fat deposition and on inhibiting inflammatory responses in both hepatocytes and macrophages.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined by fat deposition in hepatocytes (hepatic steatosis). In generally accepted concepts, NAFLD is comprised of simple steatosis, which may be benign, and non-alcoholic steatohepatitis (NASH), which is the advanced form of NAFLD. Simple steatosis progresses to NASH when the liver develops overt inflammation and necrotic damage that are not associated with alcohol consumption. It is now recognized that NASH is a leading causal factor of cirrhosis and hepatocellular carcinoma [1,2]. Additionally, hepatic steatosis is a major contributor of dyslipidemia that works with or without insulin resistance to significantly increase the incidence of atherogenic cardiovascular diseases [3]. Given this, a better understanding of how to reduce hepatic steatosis and how to decrease liver inflammation are of critical importance in effectively managing NAFLD and fatty liver-associated metabolic and inflammatory diseases.

Because NAFLD is highly prevalent in obese populations [4], obesity-associated insulin resistance is considered as a factor that critically contributes to the development of NAFLD. Mechanistically, insulin resistance at both hepatic and systemic levels, along with hyperinsulinemia, acts to increase the expression of genes for lipogenic enzymes such as acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) [5,6] and to decrease the expression of genes for fatty acid oxidation including carnitine


Editor: Jianping Ye, Pennington Biomedical Research Center, United States of America

Received: November 1, 2013; Accepted: February 6, 2014; Published: March 17, 2014

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Funding: This work was supported, in whole or in part, by American Diabetes Association (ADA) grants 1-10-JF-54, 1-13-BS-214-BR (to C.W.), and 1-10-BS-76 (to Y.H.). American Heart Association (AHA) grants 11-10-JF-54, 1-13-BS-214-BR (to C.W.), and 1-10-BS-76 (to Y.H.). National Institutes of Health (NIH) grants R01DK095828 and R01DK095862 (to C.W.), R01HL095556-01 and R21HL108922 (to Y.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Metformin treatment decreases HFD-induced hepatic steatosis and increases liver AMPK phosphorylation

NAFLD is commonly associated with obesity. Consistent with increased body weight, liver weight of HFD-fed and PBS-treated mice was much greater than that of LFD-fed mice (Figure 2A). Additionally, HFD-fed and PBS-treated mice displayed severe hepatic steatosis indicated by changes in liver sections stained with hematoxylin and eosin (H&E) and/or Oil-Red-O (Figure 2B). Compared with HFD-fed mice, serving as a mouse model of NAFLD, liver AMPK phosphorylation in HFD-fed mice was significantly increased compared with that of HFD-fed and PBS-treated mice (Figure 2C). Consistently, the phosphorylation of ACC, a substrate enzyme of AMPK, was decreased in HFD-fed and PBS-treated mice compared with that in LFD-fed mice. Upon treatment with metformin, liver ACC phosphorylation in HFD-fed mice was significantly increased compared with that of HFD-fed and PBS-treated mice (Figure 2C). We also examined changes in the mRNA levels of key enzymes that critically control the development of hepatic steatosis from livers of HFD-fed mice. In terms of regulating hepatic gene expression, the effects of HFD feeding have been previously studied in rodents by a number of investigators. However, HFD feeding increases liver FAS mRNA levels when compared with a Chow diet [24,25] and decreases liver FAS mRNA levels when compared with LFD feeding [26,27]. Additionally, HFD feeding appears to have limited effects on liver expression of ACC and sterol regulatory element-binding protein 1c (SREBP1c), the latter is a transcription factor whose active form stimulates hepatic expression of lipogenic enzymes including ACC and SREBP1c. In the represent study, we confirmed that HFD feeding increased liver mRNA levels of FAS compared with LFD feeding. Meanwhile, HFD feeding did not significantly alter the mRNA levels of ACC and SREBP1c. However, within all HFD-fed mice, treatment with metformin caused a significant decrease in the mRNA levels of ACC1 and FAS compared with PBS (Figure 2D). These results, together with decreased phosphorylation of liver ACC in metformin-treated mice, suggest a likely decrease in hepatic lipogenesis, which is consistent with the outcome of increased hepatic AMPK phosphorylation. Compared with LFD-fed and PBS-treated mice, HFD-fed and PBS-treated mice also displayed a decrease in liver AMPK phosphorylation (Figure 2C). This decrease was reversed by treatment with metformin as this was supported by the finding that liver AMPK phosphorylation in HFD-fed and metformin-treated mice was much greater than that in HFD-fed and PBS-treated mice (Figure 2C). Consistently, the phosphorylation of ACC, a substrate enzyme of AMPK, was decreased in HFD-fed and PBS-treated mice compared with that in LFD-fed mice. Upon treatment with metformin, liver ACC phosphorylation in HFD-fed mice was significantly increased compared with that of HFD-fed and PBS-treated mice (Figure 2C). We also examined changes in the mRNA levels of key enzymes that critically control the development of hepatic steatosis from livers of HFD-fed mice. In terms of regulating hepatic gene expression, the effects of HFD feeding have been previously studied in rodents by a number of investigators. However, HFD feeding increases liver FAS mRNA levels when compared with a Chow diet [24,25] and decreases liver FAS mRNA levels when compared with LFD feeding [26,27]. Additionally, HFD feeding appears to have limited effects on liver expression of ACC and sterol regulatory element-binding protein 1c (SREBP1c), the latter is a transcription factor whose active form stimulates hepatic expression of lipogenic enzymes including ACC and SREBP1c. In the represent study, we confirmed that HFD feeding increased liver mRNA levels of FAS compared with LFD feeding. Meanwhile, HFD feeding did not significantly alter the mRNA levels of ACC and SREBP1c. However, within all HFD-fed mice, treatment with metformin caused a significant decrease in the mRNA levels of ACC1 and FAS compared with PBS (Figure 2D). These results, together with decreased phosphorylation of liver ACC in metformin-treated mice, suggest a likely decrease in hepatic lipogenesis, which is consistent with the outcome of increased hepatic AMPK phosphorylation. Compared with LFD-fed and PBS-treated mice, HFD-fed and PBS-treated mice also displayed a

Palmitoyltransferase 1a (CPT1a) [7]. These changes, in turn, bring about hepatic steatosis. As a primary “hit”, fat deposition is sufficient to trigger the inflammatory responses as indicated by the results from cultured hepatocytes [8,9]. As another key characteristic of obesity, adipose tissue dysfunction has also been implicated in the development of NAFLD. Indeed, this role of dysfunctional adipose tissue is highlighted by the “second hit” hypothesis. In support of this, adipocyte-specific overexpression of monocyte chemotactrant protein-1 (MCP1), an inflammatory molecule up-regulated in adipose tissue of obese mice and human subjects, mediates the effect of adipose tissue inflammation to bring about an increase in hepatic triglyceride content [10]. These results and many others suggest that dysfunctional adipose tissue contributes to hepatic steatosis by increasing the delivery of fatty acid flux to the liver [2] and by impairing liver insulin signaling through adipose tissue-driven inflammation [11,12]. Currently, a number of approaches that are capable of improving insulin sensitivity and adipose tissue functions, i.e., weight loss, metformin treatment, and insulin sensitization by thiazolidinediones (TZDs), have been considered driven inflammation [11,12]. Currently, a number of approaches that are capable of improving insulin sensitivity and adipose tissue functions, i.e., weight loss, metformin treatment, and insulin sensitization by thiazolidinediones (TZDs), have been considered for managing NAFLD [1,13–15].

Metformin is a widely used anti-diabetic medicine that effectively lowers plasma glucose levels primarily by decreasing hepatic glucose production (HGP) and by improving lipid metabolism in both liver and muscle tissues [16–19]. At the cellular level, metformin activates AMP-activated protein kinase (AMPK). This serves as a key mechanism by which metformin treatment brings about a wide range of metabolic benefits [20]. Recent evidence also suggests that metformin is capable of inhibiting hepatic gluconeogenesis, a key flux whose increase contributes to elevation of HGP and hyperglycemia, through pathway(s) other than AMPK [21,22]. Although the mechanisms underlying metformin actions are more complicated than what were thought before, there is accumulating evidence that demonstrates the beneficial effects of metformin treatment on improving hepatic steatosis and on inhibiting liver inflammation [13,23]. At the systemic level, however, it remains to be a critical need to explore the extent to which the anti-NAFLD effects of metformin are related to alternations in both the liver and adipose tissue. Accordingly, it was hypothesized that metformin protects against NAFLD through direct effects on liver metabolic and inflammatory responses and through an indirect effect on improving adipose tissue phenotype. The results of the present study support that metformin acts directly to improve hepatocyte fat metabolism and to suppress inflammatory responses of both hepatocytes and macrophages. Interestingly, the protective role of metformin in reducing obesity-associated hepatic steatosis and inflammation appears to be independent of alterations in adipose tissue phenotype.

Results

Metformin treatment ameliorates HFD-induced systemic insulin resistance and glucose intolerance

C57BL/6j mice were fed a high-fat diet (HFD) for 12 weeks to induce obesity (diet-induced obesity, DIO). Compared with age- and gender-matched mice that were fed a low-fat diet (LFD), HFD-fed and phosphate-buffered saline (PBS)-treated mice exhibited a marked increase in body weight (Figure 1A), as well as overt hepatic steatosis and increased liver inflammatory responses (see below, Figures 2 and 3). These results demonstrate the establishment of NAFLD in obese mice. In addition, HFD-fed and PBS-treated mice displayed a significant increase in the severity of insulin resistance and glucose intolerance (Figure 1, C and D) compared with LFD-fed and PBS-treated mice. Upon treatment with metformin, body weight of HFD-fed mice remained the same compared with HFD-fed and PBS-treated mice. Also, metformin treatment did not alter food intake of HFD-fed mice (Figure 1B, HFD-Met vs. HFD-PBS). However, metformin-treated mice showed a significant decrease in the severity of HFD-induced insulin resistance and glucose intolerance (Figure 1, C and D), which were indicated by changes in plasma levels of glucose in response to a peritoneal injection of insulin and glucose, respectively. Thus, treatment with metformin ameliorates HFD-induced systemic insulin resistance and glucose intolerance without altering body weight in DIO mice.

Metformin treatment decreases HFD-induced hepatic steatosis and increases liver AMPK phosphorylation

NAFLD is commonly associated with obesity. Consistent with increased body weight, liver weight of HFD-fed and PBS-treated mice was much greater than that of LFD-fed mice (Figure 2A). Additionally, HFD-fed and PBS-treated mice displayed severe hepatic steatosis indicated by changes in liver sections stained with hematoxylin and eosin (H&E) and/or Oil-Red-O (Figure 2B). Given this, HFD-fed mice, serving as a mouse model of NAFLD, were used to examine the therapeutic effects of metformin, as well as the underlying mechanisms. Compared with HFD-fed and PBS-treated mice, HFD-fed and metformin-treated mice exhibited a significant decrease in liver weight (Figure 2A), which was accompanied by a marked decrease in the severity of hepatic steatosis (Figure 2B). Thus, treatment with metformin effectively ameliorates hepatic steatosis in obese mice. We next examined changes in liver AMPK phosphorylation, which may underlie the beneficial effects of metformin. Compared with LFD-fed and PBS-treated mice, HFD-fed and PBS-treated mice showed a significant decrease in liver AMPK phosphorylation (Figure 2C). This decrease was reversed by treatment with metformin as this was supported by the finding that liver AMPK phosphorylation in HFD-fed and metformin-treated mice was much greater than that in HFD-fed and PBS-treated mice (Figure 2C). Consistently, the phosphorylation of ACC, a substrate enzyme of AMPK, was decreased in HFD-fed and PBS-treated mice compared with that in LFD-fed mice. Upon treatment with metformin, liver ACC phosphorylation in HFD-fed mice was significantly increased compared with that of HFD-fed and PBS-treated mice (Figure 2C). We also examined changes in the mRNA levels of key enzymes that critically control the development of hepatic steatosis from livers of HFD-fed mice. In terms of regulating hepatic gene expression, the effects of HFD feeding have been previously studied in rodents by a number of investigators. However, HFD feeding increases liver FAS mRNA levels when compared with a Chow diet [24,25] and decreases liver FAS mRNA levels when compared with LFD feeding [26,27]. Additionally, HFD feeding appears to have limited effects on liver expression of ACC and sterol regulatory element-binding protein 1c (SREBP1c), the latter is a transcription factor whose active form stimulates hepatic expression of lipogenic enzymes including ACC and SREBP1c. In the represent study, we confirmed that HFD feeding decreased liver mRNA levels of FAS compared with LFD feeding. Meanwhile, HFD feeding did not significantly alter the mRNA levels of ACC and SREBP1c. However, within all HFD-fed mice, treatment with metformin caused a significant decrease in the mRNA levels of ACC1 and FAS compared with PBS (Figure 2D). These results, together with decreased phosphorylation of liver ACC in metformin-treated mice, suggest a likely decrease in hepatic lipogenesis, which is consistent with the outcome of increased hepatic AMPK phosphorylation. Compared with LFD-fed and PBS-treated mice, HFD-fed and PBS-treated mice also displayed a
decrease in liver mRNA levels of CPT1a, a rate-determining enzyme that transfers long-chain fatty acids into mitochondria for oxidation. Within HFD-fed mice, treatment with metformin tended to increase liver mRNA levels of CPT1a, but the increase was not statistically significant. Collectively, these results suggest that metformin ameliorates HFD-induced hepatic steatosis, and this effect of metformin is associated with an increase in liver AMPK phosphorylation.

Metformin treatment inhibits HFD-induced liver inflammatory responses

Inflammation is the key factor that drives the progression of simple steatosis to NASH. We examined the content of macrophages/Kupffer cells (F4/80+ cells) in livers of the mice. Unlike adipose tissue which displays a marked increase in macrophage infiltration in response to HFD feeding as established by many publications, livers of HFD-fed and PBS-treated mice contained fewer numbers of F4/80+ cells (Figure 3A, quantitative data not shown). Also, treatment with metformin tended to increase liver content of F4/80+ cells. These results suggest that liver content of F4/80+ cells is not an ideal indicator of liver inflammatory responses. We then examined liver inflammatory signaling through JNK and NF-κB p65 and quantified the mRNA levels of proinflammatory cytokines to assess liver inflammatory responses. Compared with that in livers of LFD-fed and PBS-treated mice, the phosphorylation of JNK (p46) in livers of HFD-fed and PBS-treated mice showed a decrease in liver mRNA levels of IL-1β and tumor necrosis factor α (TNFα) compared with LFD-fed and PBS-treated mice. Within all HFD-fed mice, treatment with metformin significantly decreased liver mRNA levels of IL-6, as well as IL-1β and TNFα compared with PBS (Figure 3C). Together, these results suggest that metformin decreases liver inflammatory responses while improving hepatic steatosis in DIO mice.

Metformin treatment does not alter HFD-induced adiposity and adipose tissue inflammatory responses

During obesity, dysfunctional adipose tissue critically contributes to the development of NAFLD [12]. We examined changes in adipose tissue phenotype. Consistent with DIO, all HFD-fed mice displayed a marked increase in visceral fat mass compared with LFD-fed mice (data not shown). Following treatment with metformin, HFD-fed mice did not display significant changes in visceral fat mass compared with HFD-fed and PBS-treated mice. Additionally, the size of adipocytes in HFD-fed and metformin-treated mice did not differ from that in HFD-fed and PBS-treated mice indicated by adipose tissue histology (Figure 4A). Thus, treatment with metformin did not alter HFD-induced adiposity.

Figure 1. Metformin treatment ameliorates HFD-induced insulin resistance and glucose intolerance. Male C57BL/6J mice, at 5–6 weeks of age, were fed a high-fat diet (HFD) and treated with metformin (Met, 150 mg/kg body weight/d, in phosphate-buffered saline (PBS)) or PBS for the last 4 weeks of HFD feeding. As an additional control, gender- and age-matched mice were fed a low-fat diet (LFD) for 12 weeks and treated only with PBS for the last 4 weeks. Data are means ± SE, n = 6–10. (A) Body weight was monitored weekly during the feeding/treatment regimen. (B) Food intake was calculated based on food consumption per day per mouse. (C) Insulin tolerance tests (ITT). (D) Glucose tolerance tests (GTT). For C and D, mice were fasted for 4 hr and received an intraperitoneal injection of insulin (1 U/kg body weight) (C) or glucose (2 g/kg body weight) (D). *, P<0.05 and **, P<0.01 HFD-Met vs. HFD-PBS for the same time point (C and D).

doi:10.1371/journal.pone.0091111.g001
We next examined adipose tissue inflammatory responses. As indicated by percentages of mature macrophages (F4/80\(^+\) CD11b\(^+\) cells) from the isolated adipose tissue stromal vascular cells (Figure S1, A and B in File S1), adipose tissue macrophage infiltration in HFD-fed and PBS-treated mice was markedly increased compared with that in LFD-fed and PBS-treated mice (Figure S1C in File S1). However, within HFD-fed mice, treatment with metformin did not significantly alter adipose tissue macrophage infiltration (Figure 4C and Figure S1C in File S1). These results were consistent with the finding that adipose tissue content of F4/80\(^+\) cells in HFD-fed and metformin-treated mice did not differ from that in HFD-fed and PBS-treated mice (Figure 4B). Further analyses indicated that the percentages of proinflammatory macrophages (F4/80\(^+\) CD11b\(^+\) CD11c\(^-\) CD206\(^-\) cells) among mature adipose tissue macrophages in HFD-fed and metformin-treated mice also did not differ from those in HFD-fed and PBS-treated mice (data not shown). Unlike changes in liver AMPK signaling, adipose tissue AMPK signaling (phosphorylation) was not altered by metformin. Additionally, metformin treatment did not alter adipose tissue inflammatory signaling, indicated by the phosphorylation of JNK1 (p46) and NF-kB p65 (Ser536) (Figure 4D). When proinflammatory cytokine expression was examined for HFD-fed mice, treatment with metformin did not significantly alter adipose tissue mRNA levels of IL-1\(\beta\), IL-6, and TNF\(\alpha\) (Figure 4E), as well as mRNA levels of arginase 1, adiponectin, and resistin, which all are related to adipose tissue inflammation and function. Therefore, treatment with metformin for 4 weeks appears to have limited effects on altering adipose tissue phenotype in obese mice, indicated by adiposity and inflammatory responses.

**Metformin treatment inhibits hepatocyte fat deposition and inflammatory responses**

As indicated by the results listed above (Figures 2, 3, 4), the liver is the primary target through which metformin ameliorates NAFLD in obese mice. We next examined the direct effects of metformin on metabolic and inflammatory responses in H4IIE cells, a rat hepatoma cell line commonly used as a cell model of NAFLD [28,29]. Consistent with changes in hepatocyte fat deposition, the mRNA levels of ACC1 and FAS in palmitate-treated H4IIE cells were significantly increased compared with controls (Figure 5A). This effect of palmitate, however, was partially blunted by metformin treatment, indicating a direct effect of metformin on inhibiting hepatocyte fat deposition. Consistent with changes in hepatocyte fat deposition, the mRNA levels of ACC1 and FAS in palmitated-treated H4IIE cells were significantly increased compared with controls (Figure 5A). This effect of palmitate, however, was partially blunted by metformin treatment, indicating a direct effect of metformin on inhibiting hepatocyte fat deposition.
increased compared with those in control cells (Figure 5B). Following treatment with metformin, the stimulatory effect of palmitate on the expression of ACC1 and FAS was blunted (Figure 5B). In H4IIE cells, CPT1α mRNA levels were not significantly altered by palmitate in the absence or presence of metformin (Figure 5B). We also examined the mRNA levels of proinflammatory cytokines. Compared with the control, the mRNA levels of IL-6 were increased in palmitate-treated H4IIE cells. This stimulatory effect of palmitate was completely blunted by treatment with metformin (Figure 5B).

We examined AMPK signaling and inflammatory signaling in H4IIE cells. Compared with that in cells treated with control (in the absence of metformin and lipopolysaccharide (LPS)), the phosphorylation of AMPK was increased in metformin-treated cells (in the absence of LPS) (Figure 5C). In the presence of LPS alone, AMPK phosphorylation was decreased compared with that in control cells (in the absence of metformin and LPS), and this decrease was partially reversed by treatment with metformin. When inflammatory signaling was examined, metformin treatment did not significantly alter the phosphorylation of JNK1 (p46) and NF-κB p65 under the basal conditions (in the absence of LPS). However, metformin treatment significantly blunted the effect of LPS on increasing hepatic phosphorylation of JNK1 (p46) and NF-κB p65 compared with that in control (PBS)-treated hepatocytes. Together, these results suggest that metformin directly decreases hepatocyte fat deposition and suppresses hepatocyte inflammatory responses.

Metformin treatment suppresses macrophage proinflammatory activation

Macrophages/Kupffer cells play a critical role in controlling the development of hepatic steatosis and inflammation [30]. We examined a direct effect of metformin on macrophage proinflammatory activation. In the absence of metformin, bone marrow-derived macrophages (BMDM) exhibited a significant increase in the phosphorylation of both JNK1 (p46) and NF-κB p65 (Ser536) in response to LPS stimulation (Figure 6A), indicating LPS induction of macrophage proinflammatory activation. However, upon treatment with metformin, the effect of LPS on inducing the phosphorylation of JNK1 (p46) and NF-κB p65 (Ser536) was partially blunted. When macrophage expression of proinflammatory cytokines was analyzed, the mRNA levels of IL-1β, IL-6, and TNFα were markedly increased in control (PBS)-treated BMDM after LPS stimulation. However, in metformin-treated macrophages, the effect of LPS on increasing proinflammatory cytokine mRNA levels was significantly lessened (Figure 6B). Together, these results suggest that metformin has a direct effect on inhibiting macrophage proinflammatory activation.
Discussion

On an HFD, C57BL/6J mice developed obesity-associated insulin resistance, as well as hepatic steatosis and inflammation. Accordingly, HFD-fed mice were used as a model of NAFLD to assess the therapeutic effects of metformin. Consistent with the results obtained from both human subjects and rodent models [13,23,31], metformin treatment not only improved HFD-induced systemic insulin resistance and glucose intolerance, but also brought about a marked decrease in the severity of HFD-induced hepatic steatosis and inflammation. Using in vitro systems involving hepatocytes and macrophages, the present study demonstrates that metformin is capable of directly decreasing hepatocyte fat deposition and suppressing hepatocyte and macrophage inflammatory responses, which all are associated with increased AMPK phosphorylation. However, metformin treatment did not alter obesity-associated adipose tissue phenotype. Notably, HFD-induced adiposity and adipose tissue inflammation indicated by adipose tissue histology (H&E staining), (B) Adipose tissue sections were stained for F4/80+ cells. (C) Adipose tissue macrophage infiltration. Percentages of mature macrophages (F4/80+CD11b+ cells) in adipose tissue stromal cells were calculated using FACS analyses (n = 4–6). (D) Adipose tissue AMPK signaling and inflammatory signaling were examined using Western blot analyses (n = 4–6). (E) The mRNA levels of adipose genes were quantified using real-time PCR (n = 4–6). For bar graphs (C and E), data are means ± SE.

doi:10.1371/journal.pone.0091111.g004

Metformin treatment does not alter HFD-induced adiposity and adipose tissue inflammation. Mice were treated as described in Figure 1. After the feeding/treatment regimen, mice were fasted for 4 hr prior to collection of tissue samples. (A) Adipose tissue histology (H&E staining). (B) Adipose tissue sections were stained for F4/80+ cells. (C) Adipose tissue macrophage infiltration. Percentages of mature macrophages (F4/80+CD11b+ cells) in adipose tissue stromal cells were calculated using FACS analyses (n = 4–6). (D) Adipose tissue AMPK signaling and inflammatory signaling were examined using Western blot analyses (n = 4–6). (E) The mRNA levels of adipose genes were quantified using real-time PCR (n = 4–6). For bar graphs (C and E), data are means ± SE.

Metformin Ameliorates Fatty Liver Disease

Metformin is considered as an activator of AMPK. The latter, when in active form(s), exhibits an anti-lipogenic effect through suppressing hepatic expression of lipogenic enzymes including ACC1 and FAS [32]. In addition, active AMPK phosphorylates and inhibits ACC1/2 [33]. This leads to a decrease in the production of malonyl-CoA, which in turn releases the inhibitory effect on CPT1a to favor fatty acid oxidation. In combination, these effects of active AMPK are thought to account, to a large extent, for metformin actions on reducing hepatic steatosis. In the present study, two lines of evidence support that metformin acts through stimulating AMPK phosphorylation to reduce hepatic steatosis. Firstly, in the in vivo study, changes in liver AMPK phosphorylation, indicative of AMPK activity, were positively correlated with the phosphorylation of liver ACC and reversely correlated with the degree of hepatic steatosis and with changes in the mRNA levels of ACC1 and FAS. Secondly, using an in vitro system in which palmitate-treated hepatocytes served as a cell model of hepatic steatosis, the present study further supports the direct effects of metformin on suppressing hepatocyte fat deposition and on increasing hepatocyte AMPK phosphorylation.

In terms of regulating hepatic steatosis, liver macrophages/Kupffer cells have been previously shown to critically control hepatocyte fat deposition [30]. Considering this, the in vitro results of the present study are significant in that a direct effect of metformin on suppressing hepatocyte fat deposition likely is sufficient enough to mediate the anti-hepatic steatosis actions of metformin.

The present study also suggests a potential link between AMPK and liver inflammation that is altered by metformin. In support of this, reversal of HFD-induced decrease in AMPK phosphorylation by metformin was accompanied by decreases in the phosphorylation of liver JNK1 (p46) and in the mRNA levels of proinflammatory cytokines. This observation was consistent with the results of a recent study in which metformin decreased liver...
Figure 5. Metformin treatment blunts hepatocyte fat deposition, increases AMPK phosphorylation, and decreases inflammatory responses. (A) Hepatocyte fat deposition. Bottom panels, cells were incubated with palmitate (Pal). (B) Hepatocyte mRNA levels were quantified using real-time PCR. For A and B, H4IIE cells were treated with metformin (500 μM) or PBS in the presence of palmitate (250 μM) or bovine serum albumin (BSA) for 24 hr and stained with Oil-Red-O for the last 1 hr (A). (C) Hepatocyte AMPK signaling and inflammatory signaling were examined using Western blot analyses. H4IIE cells were treated with metformin (500 μM) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min. For bar graphs (B and C), data are means ± SE, n = 6–8. *, P < 0.05 and **, P < 0.01 Pal + PBS vs. BSA + PBS (B) or LPS vs. PBS (without LPS) under the same condition (with or without Met) (C); *, P < 0.05 and **, P < 0.01 Pal + Met vs. Pal + PBS (B) or Met vs. PBS (without Met) under the same condition (with or without LPS) (C).

doi:10.1371/journal.pone.0091111.g005

Figure 6. Metformin treatment suppresses macrophage proinflammatory activation. Bone marrow-derived macrophages were treated with metformin (500 μM) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min (A) or 6 hr (B). (A) Macrophage inflammatory signaling was examined using Western blot analyses. (B) Macrophage mRNA levels of proinflammatory cytokines were quantified using real-time PCR. For bar graphs (A and B), data are means ± SE, n = 4–6. **, P < 0.01 LPS vs. PBS (without metformin) in the absence of LPS (without metformin) (A); **, P < 0.01 Met vs. PBS (without metformin) in the presence of LPS (A) or Met + LPS vs. PBS + LPS (B).

doi:10.1371/journal.pone.0091111.g006
inflammatory responses in mice fed both a methionine- and choline-deficient diet (MCD) and an HFD [23]. However, the study involving MCD/HFD-fed mice did not address how metformin suppresses liver inflammation, which could originate from hepatocyte fat deposition, macrophage/Kupffer cell proinflammatory activation, and/or adipose tissue inflammation [28–30,34]. Because metformin did not alter HFD-induced adipose tissue phenotype, the present study examined the direct anti-inflammatory effects of metformin in both hepatocytes and macrophages. Of interest, treatment with metformin partially blunted the effect of LPS on inducing the phosphorylation of JNK1 (p46) and NF-kB p65 and on increasing the mRNA levels of proinflammatory cytokine(s) in cultured hepatocytes and macrophages. These results serve as convincing evidence to support that metformin acts directly on both hepatocytes and macrophages/Kupffer cells to suppress liver inflammation in obesity-associated NAFLD. It should be noted that metformin has a direct effect on reducing hepatocyte fat deposition as discussed above. This effect of metformin is believed to decrease hepatocyte production (release) of free fatty acids including palmitate, a potent proinflammatory fatty acid [28,29]. Considering this, it is likely that in the in vivo condition metformin actions on hepatocytes also generate an indirect effect on macrophages/Kupffer cells (via paracrine) to suppress liver inflammation in NAFLD. In other words, metformin likely also acts through reducing hepatocyte fat deposition to indirectly suppress macrophage/Kupffer cell inflammatory activation. Future study is needed to examine the proportional contribution of hepatocytes vs. macrophages/Kupffer cells to the anti-inflammatory effects of metformin.

As illustrated by widely accepted concepts, dysfunctional adipose tissue during obesity contributes to the pathogenesis of NAFLD by increasing the delivery of fatty acids and inflammatory mediators to the liver to exacerbate hepatic fat deposition and inflammatory responses. Because of this, we also postulated that improved adipose tissue phenotype would contribute to the anti-NAFLD effect of metformin. However, this was not the case. In the present study, adipose tissue, unlike the liver, did not respond to metformin treatment. Notably, HFD-induced adiposity and adipose tissue inflammation in metformin-treated mice did not differ from those in PBS-treated control mice. The underlying mechanisms remained to be elucidated, but may be attributable to the effect that metformin treatment did not alter adipose tissue AMPK phosphorylation. Indeed, metformin actions on adipose tissue phenotype remain controversial. While metformin is shown to reduce body weight (adiposity) in both human and rodent models, a number of papers also demonstrate that metformin does not alter body weight, in particular in rodents fed an HFD [18,35,36]. Additionally, the weight-loss effect of metformin is tied closely to a decrease in food intake, leading to a question of whether or not metformin directly acts on adipose tissue. Considering this, the beneficial effects of metformin on features of NAFLD appear to be due largely to the direct effects of metformin on the liver. What should also be mentioned is that metformin is capable of countering insulin-induced suppression of muscle fatty acid oxidation and promoting triglyceride storage in skeletal muscle [37]. These effects may also contribute to the beneficial effects of metformin on features of NAFLD.

As mentioned above, metformin directly suppressed macrophage inflammatory activation in vitro. However, this was not the case for the in vivo study, in which metformin treatment did not alter the inflammatory status of adipose tissue macrophages. Given that metformin did not alter adipose tissue AMPK, it is possible that the treatment regimen used by the present study was not sufficient enough for metformin to be effective in adipose tissue. To verify this, future studies are required to include a relative long period of time and/or a higher dose for metformin treatment. Additional to adipose tissue inflammatory status, adiposity of HFD-fed mice was also not reduced by metformin in the present study. When adipose and liver tissues were compared, adipose tissue macrophages interacted with a highly proinflammatory local environment likely due to excessive fat deposition whereas liver macrophages/Kupffer cells did not. Considering this, it is also possible that a potential anti-inflammatory action of metformin on adipose tissue macrophages was offset by the proinflammatory effects of adipocyte-derived factors in particular saturated fatty acids [38]. While this point needs to be further validated, it appears to be clear that metformin primarily targets the liver to improve features of NAFLD, and this effect of metformin is independent of adipose tissue phenotype.

In summary, the present study provides evidence to support the beneficial effects of metformin on reducing hepatic steatosis and inflammation of NAFLD, and these effects are independent of alterations of adipose tissue phenotype. Mechanistically, metformin actions are attributable to the effects of metformin on improving hepatocyte fat metabolism and on suppressing hepatocyte and macrophage inflammatory responses. As such, metformin supplementation could be an effective approach for treatment and/or prevention of obesity-associated NAFLD.

Materials and Methods

Animal experiments

C57BL/6j mice were obtained from the Jackson Laboratory and maintained on a 12:12 h light-dark cycle (lights on at 06:00). At 5–6 weeks of age, male mice were fed a high-fat diet (HFD, 60% fat calories, 20% protein calories, and 20% carbohydrate calories) for 12 weeks and treated with metformin (150 mg/kg body weight/d, solutions in PBS) or PBS via oral gavages for the last 4 weeks. As additional controls, gender- and age-matched C57BL/6j mice were fed a low-fat diet (LFD, 10% fat calories, 20% protein calories, and 70% carbohydrate calories) for 12 weeks and treated with PBS for the last 4 weeks. Both diets are products of Research Diets, Inc (New Brunswick, NJ) and contain the same amount of casein, L-cystein, cellulose, sucrose, soybean oil, and minerals. However, the HFD contains much more lard but none corn starch compared with the LFD. During the 12-week feeding/treatment period, body weight and food intake of the mice were recorded weekly. After the feeding/treatment regimen, mice were fasted for 4 hr before sacrifice for collection of blood and tissue samples [39–41]. Epididymal, mesenteric, and perinephric fat depots were dissected and weighed as visceral fat content [40]. Liver weight was also recorded. After weighing, part of epididymal fat was subjected to isolation of stromal vascular cells as described below. Additional adipose and liver tissue samples were either fixed and embedded for histological analyses (H&E staining) or frozen in liquid nitrogen and stored at --80°C for further analyses [40,42]. Some mice were fasted similarly and used for insulin and glucose tolerance tests as described below. All animals received human care and all study protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Insulin and glucose tolerance tests

Mice were fasted for 4 hr and received an intraperitoneal injection of insulin (1 U/kg body weight) or D-glucose (2 g/kg body weight). For insulin tolerance tests, blood samples (5 μl) were collected from the tail vein before and at 15, 30, 45, and 60 min after the bolus insulin injection. Similarly, for glucose tolerance

PLOS ONE | www.plosone.org 8 March 2014 | Volume 9 | Issue 3 | e91111
tests, blood samples were collected from the tail vein before and at 30, 60, 90 and 120 min after the glucose bolus injection [43,44]. The levels of plasma glucose were measured using an enzymatic assay kit (Sigma, St. Louis, MO).

Isolation of stromal vascular cells from adipose tissue

Adipose tissue stromal vascular cells (SVC) were isolated using the collagenase digestion method as previously described [43,45]. After digestion and centrifugation, the pelleted cells were collected as SVC and subjected to FACS analyses.

Flow cytometry analysis

Adipose tissue SVC were stained with fluorescence-tagged antibodies: anti-F4/80, anti-CD11b for macrophages, and anti-CD11c and anti-CD206 for macrophage inflammatory status as previously described [46], and subjected to FACS analyses using BD FACSAria II flow cytometer (BD Biosciences, San Jose, California, USA) that is operated by Texas A&M Health Science Center College of Medicine Cell Analysis Facility.

Oil-Red-O staining and immunohistochemical analyses

Frozen liver sections were stained with Oil-Red-O as previously described [32]. The paraffin-embedded liver and adipose tissue blocks were cut into sections of 3 μm thickness and stained for the expression of F4/80 with rabbit anti-F4/80 (1:100) (AbD Serotec, Raleigh, NC) [43].

Cell culture and treatment

H4IIE cells (rat hepatoma cells) were maintained in high glucose Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin as previously described [28,34]. At 80% confluence, H4IIE cells were treated with metformin (500 μM) or PBS in the presence or absence of palmitate (250 μM) for 24 hr to induce fat deposition. To examine lipid accumulation, the treated hepatocytes were stained with Oil-Red-O for the last 1 hr. To determine changes in hepatocyte AMPK and inflammatory signaling, metformin- or PBS-treated cells were supplemented with or without LPS (100 ng/ml) for 30 min prior to harvest. Cell lysates were prepared and used to measure the levels of AMPK, p46, p65 were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU).

Western blots

Western blots

Lysates were prepared from frozen tissue samples and cultured cells using the lysis buffer containing 50 mm HEPES (pH 7.4), 1% Triton X-100, 50 mm sodium pyrophosphate, 0.1 m sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mm benzamidine, and 2 mm phenylmethylsulfonyl fluoride. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1,000 dilution. The blot was followed by a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody kit (Immobilon™ Western; EMD Millipore, Billerica, MA, USA) as previously described [40]. GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of P-AMPK/AMPK, P-ACC/ACC, P-p46/p46, and P-p65/p65 were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU).

RNA isolation, reverse transcription, and real-time PCR

The total RNA was isolated from frozen tissue samples and cultured/isolated cells. Reverse transcription was performed using the GoScript™ Reverse Transcription System (Promega) and real-time PCR analysis was performed using SYBR Green (LightCycler® 480 system; Roche) [28,41,48]. The mRNA levels were analyzed for ACC1, FAS, CPT1a, SREPB1c, IL-1α, IL-6, TNFα, arginase 1, adiponectin, and/or resistin in tissue and/or cell samples. A total of 0.1 μg RNA was used for the determination. Results were normalized to 18 s ribosomal RNA as plotted as relative expression to the average of PBS-treated control, which was set as 1.

Statistical Methods

Numeric data are presented as means ± SE (standard error). Two-tailed Student’s t tests were used for statistical analyses. Differences were considered significant at the P<0.05.

Supporting Information

File S1 Contains Figure S1. FACS analysis of adipose tissue stromal vascular cells. Male C57BL6/J mice, at 5–6 weeks of age, were fed a high-fat diet (HFD) and treated with metformin (Met, 150 mg/kg/d, in phosphate-buffered saline (PBS)) or PBS for the last 4 weeks of HFD feeding (n = 4–6). After the feeding/treatment regimen, stromal vascular cells (SVC) were isolated from epididymal fat pads and subjected to FACS analysis. (A) SVC were included for FACS analyses. (B) SVC (without staining) were analyzed for APC and FITC. (C) SVC (with staining) were analyzed for F4/80 (FITC) and CD11b (APC) expression. (PDF)

Acknowledgments

The authors thank Dr. A. Villalobos for assistance with image analyses.

Author Contributions

Conceived and designed the experiments: YH CW. Performed the experiments: SW HX HL YZ XH J. Zhao XG TG TQ RB YP YX. Analyzed the data: SW HL YZ J. Zhao XG J. Zheng YX XA LC LC QL XX YH CW. Contributed reagents/materials/analysis tools: YH CW. Wrote the paper: CW.
References

Liver Diseases and Autoimmunity

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Based on the principal clinical and pathological features, autoimmune diseases can be broadly divided into localized (involving only a single organ or tissue) and systemic (involving multiple organs). The liver is a lymphoid organ that not only participates in the immune response, but also a target of autoimmune reactions. Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are three major types of autoimmune diseases localized in the liver or autoimmune liver diseases, whereas granulomatous diseases such as sarcoidosis and connective tissue diseases such as systemic lupus erythematosus, rheumatoid arthritis and primary Sjögren syndrome (PSS) are the major systemic autoimmune diseases with liver involvement.

Over the past decade, advances have been achieved in the diagnosis and treatment of autoimmune liver diseases and liver involvement in systemic autoimmune diseases.1–5 Accordingly, in this issue of Journal of Clinical Translational Hepatology, the aim of the review articles is to update the knowledge and current management of autoimmune liver diseases and liver involvement in systemic autoimmune diseases.

AIH is a chronic inflammatory liver disease of unknown cause, with an estimated prevalence of 0.5–1.0 per 100,000; it is more common in women than in men. There have been extensive studies on this important disease, and remission of the disease is now achievable by immunosuppression; however, the prognosis is very poor if the patients are left untreated. In this issue, Kapila et al. present the clinical manifestations of AIH, update pathogenesis of liver-directed immune injury and new concepts in the understanding of immune tolerance, and finally provide insight into the development of novel therapeutic approaches.6

Sarcoidosis is a systemic, granulomatous disease that usually affects multiple organs including the liver. The estimated prevalence of sarcoidosis is 2–60 per 100,000. So far, little is known about its etiology. Liver involvement in sarcoidosis is very common. In this issue, Tadros et al. introduce the epidemiology and clinical spectrum of hepatic sarcoidosis of the disease and explore the underlying mechanism for liver injury in hepatic sarcoidosis.7 Moreover, they provide state-of-the-art knowledge on the diagnosis, differential diagnosis (with PBC and PSC) and treatment of the disease.

PSS is a systemic autoimmune disorder with secretory gland dysfunction. The estimated prevalence is 0.1–3.3%, with significant female preponderance, and it is reportedly associated with increased risk of cancers. Liver involvement is one of the commonly reported extraglandular manifestations of PSS, and two main causes of liver disease, namely chronic viral infections and autoimmune liver diseases contribute to liver involvement in PSS. Diagnosis of the causes of liver involvement in PSS is very important but challenging. In this issue, Briton-Zerón et al.8 specifically describe the causes, and provide the diagnostic strategy accordingly.

There is no doubt that the new data provided in this issue will enrich our understanding and improve clinical treatment of autoimmune liver diseases and liver involvement in systemic autoimmune diseases. This is consistent with the objective of Journal of Clinical Translational Hepatology to deliver recent advances in pathogenetic mechanisms, and developments in basic, translational and clinical studies.

Conflict of interest

None.

References

Expression and diagnostic values of calretinin and CK5/6 in cholangiocarcinoma

Zhang et al.
Expression and diagnostic values of calretinin and CK5/6 in cholangiocarcinoma

Lanjing Zhang1,2,3, Renee Frank1, Emma E Furth1, Amy F Ziober1, Virginia A LiVolsi1 and Paul J Zhang1,4*

Abstract

Background: Mesothelin, a mesothelial marker, has been found expressed in and as a potential treatment target of cholangiocarcinoma (CC). It is possible that CC may be derived from the cells sharing mesothelial markers. However, the expression of other mesothelial markers in CC is largely unknown.

Methods: Thirty CC cases (10 extrahepatic and 20 intrahepatic) were retrieved from our institutional archive. The immunohistochemical study of Calretinin (DC8), WT1 (6F-H2), Lymphatic Endothelial Marker (D2-40), CK5/6 (D5/16 B4) and CK19 (b170) was done on formalin fixed paraffin embedded sections for 2–3 blocks of each case. We compared the expression levels between CC and normal bile duct (NBD) on the same block.

Results: All of the CC and NBD are positive for CK19 (23/23) and negative for WT1 (0/23) and D2-40 (0/23), except one CC positive for D2-40 (1/23, 3.3%) and one NBD positive for WT1 (1/23, 4.3%). Calretinin immunoreactivity was detected in 52.2% (12/23) of CC, but none in NBD (0/23). CK5/6 was also detectable in 73.3% (22/30) of CC and all NBD (30/30). Increased expression of calretinin and reduced expression of CK5/6 were more likely associated with CC than NBD (P < 0.001 and P = 0.002, respectively). The sequential staining pattern of positive calretinin and negative CK5/6 in calretinin negative cases has a sensitivity of 69.57% and a specificity of 100% for differentiating CC from NBD. CK5/6 expression was also more likely associated with well-differentiated CC (7/7 versus 12/20 in moderately differentiated, and 9/10 in poorly differentiated, P = 0.019) and extrahepatic CC (10/10 versus 12/20 in intrahepatic, P = 0.029), but there was no association between the calretinin expression and the CC grade or location.

Conclusion: Calretinin and CK5/6 immunohistochemical stains may be useful for diagnosing a CC. Their immunohistochemical results should be interpreted with caution in the cases with differential diagnoses of mesothelioma and CC. A full mesothelioma panel, including WT1 and/or D2-40, is recommended to better define a mesothelial lineage. The biology of calretinin and CK5/6 expression in CC is unclear, but might shed light on identifying therapeutic targets for CC.

Keywords: Cholangiocarcinoma, Calretinin, CK5/6 and immunohistochemistry, Differentiation

Introduction

Intrahepatic cholangiocarcinoma (CC) is a relatively rare carcinoma of the biliary tree, with rising incidence and mortality [1,2]. Its 1-, 2- and 5-year survivals in US are 24.5%, 11.8% and 3.2%, respectively [1]. The diagnosis and prognostication of CC become critical for managing those patients. Studies have shown that several immunohistochemical (IHC) markers are highly expressed in CC including Annexin A1 (94.1%), CK19 (89%), MOC31 (88.2%), CK7 (83.4%), CD133 (79%), claudin4 (69.2%), high mobility group A1 (HMGAI) (31.5%) and S100P [3-6], while others has no or very low expression in CC such as glypican 3 (GPC3) (7%) and biglycan (7%) [3]. However, the markers’ expression levels are rarely compared with that of normal bile duct (NBD) which could be a morphologic mimic for CC in small lesion or small sampling. In addition to pancreatic carcinoma, a recent study shows that mesothelin, a mesothelial marker, is also found in 33% of resected CC specimens, but not hepatocellular carcinoma (HCC) or normal liver tissue [7]. Moreover, mesothelin may be used as a target for
monoclonal antibody therapy in a subset of CC in mice and as a prognostic factor for CC [8,9]. It is possible that mesothelium related proteins and/or genes may also be present in other tumors and involved in their tumorigenesis. Indeed, mesothelin and calretinin are found expressed in thymic carcinoma, thymoma, and non-keratinizing squamous cell carcinoma of lung [10,11]. But little is known regarding the expression levels, if any, of other mesothelial markers such as calretinin, CK5/6, D2-40 and WT1. Hence, we aimed to examine the IHC staining pattern of those markers in CC and NBD, and to explore the potential “mesothelial” phenotype in CC. The findings may help identify more diagnostic markers and therapeutic targets for CC.

Materials and methods
Histologically and clinically well documented CC cases were identified and retrieved from our institutional archive. The inclusion criteria included: 1. diagnosis of cholangiocarcinoma could be confirmed by review of the slides; 2. A primary tumor must clinically and pathologically arise within the hepatobiliary system, 3. patient had no past and current history of tumors in other system, 4. It was a resection specimen and had at least 3 blocks with carcinoma available for immunohistochemical (IHC) stains; 5. It had both CC and nearby NBD present on the same slide (A control H&E slide was made to confirm this, after being cut for IHC stains).

The IHC protocol and related antibodies have been described before [12]. Briefly, the IHC stains of M2A antigen (clone D2-40, 1:25, Signet Laboratories, Dedham, MA), WT1 (clone 6F-H2, 1:400, DakoCytomation, Carpinteria, CA), calretinin (DC8, 1:50, Zymed Laboratories, South San Francisco, CA), and cytokeratin 5/6 (clone D5/16 B4, 1:25, DakoCytomation) and CK19 (clone b170, 1:100, Leica/Novocastra) were conducted on formalin fixed paraffin embedded tissue sections with standard IHC protocols (BondMax, Leica Microsystems, Buffalo Grove, IL) on 2–3 blocks of each case. Appropriate positive and negative controls were performed and validated.

The protein expression levels were independently assessed by two of the authors (LZ and RF). When a disagreement was present, the two would have a discussion, consult with the senior author (PJZ) and reach an agreement upon re-review of the case. A scoring scale of 0–3 was used, with 0 for negative, 1+ for <25%, 2+ for 26-75%, and 3+ for >75%. The staining intensity was not considered for the purpose of scoring the stains. The literature search for related IHC markers’ positive rates was performed by using the marker’s names and tumors as the search term in Pubmed (NCBI, NIH, USA) in Feb. 2014.

The statistical analyses were performed by using Stata (version 11, StataCorp LP). The 95% confidence intervals (CI) were calculated by using normal distribution. Pearson Chi-square test was used to determine the association between IHC markers and tissue samples, so was Fisher exact test as a confirmation for groups with case number fewer than 6. All of the P values were calculated for 2-sided. A P value less than 5% was considered statistically significant.

Results
A total of 30 CC cases (10 extrahepatic and 20 intrahepatic) with nearby NBD met the inclusion criteria and were included in this retrospective study. They were collected between 2005 and 2011. The stained slides from the 2 to 3 blocks of each case all showed similar IHC staining pattern for each marker evaluated. The IHC stains for D2-40 and CK5/6 were performed on all of the 30 cases, and for calretinin, CK19 and WT-1 on 23 of the 30 cases due to the difficulty in obtaining additional blank slides after the dropped tissue sections in the first 2 IHC attempts. As shown in Table 1, the CC and NBD of all cases (23/23, 100% for both) were positive for WT-1, a known pancreatobiliary marker. All CC and NBD were negative for WT-1 and D2-40, both known as mesothelial markers, except 1 NBD positive for WT-1 (1/23, 4.3%) and 1 CC positive for D2-40 (1/30, 3.3%). Those unexpected positive WT-1 and D2-40 stains were focal, and scored only 1+ (less than 25%, see Table 2).

No significant difference of CK19, WT-1 or D2-40 IHC stain was found between the CC and NBD groups (Tables 1 and 2).

Of the 23 CC cases evaluated for calretinin positivity, 12 (52.2%, 95% CI 30.6-73.2%) were stained positive. CC tumor cells of the 12 cases showed dense and diffuse nuclear and cytoplasmic staining pattern of calretinin (Figure 1), while negative in the adjacent NBD in all cases (P < 0.001). For CK5/6 evaluation, 22 (73%) of CC and all 30 (100%) of NBD were positive (Table 2, Figure 2). Comparison of the CK5/6 expression in CC and the adjacent NBD revealed CC stained weaker than NBD in 18 (60.0%) cases, similar to NBD in 10 (33.3%), and stronger than NBD in 2 (6.7%) (Tables 2 and 3). Eight out of the 30 CC cases (26.7%, 95% CI 12.3-45.9%) were stained negative for CK5/6 while none of the NBD was negative (P = 0.002). The difference of CK5/6 in CC versus adjacent NBD is statistically significant (P = 0.022, Table 3). Of note, the two cases with CC stained stronger than NBD had 3+ stains in CC and 2+ in NBD (25–50 staining area difference). In the meantime, 9 (50%) of the 18 cases with CC stained weaker than NBD presented a difference of 2+ or more (50-99%) (Table 3).

We then sought the best IHC diagnostic criteria for differentiating CC versus NBD by comparing various
Table 1 Immunohistochemical profiles of the cholangiocarcinoma and accompanying normal bile ducts

<table>
<thead>
<tr>
<th></th>
<th>Negative, n (mean%, 95% confidence intervals)</th>
<th>Positive, n (mean%, 95% confidence intervals)</th>
<th>Total, n (%)</th>
<th>P value*</th>
<th>P value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calretinin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>11 (47.8, 26.8-69.4)</td>
<td>12 (52.2, 30.6-73.2)</td>
<td>23 (100)</td>
<td>&lt;0.001</td>
<td>NA</td>
</tr>
<tr>
<td>NBD</td>
<td>23 (100, 85.2-100*)</td>
<td>0 (0)</td>
<td>23 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>23 (100, 85.2-100*)</td>
<td>0 (0)</td>
<td>23 (100)</td>
<td>0.312</td>
<td>1</td>
</tr>
<tr>
<td>NBD</td>
<td>22 (95.6, 78.1-99.9)</td>
<td>1 (4.3, 0.1-21.9)</td>
<td>23 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>29 (96.7, 82.8-99.9)</td>
<td>1 (3.3, 0-17.2)</td>
<td>30 (100)</td>
<td>0.313</td>
<td>1</td>
</tr>
<tr>
<td>NBD</td>
<td>30 (100, 88.4-100*)</td>
<td>0 (0)</td>
<td>30 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK19</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>0 (0)</td>
<td>23 (100, 85.2-100*)</td>
<td>23 (100)</td>
<td>0.312</td>
<td>NA</td>
</tr>
<tr>
<td>NBD</td>
<td>0 (0)</td>
<td>23 (100, 85.2-100*)</td>
<td>23 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK5/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8 (26.7, 12.3-45.9)</td>
<td>22 (73.3, 54.1-87.7)</td>
<td>30 (100)</td>
<td>0.002</td>
<td>NA</td>
</tr>
<tr>
<td>NBD</td>
<td>0 (0)</td>
<td>30 (100, 88.4-100*)</td>
<td>30 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: CC: Cholangiocarcinoma, NBD: accompanying normal bile duct, NA: not applicable, *comparison between CC and NBD by using Pearson Chi-square test, #comparison between CC and NBD by using Fischer’s exact test, ^one-sided, 97.5% confidence interval.

cutoff values of the 2 markers (Table 4). The best sensitivity of 69.59% was achieved by using the sequential stains of calretin and CK5/6, with positive calretinin and negative CK5/6 in the calretinin negative cases as the positive result for CC (sequential calretinin and CK5/6 criteria), while maintaining 100% specificity. This sensitivity was statistically higher than that of calretinin stain more than 2+, or negative CK5/6 stain. However, we did not find the sensitivity difference between the sequential calretinin and CK5/6 criteria and the others, including positive calretinin stain alone (52.17%), CK5/6 stained negative or 1+ (60.0%) and less CK5/6 stain in CC than NBD (56.67%) (Table 4). Our Fisher exact test also revealed that CK5/6 expression was more likely associated with well-differentiated CC and extrahepatic CC, but no association between the calretinin expression and the CC grade or location (Table 5).

Discussion
Cholangiocarcinoma is an uncommon carcinoma in the developed countries, but had a rising mortality in both UK and USA [1,2]. Its carcinogenesis and diagnostic markers are not well defined. Studies have revealed some IHC markers such as CK7, CK19, MOC31, claudin4, HMGA, CD133 and Annexin A1, with variable specificities (Table 6) [3-5,13]. In particular, little is known about the IHC marks’ expression in NBD. To our best knowledge, this study is the first to explore the use of a set of known mesothelial markers for differentiating CC from accompanying NBD.

Calretinin is a 29–30 kilodalton calcium binding protein primarily expressed in the nerves [33]. Since it was found expressed in mesothelioma in 1990s [34], many types of tumor and tissue are found immunocreactive to calretinin including 22.5% of examined colonic carcinomas [35], 81.5% of ameloblastomas [36], 36% of thymic carcinomas [10], 100% of cardiac myxomas [37], 56-100% sex cord-stromal and 90-100% fibrous neoplasms of the ovaries [38-40], 95% olfactory neuroblastoma [41], 95% of adrenal cortical tumors [42], 71% of synovial sarcomas [43], 15% breast carcinomas [44], skin [45] and others [46]. Our study indicates that CC may be calretinin positive regardless of CC grade and location, and should be considered in the differential diagnoses for calretinin positive tumor. As many as 52% of our CC cases showed strong nuclear and cytoplasmic calretinin expression, while none in the NBD, suggesting a potential role of calretinin in differentiating CC from NBD and in CC carcinogenesis (Table 4). In contrast, CK19 positivity does not discriminate CC and non-cancerous NBD, and should
be used only for confirmation of a bile duct (biliary) lineage.

We also examined the expression of other mesothelial markers in CC and NBD including WT1, D2-40 and CK5/6 [12,30,47-49]. Not surprisingly, our data showed that WT1 and D2-40 remained highly specific for mesothelial lineage, and should be included in the panel to differentiate mesothelioma from CC. Of note, WT1 has also been reported positive in more than 90% in adenomatoid tumors [50], 76% ovarian sex cord-stromal tumor [39], 77% serous papillary carcinoma of the ovary [40], and 29% of endometrioid carcinoma [40]. Caution therefore should be used when interpreting a WT1 positive stain, particularly in female patients. Addition of D2-40 may also improve the sensitivity and specificity for confirming mesothelial differentiation [12].

CK5/6 is a high molecular-weight cytokeratin highly expressed in stratified epithelium and mesothelium, first found useful for distinguishing mesothelioma from adenocarcinoma in late 1990s [51,52]. Its positive rate in mesothelioma is comparable to that of calretinin [20]. A recent systemic review also confirms that CK5/6 is one of the two most sensitive IHC markers (sensitivity of 83%), and one of the two most specific IHC markers (specificity of 85%) for epitheloid mesothelioma [30]. However, many tumors other than mesothelioma are also positive for CK5/6, including but not limited to 88% of adenosquamous carcinoma of the pancreas [53], 55% of metastatic squamous carcinoma of various origin [54], 75% of lung squamous cell carcinoma in fine needle aspirate specimens [55], 100% of squamous cell carcinoma in pleural fluids [14], 98% of squamous cell carcinoma and 18% of adenocarcinoma in the lung [56], 62% of urothelial carcinoma [57], and 50% of endometrial adenocarcinoma [57]. Our study showed that all NBD and 73.3% of CC were positive for CK5/6. Further analysis found that CK5/6 expression was significant lower in the CC than the accompanying NBD (Table 3). Those findings demonstrate the differential expressions of CK5/6 in CC and NBD, and suggest a potential use of CK5/6 in differentiating CC from NBD. The association between CK5/6 expression and extrahepatic and well-differentiated CC indicates a preference of using CK5/6 in those CCs.

Of the markers we tested, the best sensitivity to identify CC is reached by using sequential criteria of positive calretinin and negative CK5/6 in the calretinin negative cases, with a 100% specificity. Using positive calretinin stain criterion is straight forward, and will result in only a 17.47% loss of sensitivity, or missing 4 out of 23 cases as shown in our study. This may be the second best strategy of using calretinin and/or CK5/6 to differentiate CC from NBD. The criterion of less CK5/6 stain in tumor than NBD requires presence of both tumor and NBD in

<p>| Table 3 Comparison of CK5/6 stains between cholangiocarcinoma and normal bile duct |
|---------------------------------|--------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>NBD</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>4</td>
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<td></td>
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</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Stain intensity comparison: CC < NBD: 18, CC = NBD: 10, and CC > NBD: 2 (<P = 0.022); CC: Cholangiocarcinoma, NBD: accompanying normal bile duct. The number in bold shows the case number with stain difference of more than 1+ (>25% difference).

Figure 1: A case showed nuclear and cytoplasmic calretinin reaction in CC (A: H&E, B&C: calretinin ×408 & ×200), and a negative cytoplasmic stain in NBD (D: calretinin ×200).

Figure 2: A case showed nuclear and cytoplasmic CK5/6 reaction in CC (A: H&E, B: CK5/6 ×200) and a weaker cytoplasmic stain in a NBD (C&D: CK5/6 ×408 & ×200).
the same specimens, which would be particularly challenging in core biopsies and cytology specimens. We therefore doubt its clinical usefulness due to specimen type limitations. Negative or 1+ CK5/6 stain gave us the second highest sensitivity, but also led to a significantly lower specificity (100% vs 73.3%, \(P = 0.007\)). Despite its single IHC stain requirement, we felt the lower specificity would be a serious concern for its clinical use. Together, we recommend sequential staining pattern of positive calretinin and negative CK5/6 in the calretinin negative cases for differentiating CC from NBD. Future work may focus on larger-scale studies and differentiating CC from HCC and mesothelioma by using these criteria.

We summarized the expression profiles of the common IHC markers for CC, mesothelioma and HCC in Table 6. Interestingly, the positive rates of those markers vary among studies. For example, the positive rate of MOC31 in mesothelioma ranged from 8% to 35%. Consistent with our summary, an excellent review and other guidelines have confirmed such a variation among reports, and made the practical yet useful recommendations on how to best utilize those markers [15,48,58]. We suggest to include at least WT1 and D2-40 in the panel to confirm a mesothelial lineage, with optional addition of calretinin, CK5/6 and/or mesothelin. Should WT1 and/or D2-40 stain be not interpretable due to technical issues or limitations (such as a fallen section), one must use other mesothelial markers to rule out the tumors with calretinin and CK5/6 reactivity from mesothelioma, such as CC as shown in this study. The reported different positive rates of the IHC markers may be in part attributed to the various IHC staining methods. For example, we noticed that CK5/6 expression was present in 2% of lung adenocarcinoma by using 1:25 dilution of D5/16B4 antibody (Boehringer-Mannheim) and Envision + biotin free detection system in Dako AutoStainer [16], but 39% by using the same 1:25 dilution of different D5/16B4 antibody (Dako) and Envision + HRP detection system in the same IHC stainer [12]. Similarly, using different dilutions of calretinin antibody (rabbit,

### Table 4 Comparison of various calretinin and CK5/6 diagnostic criteria for cholangiocarcinoma

<table>
<thead>
<tr>
<th>No.</th>
<th>Criteria</th>
<th>CC Total CC Sensitivity</th>
<th>(P) value*</th>
<th>NBD Total NBD Specificity</th>
<th>(P) value*</th>
</tr>
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<tr>
<td>1</td>
<td>Cal 1+</td>
<td>12  23</td>
<td>52.17%</td>
<td>0.227</td>
<td>23  23</td>
</tr>
<tr>
<td>2</td>
<td>Cal 2+</td>
<td>8   23</td>
<td>34.78%</td>
<td>0.018</td>
<td>23  23</td>
</tr>
<tr>
<td>3</td>
<td>Cal 3+</td>
<td>4   23</td>
<td>17.39%</td>
<td>&lt;0.001#</td>
<td>23  23</td>
</tr>
<tr>
<td>4</td>
<td>CK5/6 -</td>
<td>8   30</td>
<td>26.67%</td>
<td>0.002</td>
<td>30  30</td>
</tr>
<tr>
<td>5</td>
<td>CK5/6 - or 1+</td>
<td>18  30</td>
<td>60.00%</td>
<td>0.472</td>
<td>22 30</td>
</tr>
<tr>
<td>6</td>
<td>Less CK5/6 staining in CC than in NBD</td>
<td>17 30</td>
<td>56.67%</td>
<td>0.337</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>Cal+, and CK5/6 - in Cal - cases</td>
<td>16 23</td>
<td>69.57%</td>
<td>23 23</td>
<td>100.00%</td>
</tr>
</tbody>
</table>

Note: Cal: calretinin, CC: Cholangiocarcinoma, NBD: accompanying normal bile duct, NA: not applicable, *Comparison between criteria 7 and other criteria by using Pearson Chi-square test, #Comparison between criteria 7 and 5 by using Fischer’s exact test.

### Table 5 Association of the cholangiocarcinoma grades and locations with calrectinin and CK5/6 expression

<table>
<thead>
<tr>
<th>Calretinin</th>
<th>CK5/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg</td>
<td>1+</td>
</tr>
<tr>
<td>WD</td>
<td>3  2  1  1</td>
</tr>
<tr>
<td>%</td>
<td>42.86 14.29 28.57 14.29</td>
</tr>
<tr>
<td>MD</td>
<td>6  2  2  0</td>
</tr>
<tr>
<td>%</td>
<td>60  20  20  0</td>
</tr>
<tr>
<td>PD</td>
<td>2  1  0  3</td>
</tr>
<tr>
<td>%</td>
<td>33.33 16.67 0  50</td>
</tr>
<tr>
<td>EH</td>
<td>3  0  3  1</td>
</tr>
<tr>
<td>%</td>
<td>42.86 0  42.86 14.29</td>
</tr>
<tr>
<td>IH</td>
<td>8  4  1  3</td>
</tr>
<tr>
<td>%</td>
<td>50  25  6.25 18.75</td>
</tr>
<tr>
<td>Sum</td>
<td>11  4  4  4</td>
</tr>
<tr>
<td>%</td>
<td>47.83 17.39 17.39 17.39</td>
</tr>
</tbody>
</table>

Notes: WD = well-differentiated, MD = moderately-differentiated, PD = Poorly-differentiated, EH = extrahepatic cholangiocarcinoma, IH = intrahepatic cholangiocarcinoma, \(P\): \(P\)-value, #: \(P\) > 0.005.
Zymed, South San Francisco, CA) seemed to result in different calretinin positive rates in lung adenocarcinoma (8% versus 23%) [11,12,16]. Another study also showed that the TTF-1 positive rates in hepatocellular carcinomas vary from 0% to 70% depending on the antibody manufacturer [28]. An in-house validation of new antibodies on various tumors seems a reasonable safe-guard approach and is recommended.

On the cancer biology level, calretinin has higher positive rates in CC than mesothelin (52.17% versus 33%), and may also be a more sensitive and/or specific therapeutic target for CC. However, little is known regarding the roles of calretinin and CK5/6 in the carcinogenesis of CC and the biology of biliary epithelium. Given the recent identification of both mesothelial progenitor cells and liver stem cells [59-62], we hypothesize that the expression of calretinin and CK5/6 in CC is an aberrant differentiation of liver/bile duct stem cells, or simply reflecting the partial mesothelial phenotype of the NBD. However, much research is needed to examine our hypothesis.

This study has several limitations. First, this retrospective study may have selection bias and moderate statistical power. Second, the calretinin and CK5/6 staining profiles were not compared between CC, mesothelioma and HCC. A direct comparison of calretinin and CK5/6 expression in those tumors would be more evident. Third, the calretinin and CK5/6 IHC patterns in ductal proliferation including reactive changes or benign ductal neoplasms were not assessed, but may be of particular value in differentiating carcinoma and non-cancerous lesions. Last, the prognostic value of calretinin and CK5/6 expression in CC is not explored in this study, but may be interesting to investigate because another mesothelial marker, mesothelin, has been considered for therapeutic targets for CC. Survival studies are beyond the scope of this study, however. Future work is needed to address the unanswered questions and our study’s limitations.

Conclusions
In conclusion, we for first time showed calretinin and CK5/6 expression in CC. The sequential criterion of positive calretinin stain and negative CK5/6 stain in calretinin negative cases has a sensitivity of 69.57% and a specificity of 100% for differentiating CC from NBD. Our data also suggest to include at least one or two markers more specific for mesothelial differentiation, such as D2-40 and WT1, in the panel to define a mesothelial lineage.

Abbreviations
CC: Cholangiocarcinoma; IHC: Immunohistochemical; GPC3: Glypican 3; NBD: Normal bile duct; CI: Confidence intervals.

Competing interest
The authors declared that they have no competing interests.

Authors’ contributions
LZ and PJZ, designed the study, conducted analyses, interpreted results and drafted the manuscript. LZ, AFZ and RF conducted the study and analyses. EFF and VAL reviewed the study design, interpreted results and were involved in manuscript development. All authors read and approved the final manuscript.

Table 6 Positive rates of immunohistochemical markers for mesothelioma, cholangiocarcinoma, lung adenocarcinoma and hepatocellular carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Mesothelioma (all subtypes included)</th>
<th>Cholangiocarcinoma</th>
<th>Lung adenocarcinoma</th>
<th>Hepatocellular carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-1</td>
<td>43-93% [11,12,14-17]</td>
<td>0%#</td>
<td>0-7% [12,14,16]</td>
<td>NA</td>
</tr>
<tr>
<td>D2-40</td>
<td>86-100% [12,18,19]</td>
<td>3%#</td>
<td>0-33% [12,18,19]</td>
<td>NA</td>
</tr>
<tr>
<td>Calretinin</td>
<td>92.4-100% [11,12,16,17,20,21]</td>
<td>52%#</td>
<td>8-23% [12,16,22]</td>
<td>NA</td>
</tr>
<tr>
<td>CK5/6</td>
<td>64-100% [11,12,15-17,20]</td>
<td>0-73.9% [23]</td>
<td>0-39% [12,14-16,23]</td>
<td>NA</td>
</tr>
<tr>
<td>CK19</td>
<td>89-100% [3,24]</td>
<td>NA</td>
<td>2-10.1% [3,24]</td>
<td></td>
</tr>
<tr>
<td>Glypican-3</td>
<td>NA</td>
<td>6-7% [3,24]</td>
<td>3.6-9.6% [25,26]</td>
<td>69-87.1% [3,24-26]</td>
</tr>
<tr>
<td>Arginase</td>
<td>NA</td>
<td>0% [24]</td>
<td>0% [26]</td>
<td>94-95% [24,26]</td>
</tr>
<tr>
<td>TTF-1</td>
<td>0% [16]</td>
<td>0-10% [23,27]</td>
<td>(nuclear) 20-74% [16,23] (cytoplasmic) 50-93% [27-29]</td>
<td></td>
</tr>
<tr>
<td>HepPar-1</td>
<td>NA</td>
<td>0-7% [23,24]</td>
<td>8.1% [26]</td>
<td>74-100% [23,24,26]</td>
</tr>
<tr>
<td>EMA</td>
<td>79-93% [16,21]</td>
<td>100% [31,32]</td>
<td>100% [16]</td>
<td>125-23% [31,32]</td>
</tr>
<tr>
<td>BG 8</td>
<td>7% [16]</td>
<td>NA</td>
<td>95-96% [16,17]</td>
<td>NA</td>
</tr>
</tbody>
</table>

Note: # indicates this study; Sensitivity reported in some studies [17] is included; NA: No IHC data on the respective marker are identified by searching the Pubmed database.
Acknowledgments

We thank Kenny A. O’Rourke at Rutgers Robert Wood Johnson Library of Health Sciences for her assistance in literature retrieval. Despite of our efforts to include all publications related to the topics, we may have inadvertently omitted some and would like to apologize to their authors.

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Received: 24 February 2014 Accepted: 9 April 2014
Published: 23 April 2014

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Cite this article as: Zhang et al: Expression and diagnostic values of calretinin and CKS/6 in cholangiocarcinoma. Experimental Hematology & Oncology 2014 3:12.
Original Article
Characterization of the pathologic and endoscopic measurements of colorectal polyp sizes with a focus on sessile serrated adenoma and high-grade dysplasia

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Received February 5, 2014; Accepted February 20, 2014; Epub March 15, 2014; Published April 1, 2014

Abstract: Background: The characteristics of pathologically measured (PMS) and endoscopically measured sizes (EMS) of the colorectal polyps (CRPs) is poorly understood, particularly in polypoid unremarkable mucosa (PUM), sessile serrated adenoma (SSA), and high-grade dysplasia (HGD). Methods: To characterize the discordance and correlation between the PMS and EMS of CRPs including PUM, SSA, HGD, hyperplastic polyp (HP) and adenomas, we conducted this prospective observational study on the polyps collected between August 2012 and December 2013. Results: PMS was significantly smaller than EMS in the 497 qualified CRPs regardless of the sites (left, transverse and right colorectum) or EMS (≥1 cm and <1 cm) subgroups. The PMS and EMS discordance was associated with a diagnosis of HP and adenoma (versus PUM, SSA or HGD), single fragment (versus multiple), 3 of the 8 endoscopists and PMS<1 cm (versus ≥1 cm). Despite a good correlation between EMS and PMS in the adenomas (κ=0.626, 95% confidence intervals [CI], 0.505-0.746) and a moderate correlation in the serrated polyps (SPs) including HP and SSA, (κ=0.424, 95% CI, 0.244-0.604), 40.4% (23/57) of the adenomas and 63.6% (21/33) of the SPs with EMS≤1 cm might warrant longer follow-up intervals since their PMS were <1 cm. The PMS and EMS had linear correlations except in CRPs with HGD or EMS≤1 cm. Conclusions: The discordance between PMS and EMS is associated with the pathologic diagnosis, fragment number, endoscopists and PMS, and may lead to different follow-ups in a considerable portion of adenomas and SPs.

Keywords: Polyp, surveillance, measurement, colonoscopy, pathology, cancer

Introduction
Colorectal cancer (CRC) is one of the leading causes of cancer related deaths in the US [1]. Colonoscopy could effectively reduce CRC risks by detecting and removing the CRC precursor lesions, mainly adenomas [2, 3]. After endoscopic resection of a colorectal polyp (CRP), an endoscopic surveillance is recommended by both the US and UK guidelines [4-6]. Specifically, the CRP size, polyp number, and polyp histology (villous versus tubular adenoma, and presence of high-grade dysplasia, in the US guidelines only) are used to determine CRC risks and follow-up intervals. However, the preferred method for CRP size assessment was not specified in the US guidelines [4, 7], while the UK ones mentioned using the endoscopically measured size (EMS) [5]. EMS in fact was used in the earlier studies demonstrating that adenomas ≥1 cm had high risks for CRC [8, 9].

The preferred CRP size assessment method is controversial. EMS has been reported both larger [10, 11] and smaller [12, 13] than the corresponding pathologically measured size (PMS).
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And most of the comparison studies only included adenomas and hyperplastic polyps (HP), and had a modest to acceptable sample size (31-230 CRPs) [10, 11, 14-18]. The PMS and EMS of adenoma with high-grade dysplasia (HGD), sessile serrated adenoma (SSA), and polypoid unremarkable mucosa (PUM) to our best knowledge have not been explored yet despite their clinical significance [4-6]. In addition, only 2 studies investigated the factors associated with the discordance and correlation between PMS and EMS of CRP [11, 19]. Large-scale, prospective studies ideally also including PUM, SSA and HGD are needed to better characterize the PMS and EMS of CRPs.

EMS was originally used in the hallmark trials on optimal CRP surveillance intervals [3, 9]. In the meantime, PMS is preferred by some for its higher reliability, accuracy and simplicity, and has been recommended by the European colorectal cancer screening pathology quality assurance guidance [11, 14, 15, 17]. Before the trials stratifying CRC risk with PMS become available, EMS seems more directly associated with CRP classification, risk prognostication and follow-up interval determination. Therefore, a potential linear correlation between PMS and EMS would help estimate EMS and determine proper follow-up interval, when EMS is not available. No studies have examined the linear correlation between PMS and EMS.

We therefore conducted this prospective study to investigate the discordance and potential linear correlation between the PMS and EMS in 497 qualified CRPs. HGD, adenoma, SSA, HP and PUM were all included to fill the aforementioned knowledge gap on SSA and HGD.

Materials and methods

Patients and study design

An IRB approval was obtained from University Medical Center of Princeton at Plainsboro. We prospectively included all qualified colonoscopy cases, for symptoms or screening, incurred at the same institution from August 2012 to December 2013. All of the patients had provided written informed consents for the colonoscopic procedures. The inclusion criteria were: 1. No history of or current diagnosis of inflammatory bowel diseases; 2. EMS data were available in the endoscopic report; 3. The endoscopic report indicated that the CRP was completely removed and retrieved; 4. All CRPs were collected between August 2012 to December 2013, except the cases with PUM between March to December 2013. No patients underwent additional or unnecessary colonoscopies or had alterations in their management as part of the study. The following information of the qualified cases was collected: patient’s age, gender, lesion site, pathologic diagnosis, EMS, PMS and the endoscopist initials.

Endoscopic procedure and pathological evaluation

Video endoscopes (Olympus Optical Co., Tokyo, Japan) were used for all procedures. The patients were prescribed polyethylene glycol lavage bowel preparation or equivalent and the examiners cleaned the colon during instrument insertion and withdrawal as much as possible. The CRPs were identified mainly during withdrawal. The EMS was rendered by using the largest diameter of each CRP in situ (to the nearest mm) with wide-open biopsy forceps as the reference. Eight endoscopists (four with a more-than-ten-year colonoscopy experience) with qualified adenoma detection rates participated in the study. The method of retrieval was at the endoscopist’s discretion: retrieval net or tripod.

Pathologic evaluations were performed by a gastrointestinal and liver pathology fellowship trained pathologist (LZ). All collected specimens were fixed in 10% formalin within 1 hour of the removal and then fixed for a minimum of
4 hours. Then the fixed specimens were cut into 2-mm slices according to standard pathology laboratory protocols. By using a micrometer, the PMS for the CRPs with single tissue fragment was the greatest dimension of the lesions, and the PMS for the CRPs with multiple fragments were the aggregation of the lesions’ greatest dimensions. The histological diagnoses, including PUM, HP, SSA, adenoma and HGD, were determined according to the Vienna classification system [20]. The endoscopists and the pathologists were blinded to each other’s measurements.

Statistical analysis

The data were entered manually and analyzed by using STATA version 12.0 (Stata Corp, College Station, Texas, USA). The paired continuous data were compared by using the paired Student’s t test. Linear regression analysis (LRA) was used to assess the correlation between PMS and EMS. The association between categorical data and interrater agreement were analyzed by using χ² test and κ statistics respectively. A two-tailed P<0.05 was considered statistically significant.

Results

As shown in Figure 1, 1073 CRPs were identified through the colonoscopic procedures. Of them, 12 were not assessed for PMS and 564 cases had no available endoscopic reports. Finally, 497 CRPs with both EMS and PMS were included for the analysis. The average patient age was 60.64 year old (±10.73 SD) and 273 (54.9%) of CRPs were taken from a male. We found 149 (30.0%) CRPs located in the left colon, 108 (21.7%) in the transverse colon and 240 (48.3%) in the right colon and rectum.

In the qualified 497 CRPs, the EMS (0.71±0.995 cm, Mean±SD) was significantly larger than the PMS (0.549±0.455 cm) (P<0.001). Compared with HP, the PMSs of HGD, adenoma and SSA were larger (P<0.001 for all). Compared with adenoma, the PMS of HGD (P=0.1343) and SSA (P=0.1602) were not statistically different. Our LRA showed that EMS and PMS significantly correlated with each other (Figure 2, coefficient 0.180 and P<0.001).

We classified 37 CRPs as PUM, 105 as HP, 84 as SSA, 262 as adenoma and 9 as HGD. All of the PUMs were described as a polyp or prominent fold in the endoscopic report. The mean EMS of HP and adenoma were 0.458 cm (±0.262 SD) and 0.784 cm (±1.183 SD) respectively, significantly larger than the paired PMS (0.372±0.218 cm, P<0.001 and 0.597±0.486 cm, P=0.007; respectively). No statistical differences between EMS and PMS were detected in the PUMs (P=0.097), SSA (P=0.197) or HGD (P=0.115). By using LRA, linear correlations between EMS and PMS were found in the PUM (coefficient 0.056, P=0.003), HP (coefficient 0.466, P<0.001), SSA (coefficient 0.882, P<0.001) and adenoma groups (coefficient 0.137, P<0.001), but not in the HGD group (coefficient 0.049, P=0.686).

The 397 CRPs with an EMS≤1 cm had a mean EMS of 0.46 cm (±0.191SD), significantly larger than the paired PMS (0.41±0.223 cm, P<0.001, Table 1). For the 100 CRPs with an EMS≥1 cm, there was also a statistical difference betw-
Pathologic and endoscopic measurements of colorectal lesion sizes

Table 1. Factors associated with the discordance and correlation between the pathologic and endoscopic measurements of colorectal polyp sizes

<table>
<thead>
<tr>
<th>Category (n, %)</th>
<th>PMS (mean (SD))</th>
<th>EMS (mean (SD))</th>
<th>P value</th>
<th>LRA Coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (497, 100%), mean (SD)</td>
<td>0.549 (0.455)</td>
<td>0.71 (0.995)</td>
<td>0.000</td>
<td>0.180</td>
<td>0.000</td>
</tr>
<tr>
<td>Sites (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left colon (149, 30.0%)</td>
<td>0.645 (0.568)</td>
<td>0.746 (0.736)</td>
<td>0.013</td>
<td>0.579</td>
<td>0.000</td>
</tr>
<tr>
<td>Transverse (108, 21.7%)</td>
<td>0.506 (0.348)</td>
<td>0.613 (0.434)</td>
<td>0.000</td>
<td>0.581</td>
<td>0.000</td>
</tr>
<tr>
<td>Right colon and rectum (240, 48.3%)</td>
<td>0.510 (0.409)</td>
<td>0.740 (1.273)</td>
<td>0.004</td>
<td>0.073</td>
<td>0.000</td>
</tr>
<tr>
<td>Pathologic Diagnosis (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUM (37, 7.4%)</td>
<td>0.338 (0.150)</td>
<td>0.678 (1.279)</td>
<td>0.097</td>
<td>0.056</td>
<td>0.003</td>
</tr>
<tr>
<td>HP (105, 21.1%)</td>
<td>0.372 (0.218)</td>
<td>0.458 (0.262)</td>
<td>0.000</td>
<td>0.466</td>
<td>0.000</td>
</tr>
<tr>
<td>SSA (84, 16.9%)</td>
<td>0.686 (0.558)</td>
<td>0.726 (0.549)</td>
<td>0.197</td>
<td>0.882</td>
<td>0.000</td>
</tr>
<tr>
<td>Adenoma (262, 52.7%)</td>
<td>0.597 (0.486)</td>
<td>0.784 (1.183)</td>
<td>0.007</td>
<td>0.137</td>
<td>0.000</td>
</tr>
<tr>
<td>HGD (9, 1.8%)</td>
<td>0.844 (0.456)</td>
<td>1.711 (1.473)</td>
<td>0.115</td>
<td>0.049</td>
<td>0.686</td>
</tr>
<tr>
<td>Fragment number (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single (278, 55.9%)</td>
<td>0.387 (0.326)</td>
<td>0.56 (0.626)</td>
<td>0.000</td>
<td>0.236</td>
<td>0.000</td>
</tr>
<tr>
<td>Multiple (219, 44.1%)</td>
<td>0.755 (0.510)</td>
<td>0.91 (1.297)</td>
<td>0.060</td>
<td>0.127</td>
<td>0.000</td>
</tr>
<tr>
<td>Endoscopists (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (51, 10.3%)</td>
<td>0.48 (0.306)</td>
<td>0.48 (0.322)</td>
<td>1.000</td>
<td>0.465</td>
<td>0.000</td>
</tr>
<tr>
<td>B (72, 14.5%)</td>
<td>0.57 (0.422)</td>
<td>0.78 (0.571)</td>
<td>0.000</td>
<td>0.667</td>
<td>0.000</td>
</tr>
<tr>
<td>C (35, 7.0%)</td>
<td>0.53 (0.356)</td>
<td>0.68 (0.433)</td>
<td>0.001</td>
<td>0.67</td>
<td>0.000</td>
</tr>
<tr>
<td>D (147, 29.6%)</td>
<td>0.46 (0.380)</td>
<td>0.68 (1.525)</td>
<td>0.071</td>
<td>0.050</td>
<td>0.015</td>
</tr>
<tr>
<td>E (23, 4.6%)</td>
<td>0.49 (0.198)</td>
<td>0.60 (0.158)</td>
<td>0.000</td>
<td>0.949</td>
<td>0.000</td>
</tr>
<tr>
<td>F (12, 2.4%)</td>
<td>0.63 (0.407)</td>
<td>0.80 (0.724)</td>
<td>0.152</td>
<td>0.51</td>
<td>0.000</td>
</tr>
<tr>
<td>G (62, 12.5%)</td>
<td>0.62 (0.363)</td>
<td>0.89 (1.035)</td>
<td>0.031</td>
<td>0.123</td>
<td>0.005</td>
</tr>
<tr>
<td>H (12, 2.4%)</td>
<td>0.49 (0.315)</td>
<td>0.66 (0.231)</td>
<td>0.005</td>
<td>1.164</td>
<td>0.000</td>
</tr>
<tr>
<td>EMS (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMS≥1 cm (100, 20.1%)</td>
<td>1.09 (0.690)</td>
<td>1.73 (1.871)</td>
<td>0.001</td>
<td>0.028</td>
<td>0.457</td>
</tr>
<tr>
<td>EMS&lt;1 cm (397, 79.9%)</td>
<td>0.41 (0.223)</td>
<td>0.46 (0.191)</td>
<td>0.000</td>
<td>0.703</td>
<td>0.000</td>
</tr>
<tr>
<td>PMS (n, %)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMS≥1 cm (60, 12.1%)</td>
<td>1.45 (0.704)</td>
<td>1.45 (0.816)</td>
<td>0.968</td>
<td>0.564</td>
<td>0.000</td>
</tr>
<tr>
<td>PMS&lt;1 cm (437, 87.9%)</td>
<td>0.43 (0.206)</td>
<td>0.61 (0.975)</td>
<td>0.000</td>
<td>0.050</td>
<td>0.000</td>
</tr>
</tbody>
</table>

PUM: Polypoid unremarkable mucosa; PMS: pathologically measured size; EMS: endoscopically measured size; LRA: linear regression analysis; SD: standard deviation; HP: hyperplastic polyp; SSA: sessile serrated adenoma; HGD: high-grade dysplasia.

Table 2. The impact of the pathologically and endoscopically measured lesion sizes on the surveillance intervals (based on the size cutoff of 1 cm)

<table>
<thead>
<tr>
<th>Category</th>
<th>Adenoma</th>
<th>Serrated polyps</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS&lt;1 cm (n=221)</td>
<td>PMS≥1 cm (n=41)</td>
<td>Total (n=262)</td>
</tr>
<tr>
<td>EMS&lt;1 cm, n (%)</td>
<td>198 (96.6%)</td>
<td>7 (3.4%)</td>
</tr>
<tr>
<td>EMS≥1 cm, n (%)</td>
<td>23 (40.4%)</td>
<td>34 (59.6%)</td>
</tr>
</tbody>
</table>

PMS: pathologically measured size; EMS: endoscopically measured size; Serrated polyps include hyperplastic poly; traditional serrated adenoma and sessile serrated adenoma; *: a P value less than 0.001 in the chi² tests; #: k=0.626 (95% confidence intervals [0.505 to 0.746]; ^: k=0.424 (95% CI, 0.244 to 0.804).

Table 2. The impact of the pathologically and endoscopically measured lesion sizes on the surveillance intervals (based on the size cutoff of 1 cm)

Even the EMS and PMS (1.73±1.871 cm vs. 1.09±0.690 cm, P=0.001). Our LRA showed a linear correlation between the EMS and PMS in the CRPs with an EMS<1 cm (coefficient 0.703, P<0.001), but not in the CRPs with an EMS≥1 cm (coefficient 0.028, P=0.457). When taken 1 cm as a cutoff in PMS per the US and British guidelines and recommendation [4-6], 337
CRPs were classified into the small-size group, with a mean PMS of 0.43 cm (±0.223 SD) and a mean EMS of 0.61 cm (±0.975 SD) (P<0.001). Among the rest 60 CRPs with their PMS≥1 cm, the EMS was similar to the PMS (1.45±0.816 cm versus 1.45±0.704 cm, P=0.968). However, the EMS had a linear association with the PMS in the CRPs with a PMS<1 cm or ≥1 cm, with different coefficients (coefficient 0.050, P<0.001 and coefficient 0.564, P<0.001, respectively).

Our results showed that 278 CRPs had single fragment and 201 had multiple fragments. In the single-fragment group, the EMS was larger than the PMS (P<0.001, Table 1) with a linear correlation between the two (coefficient 0.236 and P<0.001). Among the 219 multiple-fragment CRPs, the EMS was also possibly larger than the PMS (0.91±1.297 cm vs. 0.755±0.510 cm), but with no statistical significance (P=0.060), while LRA showed that the EMS and PMS correlation was statistically significant (coefficient 0.127, P<0.001).

The correlation and discordance between EMS and PMS were operator-dependent. Five of the 8 (62.5%) participated endoscopists estimated an EMS significantly larger than the PMS, while the other 3 estimated an EMS similar to the corresponding PMS (Table 1). The EMS were correlated with the PMS with regards to each individual endoscopist (coefficient 0.050 to 1.164 and P=0.001 to 0.015). The endoscopists’ practicing years (≥10 years versus <10 years) were not associated with the linear correlation between EMS and PMS (data not shown).

We last sought the association between the follow-up recommendations based on the PMS and EMS with 1 cm as the size cutoff, according to the US and British guidelines and recommendation [4-6, 21]. The EMS and PMS were found associated with each other in the adenoma and serrated polyps (SPs) by using χ² test (P<0.001, Table 2). There was a good correlation between the EMS and PMS in the adenomas (κ=0.626, 95% confidence intervals [CI], 0.505 to 0.746) and a moderate correlation in the SPs (κ=0.424, 95% CI, 0.244 to 0.604). However, 40.4% (23/57) of the adenomas and 63.6% (21/33) of the SPs with EMS≥1 cm would be reclassified as low-risk lesions based on a PMS<1 cm, and hence warrant a longer follow-up interval, suggesting that a considerable portion of adenomas and SPs may have been mis-sized and hence misclassified.

Discussion

Comparison and linear correlation between the EMS and PMS of CRPs

PMS of adenomas is an important factor for predicting cancer risks [8, 9] and determining the appropriate follow-up strategy [4, 5, 21]. Recent studies consider PMS as the “golden standard” and preferred it over EMS [10, 15, 18, 22]. However, most of the prior studies are limited by a modest to acceptable study size and/or retrospective study in nature. Not surprisingly, some studies showed EMS was larger than PMS [10, 11] and the others showed otherwise [12, 16]. Our large sample size (497 CRP) may offer more statistical power and shed light on the best measurement method for CRP CRC risk stratification.
We found that EMS was larger than PMS regardless of the CRP site (left, transverse and right colon) or PMS subgrouping (≥ and <1 cm). The definition/nature of the EMS and PMS may have contributed to their difference. PMS is based on the histologic evidence of CRP lesions, and may be different from the EMS which is endoscopic findings. In fact, we have noted that adenomas often were only a portion of the resected CRP (Figure 3). We propose that PMS is the bona fide lesion size of the completely resected CRPs, would better predict their biological behaviors than EMS, and therefore should be preferred over EMS for CRC risk stratification. Our proposal is supported by majority of the studies comparing PMS and EMS of adenomas and HPs [14-16, 22] and the European guidelines for CRP quality assurance [23], and may also be applied to PUM, SSA and HGD. In the cases of incomplete polypectomy, PMS may be smaller than bona fide CRP size and EMS may be more reliable.

There are several other possible reasons for the PMS and EMS differences. First, the optical distortion caused by distance and visual angle would affect the endoscopic and microscopic measurements. Second, the wide-opened forceps, commonly used reference for EMS, may not always be well with the largest dimension of the lesion, causing measurement bias. Its guiding effect may also be limited when comparing a CRP much larger than the forceps’ wide-opened mouth. A ruler may help but will still face the problem of alignment. Therefore, it is not surprising that PMS has less variation than the EMS assisted by a ruler [14]. Third, the grasp and removal of an excised CRP may sometimes cause deformation, probably leading to a different PMS. Fourth, it has been reported that there would be a shrinkage after fixation [14]. Of note, recent studies also found formalin fixation of CRP specimens did not affect CRP sizes [10, 11, 16]. Last, in this single-center study, the institutional endoscopists’ and/or the pathologist’s performance and environmental factors may have potentially biased the results. A multi-center may address these biases.

The different PMS and EMS may cause mis-measurement and reclassification of CRPs despite the good and moderate agreements between PMS and EMS in adenomas and SPs, respectively. Consistent with an early report of 61 CRPs [11], we showed 40% of the adenomas with EMS≥1 cm had a PMS<1 cm and would be reclassified from a high-risk adenoma to a low-risk one based on the size, while only 3.4% of the adenomas would be upgraded from a low-risk adenoma to a high-risk one. The recommended follow-up interval for those downgraded adenomas would therefore be changed from 3 years to 5-10 years [4, 21]. Similarly, 63.6% of SP with EMS≥1 cm would warrant a longer follow-up interval (from 3 years to 10 years) for their PMSs<1 cm, according to the US guidelines [6, 21]. Considerable economic and clinical consequences may be resulted in. Moreover, EMS of 1 cm was used in the earlier trials stratifying adenoma and HP risks for CRC [3, 9]. Our data hence suggest that the bona fide adenoma size cutoff (PMS) for higher CRC risk may be smaller than 1 cm. Despite the enthusiasm in and supporting data for using PMS as the preferred CRP size measurement method, more high quality evidence (i.e. randomized, prospective, multi-center studies) in our opinion are needed to demonstrate the usefulness of PMS for CRC risk assessment. Before those lines of evidence become available, one feasible approach is to use the here described linear association between PMS and EMS and related factors to predict the EMS with a known PMS. In fact, our linear regression models allow prediction of EMS based on the CRP types (pathologic diagnosis), PMS, and endoscopists, and hence a direct use of PMS for CRC risk prediction.

Factors associated with the PMS and EMS discordance and correlation

The EMSs of PUM and SSA were found similar to the PMSs in this study and had a linear correlation with the PMSs, which to our best knowledge has not been reported before. Compared with PUM, SSAs were more precisely measured under the endoscopy, probably due to a sessile endoscopic morphology and the larger sizes. Given the important role of SSA in CRC development [24-26], large sessile lesions may have been examined more carefully under endoscopy, and given additional examinations, such as staining and narrow band image. In addition, the border of SSA may be easier to identify for its sessile endoscopic morphology than that of pedunculated CRPs.
HGD is considered a criterion for high-risk adenoma by the US, but not the UK, guidelines and warrants a follow-up in 3 years [4, 21]. However, the adenomas with HGD have not been subject to the PMS and EMS comparison. Despite only 9 (1.8%) cases of HGD included in this study, we for the first time demonstrated no significant differences between the PMS and EMS in the adenomas with HGD, suggesting the PMS and EMS may be interchangeable in those cases. Interestingly, no linear correlation between those two was established in our LRA. Because HGD histology trumps the size of adenoma, at least in the US guidelines, the sizes of adenomas with HGD seem less important. However, there are no data on the potential synergistic effects of adenoma size and presence of HGD on CRC risk. It would be very interesting to study whether the CRPs with adenomas ≥1 cm and HGD will have higher CRC risks than that with HGD or adenomas ≥1 cm alone.

Besides the pathologic diagnosis of CRP, we also identified 3 factors associated with the PMS and EMS discordance including tissue fragment number, endoscopist and PMS sub-grouping (≥1 cm versus <1 cm). Those factors may help predict the PMS based on EMS or vice versa, and identify the causes of PMS and EMS discordance. In contrast to our findings, a study of 61 CRPs including only HP and adenoma showed that PMS and EMS discordance is not dependent on endoscopist and pathologic diagnosis [11]. We speculate the different findings may be attributed to addition of PUM, SSA and HGD in our study, different sample sizes, and different measurement performances of the pathologists and the endoscopists in the studies. Our data and the earlier work, however, agreed on that the PMS and EMS discordance is independent of CRP location [11].

There was a significant difference between the EMS and the PMS in the single-fragment CRP, but not in the multiple-fragment ones. The multiple fragments in a CRP polypectomy specimen imply that the lesion could not be excised as a whole, probably due to the difficulties in endoscopic manipulation and/or a size larger than the single-fragmented CRP ones (P<0.0001). Both of the manipulation difficulties and larger sizes may result in incomplete resection and retrieval of the CRP. Meanwhile, the pathologists had to deal with the problems of aggregating the fragments together for PMS, leading to an additional bias. So our results on multiple-fragment CRPs should be interpreted and applied to clinical practice with caution.

Study limitations and conclusions

There are several limitations of this study. First, this study was conducted at a single medical center, causing potential environmental and operator biases. Second, we did not measure the size of excised CRPs before fixation, so we could not render any information on the effect of the fixation. However, previous work has shown that fixation had little impact on the PMS [14, 16]. Third, we did not record the methods of CRP resection/polypectomy, which might also influence the PMS. Last, the sample size of HGD is relatively small although this is the first report in this regard.

In summary, this prospective study of PUM, SSA, HP, adenoma and HGD show that PMS is significantly smaller than EMS despite the linear correlation between PMS and EMS. Therefore, the bona fide CRPs size (PMS) cutoff for CRC risk stratification may be smaller than the currently recommended 1 cm. The discordance between PMS and EMS is associated with several factors including pathologic diagnosis, fragment number, endoscopists and PMS, and may contribute to different follow-ups in a considerable portion of adenomas and SPs.

Acknowledgements

We thank Kerry A. O’Rourke at Rutgers Robert Wood Johnson Library of Health Sciences for her assistance in literature retrieval.

Disclosure of conflict of interest

None.

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References

Pathologic and endoscopic measurements of colorectal lesion sizes


Pathologic and endoscopic measurements of colorectal lesion sizes


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Her study addressed the feasibility of utilizing manganese-enhanced MRI (MEMRI) to assess more complex cognitive and emotional processes in the neural circuitry involved in Amygdala, Prefrontal Cortex, Cingulate Cortex, Bed Nucleus Stria Terminalis (BNST), Caudate Putamen, Substantia Nigra, Hippocampus, Thalamus and Hypothalamus. Her study with paper published in the authoritative journal of NeuroImage established the validity of using MEMRI in the exploration of highly relevant complex neural circuitries associated with cognition and emotion, which was cited by many peers. So far, She has published over 20 peer-reviewed articles.
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Dr. Li Li is Professor of Family Medicine, Epidemiology and Biostatistics at Case Western Reserve University and an attending physician of family medicine at University Hospitals Case Medical Center. His research interests primarily involve cancer molecular/genetic epidemiology and prevention with focuses on gene-environment interaction, understanding the mechanistic link between energy imbalance and colon carcinogenesis, screening and early detection of colon neoplasia, and risk prediction modeling in breast cancer. Dr. Li has received multiple NIH research grants and contributed significantly to many large multi-investigator efforts, including the Case Center for Transdisciplinary Research on Energetics and Cancer (TREC) program and the Case GI malignancy Special Program of Research Excellence (SPORE) program. Dr. Li is also Associate Director for Prevention Research at the NCI-designated Case Comprehensive Cancer Center, overseeing population research that cuts across all aspects of translational cancer research ranging from epidemiology, psychosocial and behavioral sciences, genomics, biomarker discovery and validation, to the application of novel discoveries from both wet and dry laboratories to screening and early detection, prevention and intervention. He is also founding director of a new Clinical Translational Science PhD program at Case Western Reserve University School of Medicine.
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Dr. Peng obtained his Bachelor Degree of Medicine in 1986 from Jiangxi Medical College and Master Degree of Medicine in 1989 from Tongji Medical University. He practiced Anesthesia in Union Hospital of Tongji Medical University and was promoted to Attending physician/Instructor in 1992 and Associate Professor in 1996, and then was appointed as vice-director of the Department of Anesthesia & Intensive Care in 1996. He was the founder and vice-Chair of Hubei Society of Critical Care Medicine (1998-2001). He received his critical care training in 1999 and 2002-2004 in Hong Kong, respectively. He obtained his Doctor Degree of Medicine (Anesthesiology) from Tongji Medical University in 2001, and the second Doctor Degree of Medicine (Critical Care Medicine) from the Chinese University of Hong Kong in 2004. Dr. Peng moved to USA in 2005 and worked as a postdoctoral researcher at the University of Colorado Health Science Center (Denver). He joined the University of Pittsburgh in 2006, and worked as Postdoctoral Fellow/ CRISMA Fellow, Research Associate, and Assistant Professor, respectively.

Dr. Peng’s research interest is cellular metabolism, oxidative injury in the development of organ dysfunction during sepsis and organ protection, immuno-modulation of sepsis. He is also interested in the biomarkers for predicting organ injury and recovery. He published more than thirty original papers in the international peer journals and also published more than ten book chapters and invited reviews. He was the recipients of Harry M. Vars Research Award (USA, 2006), American Society for Parenteral and Enteral Nutrition Promising Investigator Award (USA, 2006) and Society of Critical Care Medicine Annual Award (USA, 2009).

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Dr. Wang’s major research interests are focused on tumor biology, animal models, tumor markers for early detection and prognosis, and identification of new therapeutic targets for pancreatic cancer, which is one of the most lethal human tumors. Dr. Wang received many awards for his research and was invited as one of the experts to serve on the American Joint Committee on Cancer (AJCC) Foregut Task Force for the 7th edition of the AJCC Cancer Staging Manual. Dr. Wang has authored more than 150 peer-reviewed publications including Nature, Cell, Cancer Cell, Journal of Clinical Oncology, Proceedings of National Academy of Sciences USA, Gastroenterology, Journal of Clinical Investigation etc. and received more than 20 research grants including NIH/NCI SPORE on pancreatic cancer, RO1, KO8 and R21.
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Dr. Chunbin Zou received his MD from Hengyang Medical College and a Msc. in medical science from Tongji U Medical University (88G), China and PhD in Molecular and cellular parasitology from Juntendo University School of Medicine, Japan. He completed his post-doctoral fellow training in Pharmaceutical biochemistry at University of California at San Francisco school of Pharmacy and in molecular and cellular pathology at University of Pittsburgh School of Medicine. In March 2010, Dr. Zou joined the Pulmonary, Allergy, and Critical Care faculty at the University of Pittsburgh as a Research Assistant Professor.

Dr. Zou’s laboratory focuses on a novel epigenetic code histone O-palmitoylation and its pathophysiological significances. Dr. Zou serendipitously discovered a novel histone modification called histone O-palmitoylation that acts as a new epigenetic mark to regulate gene transcription. This discovery was published in J. Biol. Chem., was commented and recommended by Faculty of 1000 organization as the top 2% biological research article. Continuing on this line of research will add ample knowledge of a novel layer of epigenetic regulation both in basic and medical scientific fields.

The related research interest in Dr. Zou’s laboratory is protein degradation. In the living cells, functional proteins are timely turnover to ensure the life process to go smoothly. Protein degradation also plays a crucial role in the pathogenesis of many diseases. Utilizing the state-of-art interdisciplinary approaches, he is studying the signaling transduction in regulation of histone modification enzymes in terms of protein degradation. He has uncovered several unstudied enzymes called E3 ubiquitin ligases that control the protein degradation of several important epigenetic histone modification enzymes in pneumonia lung tissues. He is actively to seek the possible small molecules that may intervent the protein degradation thus control the pathological progress of specified diseases.

Please see the website for detailed information:
http://www.dept-med.pitt.edu/paccm/faculty/ZouChunbin.html
让中国生物医学科研论文走向世界

——美捷登（Medjaden）简介

美捷登生物科技有限公司 2005 年成立于中国香港，公司秉承“
您不放弃，我们决不放弃”的服务理念，依托强大的专业团队，在业
界树立起良好的信誉口碑。九年多来，公司从单纯修改发表论文服
务，发展到如今集论文修改、课题设计、基金申请、论文写作培训为
一体的生物科技有限公司，为广大医务、科研工作者提供了全面而有
力的专业保障。

美捷登拥有一站式全程服务的专业理念和操作模式：评估→修改→编辑→润色→校对→代投，直至最终发表，各环节均有专人负责，环环相扣。迄今为止，美捷登已评估过数以万计的论文，并向一万多篇论文提供了正式服务，经美捷登服务后的论文一年发表率为74%，两年发表率高达93%，其中单篇影响因子最高为30.387（JAMA, IF2013）。这些论文均被SCI/SCIE, Medline, PubMed, EMBASE等检索系统或数据库收录。同时，“一次付费，服务到论文发表为止”的服务理念，也赢得了广大客户的一致信赖与广泛好评。

近年来，美捷登规模日趋壮大，各项经验日益丰富。应广大客户要求，相继开展论文撰
写、实验设计、基金申请服务，并对外开展了一系列培训讲座。先后在北京、南京、上海、
广州、西安、长沙、苏州、武汉、新疆、大连、重庆、成都、沈阳、温州、惠州等地成功举办
了SCI论文写作和发表方面的讲座及培训班100余场；值得一提的是，美捷登通过与北京友谊
医院合作，成功举办了两届“临床高水平SCI论文撰写与修改高级培训班”（国家继续教育项
目），受到国内生物医学科研人员的广泛赞誉。此外，美捷登还积极参与国内科研及教育相
关的论坛和峰会，包括“SCI论文与医疗、教学”全国讨论会（《医学与哲学》杂志社主办），以
及《第9届武汉炎症性肠病高峰论坛》等等。美捷登的资深编辑们为在中国普及论文写作与发
表的知识也做出了相当大的贡献，尤其是主编夏华向长期坚持活跃在丁香园论文写作版，并
针对园友提出的问题进行专业解答。美捷登作为“丁香园金牌供应商”，是丁香园论坛最受信
赖的论文合作伙伴。目前，美捷登与国内许多科研单位都建立了良好且稳定的科研和论文发
表合作关系。2010年，美捷登与著名生物医学出版社Landes Bioscience正式合作，成为该出
版社旗下30多份杂志首推的论文编辑公司，为美捷登的进一步发展提供了更为广阔的平台。

美捷登立足于香港，兼具国际化视野和本土经验，拥有国际化的编辑团队，采用先进的
管理理念，充分利用自身专业优势，帮助科研工作者将科研成果展示在国际平台。美捷登全体
员工将用热情周到的态度，向合作团体提供专业、优质、高效的服务，衷心希望与您有进一步
的合作。更多详情，请登陆美捷登公司网站（www.medjaden.com）了解。
华誉出版社有限公司（XIA & HE Publishing Ltd.）由美捷登生物科技有限公司创始人夏华向和何华于2011年创办成立，总部位于香港。出版社致力于为生物医学研究工作者提供一个可以实时分享和交流生物医药领域最新信息的平台，使全球的科学家和临床医生都能更好地为人类健康进行研究工作。本出版社出版的所有杂志都使用同行评审和开放索取（Open Access）模式，以实现出版社的宗旨：自由无界地分享生物医学信息，使作者和读者能更有效的互动沟通，并促进已发表信息在实验研究和临床操作过程中的实际应用。

本出版社寻求与医院、机构或个人合作出版生物医药类杂志。我们的目标是变成国内最具影响力的英文杂志出版商，以期能覆盖生物医药的各个领域，为全球的读者服务。

Journal of Clinical and Translational Hepatology (JCTH) 是本出版社与重庆医科大学第二附属医院合作出版的第一本期刊，于2013年9月发行第一期。迄今为止，已成功发行5期。此外 Exploratory Research and Hypothesis in Medicine (ERHM) 和 Exploratory Research and Hypothesis in Pharmacology (ERHP) 也正在筹备中，计划将于明年正式发行。

JCTH主要收录肝病领域基础、临床或转化相关的研究文章。ERHM和ERHP主要收录探索性的研究论文和最新的综述。杂志内容优先选取最新发现以及有理有据的研究假设。ERHM和ERHP正在招募编辑队伍，您如果有兴趣可访问出版社网站查看详细内容（http://www.xiahepublishing.com/a/Careers/index.html）

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