Published in final edited form as:

Gastroenterology. 2011 June ; 140(7): 2084–2094. doi:10.1053/j.gastro.2011.02.056.

Microsomal Prostaglandin E Synthase-1 Inhibits PTEN and Promotes Experimental Cholangiocarcinogenesis and Tumor Progression

Dongdong Lu^{1,2}, Chang Han¹, and Tong Wu¹

¹ Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA 70112

² Tongji University School of Life Science and Technology, Shanghai 200092, China

Abstract

Background & Aims—Microsomal prostaglandin E synthase-1 (mPGES-1) is a rate-limiting enzyme that is coupled with cyclooxygenase-2 (COX-2) in the synthesis of prostaglandin E₂ (PGE₂). Although COX-2 is involved in development and progression of various human cancers, the role of mPGES-1 in carcinogenesis has not been determined. We investigated the role of mPGES-1 in human cholangiocarcinoma growth.

Methods—We used immunohistochemical analyses to examine the expression of mPGES-1 in formalin-fixed, paraffin-embedded human cholangiocarcinoma tissues. The effects of mPGES-1 on human cholangiocarcinoma cells were determined in vitro and in SCID mice. Immunoblotting and immunoprecipitation assays were performed to determine the levels of PTEN and related signaling molecules in human cholangiocarcinoma cells with overexpression or knockdown of mPGES-1.

Results—mPGES-1 is overexpressed in human cholangiocarcinoma tissues. Overexpression of mPGES-1 in human cholangiocarcinoma cells increased tumor cell proliferation, migration, invasion, and colony formation; in contrast, RNAi knockdown of mPGES-1 inhibited tumor growth parameters. In SCID mice with tumor xenografts, mPGES-1 overexpression accelerated tumor formation and increased tumor weight ($P < 0.01$), whereas mPGES-1 knockdown delayed tumor formation and reduced tumor weight ($P < 0.01$). mPGES-1 inhibited the expression of PTEN, leading to activation of the EGFR–PI3K–AKT–mTOR signaling pathways in cholangiocarcinoma cells. mPGES-1–mediated inhibition of PTEN is regulated through blocking of EGR-1 sumoylation and binding to the 5'-UTR of the *PTEN* gene.

Conclusions—mPGES-1 promotes experimental cholangiocarcinogenesis and tumor progression by inhibiting PTEN.

© 2011 The American Gastroenterological Association. Published by Elsevier Inc. All rights reserved.

Address correspondence to: Tong Wu, M.D., Ph.D., Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112. Tel: 504-988-5210, Fax: 504-988-7862, twu@tulane.edu.

DL: Performing experiments, analysis and interpretation of data, drafting of the manuscript.

CH: Assisting key experiments, critical revision of the manuscript

TW: Study concept and design, data interpretation, critical revision of the manuscript, obtained funding.

No conflicts of interest exist

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

cancer cell signaling; biliary tract cancer; bile duct; liver

Introduction

Cyclooxygenase-2 (COX-2)-derived prostaglandin E₂ (PGE₂) is implicated in the development and growth of various human and animal cancers including cholangiocarcinoma¹. The COX-2/PGE₂ signaling pathway regulates multiple processes of carcinogenesis, including tumor cell proliferation, survival, invasion and angiogenesis. The synthesis of PGE₂ is tightly controlled by cyclooxygenase (COX) and microsomal prostaglandin E synthase-1 (mPGES-1). Whereas the COX enzymes catalyze the formation of endoperoxide prostaglandin H₂ (PGH₂) from membrane arachidonic acid, mPGES-1 catalyzes the formation of PGE₂ from COX-derived PGH₂²⁻⁶. It is of note that mPGES-1 is functionally coupled with COX-2 in marked preference to COX-1^{3, 7}. Similar to COX-2, mPGES-1 is a cytokine-inducible enzyme and is critically involved in pain and inflammation-related diseases. However, the potential role of mPGES-1 in tumorigenesis is far less recognized in comparison to COX-2. Recently, mPGES1 has been reported to be overexpressed in several human cancers including hepatocellular carcinoma⁸, although the exact role of mPGES-1 in carcinogenesis remains to be defined.

Cholangiocarcinoma is highly malignant cancer of the biliary tract with poor prognosis. The incidence and mortality of cholangiocarcinoma is rising worldwide^{9, 10} and currently there is no effective chemoprevention or treatment. The tumor often arises from background conditions that cause long-standing inflammation, injury and reparative biliary epithelial cell proliferation, such as primary sclerosing cholangitis, clonorchiasis, hepatolithiasis or complicated fibropolycystic diseases^{1, 10-13}. Intrahepatic cholangiocellular carcinoma is also associated with hepatitis C viral infection and liver cirrhosis secondary to other nonbiliary causes¹⁴. Cholangiocarcinogenesis is believed to involve a series of sequential events including chronic inflammation, cholangiocyte proliferation, dysplasia and ultimately malignant transformation¹¹. Consistent with the strong association between bile duct chronic inflammation and cholangiocarcinoma, recent studies have demonstrated an important role of COX-2-derived PGE₂ in cholangiocarcinogenesis¹. Elevated expression of COX-2 has been documented in cholangiocarcinoma cells and pre-cancerous bile duct lesions but not in normal bile duct epithelial cells¹⁵⁻¹⁷. Overexpression of COX-2 in cultured human cholangiocarcinoma cells enhances PGE₂ production and promotes tumor growth, whereas depletion of COX-2 attenuates growth^{18, 19}. PGE₂ treatment increases cholangiocarcinoma cell growth and prevents apoptosis¹⁸⁻²⁴. These findings suggest an intriguing opportunity of inhibiting COX-2/PG pathway to disrupt the inflammation, dysplasia and malignant transformation processes in high-risk patients, such as those with primary sclerosing cholangitis (PSC). Indeed, inhibition of COX-2 has been found to efficiently prevent the growth and invasion of cholangiocarcinoma cells *in vitro* and in animal models¹. These findings provide important preclinical evidence for targeting COX-2 for prevention and treatment of human cholangiocarcinoma. However, as some COX-2 inhibitors are known to be associated with increased cardiovascular side effect²⁵⁻²⁸, there is an urgent and practical need to identify COX-2 downstream target for effective anti-tumor therapy with less side effect.

In addition to prostaglandin pathway, recent studies have shown an important role of PTEN (phosphatase and tensin homolog deleted on chromosome 10) in cholangiocarcinogenesis. PTEN is a key tumor suppressor gene that is disrupted or suppressed in various human cancers^{29, 30} including cholangiocarcinoma³¹. The *PTEN* gene encodes a plasma membrane

lipid phosphatase that hydrolyzes the 3' phosphate of phosphoinositides and thereby negatively regulates phosphoinositide 3-kinase (PI3K)³². While progressive reduction of PTEN is frequently associated with more aggressive cancers, subtle variations in PTEN levels are recognized to have critical consequences for tumor progression³³. It has been shown that the absence of functional PTEN leads to constitutive activation of downstream components of the PI3K pathway including AKT (protein kinase B)³⁴. This study provides novel evidence that mPGES-1 promotes human cholangiocarcinoma cell growth through downregulation of PTEN. Our findings depict a novel mechanism by which mPGES-1 promotes cholangiocarcinogenesis and tumor progression and suggest that mPGES-1 may represent a novel effective therapeutic target with lesser side effect compared to targeting COX-2.

Methods

Immunohistochemical analysis was performed to determine the expression of mPGES-1 and PTEN in 23 formalin-fixed, paraffin-embedded human cholangiocarcinoma tissues.

To establish human cholangiocarcinoma cell lines with overexpression or knockdown of mPGES-1, CCLP1 cells were stably transfected with the mPGES-1 expression vector, mPGES-1 RNAi vector, and two corresponding control vectors. Immunofluorescence staining and western blotting were performed to verify transfection efficiency. The growth curves of the cell lines with overexpression or knockdown of mPGES-1 were determined by direct cell counting and WST-1 assay. Colony formation efficiency was determined by using cells grown in soft agar and culture plates. Flow cytometry was performed to determine cell cycle progression. BrdU staining was performed to determine DNA synthesis. Standard transwell and wound healing assays were performed to determine cell migration, invasion and injury repair ability. Immunofluorescence microscopy, western blotting and RT-PCR were performed to determine the protein level of PTEN as well as the phosphorylation of PTEN. Western blotting analysis was performed to determine the level of PI3K and its related signaling molecules including epidermal growth factor receptor (EGFR), pEGFR(Tyr845), PI3K, pPI3K, AKT, pAKT, mammalian target of rapamycin (mTOR), pmTOR, Toll-like receptor 4 (TLR4), nuclear factor- κ B (NF κ B), P62, cyclinD1, survivin, and myeloid cell leukemia-1 (Mcl-1) in cells with overexpression or knockdown of mPGES-1.

To determine the effect of mPGES-1 on cholangiocarcinoma growth *in vivo*, the stably transfected cholangiocarcinoma cell lines were injected subcutaneously at armpit into SCID mice. The mice were observed over 4 weeks for tumor formation. Upon sacrifice, the tumors were recovered. The wet weight of each tumor and tumor appearance time were determined for each mouse. Portion of each tumor was used for western blotting, hematoxylin-eosin (HE) staining and immunohistochemical staining of PCNA, Ki67, and PTEN.

To determine the role of EGR1 in mPGES-1-induced suppression of PTEN, cultured human cholangiocarcinoma cells were transfected with vectors expressing PTEN, EGR1, mPGES-1, or the control vector (pCMV5) and the cells were analyzed for PTEN protein and mRNA levels as well as the binding between EGR1 and PTEN proteins. As a parallel approach, immunoprecipitation and western blotting analysis were performed to determine the potential effect of mPGES1 on EGR1 binding to PTEN protein. The role of EGR1 sumoylation was examined by immunoprecipitation with SUM1 antibody followed by EGR1 western blotting, or vice versa. DNA pull down and chromatin immunoprecipitation (CHIP) assays were carried to examine whether mPGES-1 might influence the binding of EGR1 and SUM-EGR1 to the 5'-UTR of the PTEN gene.

Results

Expression of mPGES-1 in human cholangiocarcinoma cells

We first performed immunohistochemical analysis to determine the expression of mPGES-1 in human cholangiocarcinoma tissues. Formalin-fixed paraffin-embedded tissue specimens from 23 surgically resected cholangiocarcinoma patients were utilized. Our data showed that mPGES-1 was expressed in 18 of 23 cholangiocarcinoma cases (78.26%), whereas 5 of 23 cholangiocarcinoma cases were negative or showed trace staining (21.74%) ($p < 0.01$) (Figure 1A). The staining pattern and intensity were not significantly different between intrahepatic and extrahepatic cholangiocarcinomas (positive staining was observed in 14/18 intrahepatic tumors and 4/5 extrahepatic tumors). We next performed immunoblotting analysis to determine the level of mPGES-1 in human intrahepatic biliary epithelial cells (HiBECs) and three intrahepatic human cholangiocarcinoma cell lines (SG231, CCLP1, HuCCT1). As shown in Figure 1A, although low level of mPGES-1 protein was detected in HiBECs, high expression of mPGES-1 was observed in three human cholangiocarcinoma cell lines (CCLP1 cell line expresses higher mPGES1 than two other CC cell lines). These observations indicate that mPGES-1 is overexpressed in human cholangiocarcinoma cells. It is of note that the three human cholangiocarcinoma cell lines exhibit lower level of PTEN compared to primary intrahepatic biliary epithelial cells (HiBECs) (Supplementary Figure S1). Correlation coefficient analysis showed a modest inverse correlation between mPGES-1 and PTEN expression in human cholangiocarcinoma tissues ($r = -0.313$) (Supplementary Table 1).

mPGES-1 accelerates cholangiocarcinogenesis *in vitro* and *in vivo*

To investigate the role of mPGES-1 in cholangiocarcinoma cell growth, we constructed cholangiocarcinoma cell lines with stable overexpression or depletion of mPGES1. Successful alteration of mPGES-1 expression in the established cell lines was verified by immunofluorescence staining and western blotting (Figure 1B & Supplementary Figure S2). Under immunofluorescence, the cellular mPGES-1 expression was significantly higher in mPGES-1 overexpressed stable cell line than in the corresponding GFP control vector cells. Accordingly, mPGES-1 immunofluorescence in mPGES-1 RNAi stable cell line was substantially lower (essentially absent) in comparison to the visible baseline mPGES-1 immunofluorescence in the corresponding RNAi control vector cells. Western blotting showed that the level of mPGES-1 was increased in mPGES-1 overexpressed cells (44KD, GFP-mPGES-1 fusion protein) and reduced in mPGES-1 knockdown cells (17KD). Accordingly, the synthesis of PGE₂ was significantly increased in mPGES1 overexpressed cells and decreased in mPGES-1 knockdown cells (Figure 1C).

We next examined the growth curves of the four established cholangiocarcinoma cell lines by the direct cell count experiment. As shown in Figure 1D, overexpression of mPGES-1 significantly increased the growth of cholangiocarcinoma cells when compared to the GFP control cells. In contrast, RNAi knockdown of mPGES-1 significantly inhibited tumor cell growth. Similar results were also obtained by using the WST-1 assay (Supplementary Figure S3). Accordingly, mPGES-1 overexpressed cells possessed significantly higher colony-formation efficiency, whereas mPGES-1 depleted cells exhibited lower colony-formation efficiency (Figure 1E and Supplementary Figure S4). These results suggest a potential role of mPGES-1 in cholangiocarcinoma cell growth.

To further determine whether mPGES-1 could affect the tumorigenesis *in vivo*, the aforementioned four cholangiocarcinoma cell lines were injected subcutaneously at armpit into SCID mice. Significant differences in tumor weights were observed among different groups. As shown in Figure 2A, when mPGES-1 was overexpressed, the tumor weight

increased approximately three folds compared to the control group (2.11 ± 0.40 grams versus 0.67 ± 0.11 grams, $p < 0.01$). Conversely, when mPGES-1 was knocked down, the tumor weight decreased to approximately half of the control tumor weight (0.23 ± 0.04 grams versus 0.57 ± 0.15 grams, $p < 0.01$). We also noticed early tumor formation in the mPGES-1 overexpressed group (5.41 ± 1.49 days) in comparison to the control group (9.87 ± 2.52 days) ($p < 0.01$). On the other hand, the time of tumor appearance was prolonged in the mPGES-1 knockdown group (16.21 ± 3.99 days) compared to the control group (10.81 ± 3.08 days) ($p < 0.01$).

Pathological examination (H&E stain) of the resected tumor tissues revealed more poorly-differentiated tumor cells and fewer moderately or well-differentiated tumor cells in mPGES-1 overexpression group in comparison to the mock group. Conversely, the mPGES-1 knockdown tumors showed more moderately or well-differentiation cells and fewer poor-differentiation cells in comparison to the corresponding control tumors (Figure 2B). Thus, mPGES1 overexpression appears to enhance the malignant grade of the transplanted tumors whereas mPGES-1 knockdown lowers the malignant grade of the transplanted tumors. Immunohistochemical stains for two cell proliferation markers, Ki67 and PCNA, were performed in the transplanted tumors. As shown in Figure 2B, the number of Ki67 positive cells is higher in mPGES-1 overexpression group ($79.83 \pm 15.22\%$ compared to $35.12 \pm 6.97\%$ in the control group, $p < 0.01$) and lower in mPGES-1 knockdown group ($8.79 \pm 2.31\%$ compared to $32.57 \pm 5.95\%$ in the control group, $p < 0.01$). Accordingly, mPGES-1 overexpressed tumors show more PCNA-positive cells ($91.67 \pm 8.05\%$ versus $42.24 \pm 5.97\%$ in control tumors, $p < 0.01$) and lower in mPGES-1 knockdown tumors ($17.86 \pm 3.13\%$ versus $38.78 \pm 6.89\%$ in control tumors, $p < 0.01$). Taken together, results of the above *in vitro* and *in vivo* experiments suggest an important role of mPGES-1 in cholangiocarcinoma cell growth.

mPGES-1 regulates cell cycle progression at G1/S checkpoint and enhances tumor cell migration and invasion

Given that mPGES-1 expression was positively correlated with the proliferation index as calculated by the PCNA- and Ki67-nuclear labeling ratio in SCID mouse xenograft tumors, we postulated that mPGES-1 might be implicated in the regulation of cell proliferation. To further address this, flow cytometry was performed in the stably transfected cell lines with mPGES-1 overexpression or knockdown. As shown in Figure 3A, the cells stably transfected with mPGES-1 showed a significant decrease in the G0/G1 phase cells compared to control mock cell lines ($15.6 \pm 3.1\%$ versus $38.8 \pm 6.9\%$, $p < 0.01$) and a significant increase in the S phase cells ($75.1 \pm 14.3\%$ versus $52.3 \pm 7.8\%$, $p < 0.05$). Conversely, the cells with mPGES-1 knockdown showed a significant increase in G0/G1 phase cells ($70.1 \pm 15.5\%$ versus $41.3 \pm 7.1\%$, $p < 0.01$) and a significant decrease in S phase cells ($20.3 \pm 3.2\%$ versus $49.5 \pm 10.7\%$). We observed that the percentages of G2 phase cells were not significantly altered in cells with mPGES-1 overexpression or knockdown ($p > 0.05$). These data suggest that mPGES-1 likely regulates cell cycle at the G1/S check point. Consistent with these results, we observed more BrdU positive cells in mPGES-1 overexpression cells ($71.44 \pm 12.47\%$ versus $31.78 \pm 5.96\%$ in mock control, $p < 0.01$) and fewer BrdU positive cells in mPGES-1 knockdown cell line ($10.81 \pm 2.09\%$ versus $28.79 \pm 8.21\%$ in corresponding control, $p < 0.01$) (Figure 3B). These findings suggested that mPGES-1 is able to accelerate DNA synthesis by promoting cell cycle progression via altering G1/S check point.

Standard transwell and wound healing assays were next performed to determine whether mPGES-1 might influence cell migration, invasion and injury repair ability. As shown in Figure 3C, the Transwell assay showed more migratory cells in mPGES-1 overexpressed cell line (160.56 ± 25.78 versus 70.34 ± 7.71 in mock control, $p < 0.01$) and fewer migratory cells in mPGES-1 knockdown cell line (34.11 ± 9.05 versus 75.67 ± 9.58 in corresponding

control, $p < 0.01$). The wound healing assay showed that mPGES-1 overexpression promoted cellular injury repair and/or migration at 8 and 24h, while mPGES-1 knockdown inhibited this process (Figure 3D). These findings suggest that mPGES-1 enhances cell migration, invasion and injury repair ability in cholangiocarcinoma cells.

mPGES-1 inhibits PTEN expression in cholangiocarcinoma cells

PTEN is an important tumor suppressor which has been implicated in the tumorigenesis of several human cancers including cholangiocarcinoma³¹. Given the clear effect of mPGES-1 on cholangiocarcinoma cell growth as shown above, we further examined whether mPGES-1 might regulate PTEN expression in cholangiocarcinoma cells. In the SCID mouse tumor xenograft model, we observed that the expression of PTEN was suppressed in mPGES-1 overexpressed tumors but increased in mPGES-1 depleted tumors, as determined by immunohistochemical stain and immunoblotting analysis (Figure 2B). In cholangiocarcinoma cells cultured *in vitro*, immunofluorescence microscopy revealed that mPGES-1 overexpression inhibited the protein level of PTEN as well as the phosphorylation of PTEN [pPTEN(Ser380)], whereas mPGES-1 depletion increased the levels of PTEN and p-PTEN (Figure 4A). Western blot analysis showed reduced PTEN/p-PTEN in mPGES-1 overexpressed cells but elevated PTEN/p-PTEN in mPGES-1 depleted cells (Figure 4B). These results suggest that PTEN is a downstream target of mPGES-1 in cholangiocarcinoma cells. It is of note that the level of PTEN mRNA was reduced in mPGES-1 overexpressed cells but elevated in mPGES-1 knockdown cells as determined by RT-PCR, suggesting that mPGES-1 may inhibit PTEN mRNA transcription or induce its degradation (Figure 4C).

Our further experiments showed that overexpression of PTEN prevented mPGES-1-induced tumor cell growth (Figure 4D). Accordingly, depletion of PTEN by siRNA partially prevented mPGES-1 knockdown-induced inhibition of tumor cell growth (Figure 4E). These observations indicate the involvement of PTEN in mPGES-1-induced cholangiocarcinoma cell growth.

mPGES-1 inhibits EGR1-mediated cis-activation and trans-activation of PTEN

Since EGR1 is a key factor that controls PTEN expression via cis-activation (EGR1 binding to PTEN promoter) and/or trans-activation (EGR1 binding to PTEN protein)^{35, 36}, we further investigated the potential role of EGR1 in mPGES-1-induced suppression of PTEN. In this approach, we transfected the parent CCLP1 cells with vectors expressing PTEN, EGR1, mPGES1, and the control vector (pCMV5) (the PTEN expression vector encodes full-length PTEN cDNA with 5'-UTR containing consensus EGR1 binding site); these cells were analyzed for PTEN protein and mRNA levels as well as the binding between EGR1 and PTEN proteins. As shown in Figure 5A, forced expression of EGR1 enhanced PTEN protein expression (via cis-activation); this effect was attenuated when mPGES-1 was overexpressed. Accordingly, RT-PCR analysis revealed that mPGES-1 overexpression inhibited EGR1-induced accumulation of PTEN mRNA. We further performed immunoprecipitation and western blotting analysis to determine the potential effect of mPGES1 on EGR1 binding to PTEN protein (trans-activation). For this purpose, the CCLP1 cells were transfected with vectors to overexpress full length PTEN in conjunction with mPGES-1 overexpression or knockdown. As shown in Figure 5B, the amount of EGR1 bound to PTEN protein was significantly decreased when mPGES-1 was overexpressed but increased when mPGES-1 was knocked down. In addition, we also found that the amount of EGR1 bound to phosphorylated PTEN (ser380) was also decreased when mPGES-1 was overexpressed but increased when mPGES-1 was knocked down (Figure 5C). These findings suggest that EGR1-mediated cis-activation and trans-activation of PTEN are impaired in mPGES-1 overexpressed cells and restored in mPGES-1 depleted cells.

mPGES-1 inhibits EGR1 sumoylation and prevents SUM-EGR1 binding to PTEN gene promoter

As sumoylation of EGR1 is critical for its transcriptional regulation of PTEN³⁷, we next examined whether mPGES-1 might regulate PTEN expression via sumoylation of EGR1. As shown in Figures 6A-C, the level of SUM-EGR1 is decreased in mPGES-1 overexpressed cells but increased in mPGES-1 depleted cells. This phenomenon was observed by immunoprecipitation with SUM1 antibody followed by EGR1 western blotting, or vice versa. We next employed DNA pull down and chromatin immunoprecipitation (CHIP) assays to examine whether mPGES-1 might influence the binding of EGR1 and SUM-EGR1 to 5'-UTR of the PTEN gene. As shown in Figure 6D, EGR1 bound to 5'-UTR of PTEN was increased in mPGES-1 depleted cells but decreased in mPGES-1 overexpressed cells. Accordingly, SUM-EGR1 bound to PTEN 5'-UTR was also significantly increased in mPGES-1 depleted cells and decreased in mPGES-1 overexpressed cells (Figure 6E). These findings were further corroborated by the data from the chromatin immunoprecipitation (CHIP) assays showing higher EGR1 binding to PTEN promoter in mPGES-1 depleted cells compared to mPGES-1 overexpressed cells (Supplementary Figure S5). Likewise, the CHIP assay in EGR1 overexpressed cells also showed higher sumoylated protein binding to PTEN promoter in cells with mPGES-1 knockdown compared to cells with mPGES-1 overexpression (Supplementary Figure 6). Similar findings were also observed by real-time PCR based CHIP assay (Supplementary Figure 7). Taken together, these findings suggest that mPGES-1 is able to inhibit EGR1 sumoylation and prevent EGR1 binding to PTEN 5'-UTR, thus leading to inhibition of PTEN expression.

mPGES-1 regulates PTEN downstream signaling molecules

Since PTEN is known to dephosphorylate and inhibit PI3K³², we further examined the potential effect of mPGES1 on PI3K and its related signaling molecules. As shown in Figure 7A, the levels of epidermal growth factor receptor (EGFR), pEGFR(Tyr845), PI3K, pPI3K, AKT, pAKT, mammalian target of rapamycin (mTOR), pmTOR, Toll-like receptor 4 (TLR4), nuclear factor- κ B (NF κ B), P62, cyclinD1, survivin, and myeloid cell leukemia-1 (Mcl-1) were all increased in cells stably transfected with mPGES-1; conversely, the levels of these molecules were decreased in cells stably transfected mPGES1 RNAi. It is of note that the level of ARF/P14, a critical cell cycle relative gene implication in PTEN regulation³⁷, was increased when mPGES-1 was knocked down but decreased when mPGES-1 was overexpressed. In transiently transfected cells, the levels of PI3K and p-PI3K were increased when mPGES-1 was overexpressed but decreased when mPGES1 was knocked down (Figure 7B). Taken together, these findings suggest that mPGES-1 inhibits PTEN expression and activates the EGFR-PI3K-AKT-mTOR pathway in cholangiocarcinoma cells.

Discussion

This study provides novel evidence that mPGES-1 is overexpressed in human cholangiocarcinoma cells and plays an important role in cholangiocarcinoma growth. We have shown that mPGES-1 is expressed in most human cholangiocarcinoma tissues. Furthermore, the level of mPGES-1 protein is increased in human cholangiocarcinoma cell lines compared to the primary intrahepatic biliary epithelial cells. In cultured human cholangiocarcinoma cells, overexpression of mPGES-1 promoted tumor cell proliferation and cell cycle progression, whereas knockdown of mPGES-1 prevented tumor growth parameters. Notably, mPGES-1 overexpressed tumor cells were found to possess significantly higher colony-formation efficiency; this ability was greatly decreased in the mPGES-1 knockdown cells. Furthermore, mPGES-1 overexpression increased tumor cell migration and repair ability, whereas mPGES-1 knockdown reduced tumor cell migration

and repair. These findings demonstrate that mPGES-1 enhances the malignant behavior of cholangiocarcinoma cells. The above *in vitro* data were further corroborated by the results from the SCID mouse model showing that mPGES-1 overexpression accelerated tumor onset and increased tumor weight, whereas mPGES-1 knockdown delayed tumor formation and reduced tumor weight.

Our results provide novel evidence that mPGES-1 enhances cholangiocarcinogenesis through inhibition of PTEN. This finding is noteworthy, as disruption of PTEN has been implicated in the development and progression of a number of human cancers^{29, 30, 32-34, 38, 39}. Although mutations of *PTEN* have not been reported in human cholangiocarcinomas, loss of functional PTEN has been implicated in cholangiocarcinogenesis^{31, 40}. In a murine model of intrahepatic cholangiocarcinoma, disruption of the PTEN gene was associated with increased level of p-AKT and mTOR, suggesting that PTEN may regulate cholangiocarcinogenesis by affecting its downstream genes³¹. Consistent with the observations that loss of PTEN protein is a prognostic factor in other malignancies, Chung et al showed that loss of PTEN expression is associated with more aggressive tumor growth parameters and worse survival outcome in cholangiocarcinoma patients⁴⁰. In the current study, we observed that overexpression of PTEN prevented mPGES-1-induced cholangiocarcinoma cell growth; this finding supports the involvement of PTEN in mPGES-1-induced cholangiocarcinoma cell growth. Our data presented in this study, along with the documented role of PTEN in tumorigenesis, indicate a crucial role of mPGES-1-mediated inhibition of PTEN in cholangiocarcinogenesis.

Silencing of PTEN expression in cholangiocarcinoma cell lines has been described to occur through mechanisms including homozygous deletion⁴¹ and interference by microRNA (miR-21)⁴². The current study shows that upregulation of mPGES-1 is another important molecular alteration that leads to PTEN inhibition and cholangiocarcinoma growth. The observation that mPGES-1 expression status alters PTEN mRNA level suggests regulation of PTEN gene transcription and/or mRNA degradation. Our results provide evidence for the involvement of early growth response-1 (EGR1) in mPGES-1-mediated inhibition of PTEN expression. DNA pull down and chromatin immunoprecipitation (CHIP) assays revealed that the amounts of EGR1 as well as SUM-EGR1 bound to 5'-UTR of PTEN were significantly increased in mPGES-1 depleted cells but decreased in mPGES-1 overexpressed cells. These results suggest that mPGES-1 attenuates EGR1 sumoylation and inhibit EGR1 binding to PTEN 5'-UTR, which may be crucial for suppression of PTEN expression. Our findings are consistent with the documented phenomenon that EGR-1 induces PTEN gene transcription through binding to its specific consensus site at the 5'-UTR of the PTEN gene^{35, 36}. As EGR1 is able to regulate PTEN through transcriptional regulation (cis-activation) as well as direct interaction with PTEN protein (trans-activation), we further examined the effect of mPGES-1 on EGR1-PTEN binding. We observed that mPGES-1 overexpression noticeably decreased EGR1-PTEN binding; in contrast mPGES-1 knockdown enhanced EGR1-PTEN binding. These results suggest that mPGES-1 is able to inhibit PTEN through blocking EGR1 cis-activation as well as trans-activation of PTEN in cholangiocarcinoma cells. Additionally, we observed that mPGES-1 overexpression led to reduction of ARF/P14 whereas mPGES-1 depletion enhanced ARF/P14 level in CCLP1 cells; this finding indicates the involvement of ARF/P14 in mPGES-1 mediated inhibition of PTEN and is consistent with the documented role of ARF/P14 in EGR-1 sumoylation and PTEN gene transcription³⁷.

In summary, this study shows that mPGES-1 inhibits sumoylation of EGR1 and reduces the binding of SUM1-EGR1 to the 5'-UTR of PTEN, leading to inhibition of PTEN transcription. Our findings indicate that inhibition of PTEN by mPGES-1 leads to activation of EGRF-PI3K-AKT-mTOR pathway, which facilitates the expression of several

downstream genes (including TLR4, NF κ B, P62, cyclin D, survivin, Mcl-1) (illustrated in Figure 7C). These results document a pivotal role of mPGES-1 in cholangiocarcinogenesis and tumor progression. Consequently, mPGES-1 may represent a promising therapeutic target for future chemoprevention and treatment of human cholangiocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by National Institutes of Health grants CA102325, CA106280, CA134568, DK077776 (to T.W.) and CA137729 (to C.H.).

References

1. Wu T. Cyclooxygenase-2 and prostaglandin signaling in cholangiocarcinoma. *Biochim Biophys Acta*. 2005; 1755:135–50. [PubMed: 15921858]
2. Jakobsson PJ, Thoren S, Morgenstern R, et al. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A*. 1999; 96:7220–5. [PubMed: 10377395]
3. Murakami M, Kudo I. Prostaglandin E synthase: a novel drug target for inflammation and cancer. *Curr Pharm Des*. 2006; 12:943–54. [PubMed: 16533161]
4. Nebert DW, Vasiliou V. Analysis of the glutathione S-transferase (GST) gene family. *Hum Genomics*. 2004; 1:460–4. [PubMed: 15607001]
5. Kudo I, Murakami M. Prostaglandin E synthase, a terminal enzyme for prostaglandin E2 biosynthesis. *J Biochem Mol Biol*. 2005; 38:633–8. [PubMed: 16336776]
6. Samuelsson B, Morgenstern R, Jakobsson PJ. Membrane prostaglandin E synthase-1: a novel therapeutic target. *Pharmacol Rev*. 2007; 59:207–24. [PubMed: 17878511]
7. Murakami M, Naraba H, Tanioka T, et al. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem*. 2000; 275:32783–92. [PubMed: 10869354]
8. Takii Y, Abiru S, Fujioka H, et al. Expression of microsomal prostaglandin E synthase-1 in human hepatocellular carcinoma. *Liver Int*. 2007; 27:989–96. [PubMed: 17696939]
9. Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. *Semin Liver Dis*. 2004; 24:115–25. [PubMed: 15192785]
10. Gores GJ. Cholangiocarcinoma: current concepts and insights. *Hepatology*. 2003; 37:961–9. [PubMed: 12717374]
11. Sirica AE. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. *Hepatology*. 2005; 41:5–15. [PubMed: 15690474]
12. Berthiaume EP, Wands J. The molecular pathogenesis of cholangiocarcinoma. *Semin Liver Dis*. 2004; 24:127–37. [PubMed: 15192786]
13. Lazaridis KN, Gores GJ. Cholangiocarcinoma. *Gastroenterology*. 2005; 128:1655–67. [PubMed: 15887157]
14. Okuda K, Nakanuma Y, Miyazaki M. Cholangiocarcinoma: recent progress. Part 2: molecular pathology and treatment. *J Gastroenterol Hepatol*. 2002; 17:1056–63. [PubMed: 12201864]
15. Endo K, Yoon BI, Pairojkul C, et al. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology*. 2002; 36:439–50. [PubMed: 12143054]
16. Hayashi N, Yamamoto H, Hiraoka N, et al. Differential expression of cyclooxygenase-2 (COX-2) in human bile duct epithelial cells and bile duct neoplasm. *Hepatology*. 2001; 34:638–50. [PubMed: 11584358]
17. Chariyalertsak S, Sirikulchayanonta V, Mayer D, et al. Aberrant cyclooxygenase isozyme expression in human intrahepatic cholangiocarcinoma. *Gut*. 2001; 48:80–6. [PubMed: 11115827]

18. Han C, Leng J, Demetris AJ, et al. Cyclooxygenase-2 promotes human cholangiocarcinoma growth: evidence for cyclooxygenase-2-independent mechanism in celecoxib-mediated induction of p21waf1/cip1 and p27kip1 and cell cycle arrest. *Cancer Res.* 2004; 64:1369–76. [PubMed: 14973068]
19. Wu T, Leng J, Han C, et al. The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells. *Mol Cancer Ther.* 2004; 3:299–307. [PubMed: 15026550]
20. Wu T, Han C, Lunz JG 3rd, et al. Involvement of 85-kd cytosolic phospholipase A(2) and cyclooxygenase-2 in the proliferation of human cholangiocarcinoma cells. *Hepatology.* 2002; 36:363–73. [PubMed: 12143044]
21. Nzeako UC, Guicciardi ME, Yoon JH, et al. COX-2 inhibits Fas-mediated apoptosis in cholangiocarcinoma cells. *Hepatology.* 2002; 35:552–9. [PubMed: 11870367]
22. Zhang Z, Lai GH, Sirica AE. Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology.* 2004; 39:1028–37. [PubMed: 15057907]
23. Lai GH, Zhang Z, Sirica AE. Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin to suppress rat cholangiocarcinoma growth in vitro through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. *Mol Cancer Ther.* 2003; 2:265–71. [PubMed: 12657721]
24. Han C, Wu T. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *J Biol Chem.* 2005; 280:24053–63. [PubMed: 15855163]
25. Vanchieri C. Vioxx withdrawal alarms cancer prevention researchers. *J Natl Cancer Inst.* 2004; 96:1734–5. [PubMed: 15572751]
26. Couzin J. Clinical trials. Nail-biting time for trials of COX-2 drugs. *Science.* 2004; 306:1673–5. [PubMed: 15576585]
27. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest.* 2006; 116:4–15. [PubMed: 16395396]
28. Baron JA, Sandler RS, Bresalier RS, et al. A randomized trial of rofecoxib for the chemoprevention of colorectal adenomas. *Gastroenterology.* 2006; 131:1674–82. [PubMed: 17087947]
29. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol.* 2004; 22:2954–63. [PubMed: 15254063]
30. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. *Cell.* 2008; 133:403–14. [PubMed: 18455982]
31. Xu X, Kobayashi S, Qiao W, et al. Induction of intrahepatic cholangiocellular carcinoma by liver-specific disruption of Smad4 and Pten in mice. *J Clin Invest.* 2006; 116:1843–52. [PubMed: 16767220]
32. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3 kinases as regulators of growth and metabolism. *Nat Rev Genet.* 2006; 6:606–619. [PubMed: 16847462]
33. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem.* 1998; 273:13375–78. [PubMed: 9593664]
34. Downward J. Mechanisms and consequences of activation of protein kinase B/Akt. *Curr Opin Cell Biol.* 1998; 10:262–7. [PubMed: 9561851]
35. Virolle T, Adamson ED, Baron V, et al. The Egr-1 transcription factor directly activates PTEN during irradiation-induced signaling. *Nat Cell Biol.* 2001; 3:1124–1128. [PubMed: 11781575]
36. Moorehead RA, Hojilla CV, De Belle I, et al. Insulin-like growth factor-II regulates PTEN expression in the mammary gland. *J Biol Chem.* 2003; 278:50422–50427. [PubMed: 14517213]
37. Yu J, Zhang SS, Saito K, et al. PTEN regulation by Akt-EGR1-ARF-PTEN axis. *EMBO J.* 2009; 28:21–33. [PubMed: 19057511]
38. Alimonti A, Carracedo A, Clohessy JG, et al. Subtle variations in Pten dose determine cancer susceptibility. *Nat Genet.* 2010; 42:454–8. [PubMed: 20400965]

39. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell*. 2007; 12:9–22. [PubMed: 17613433]
40. Chung JY, Hong SM, Choi BY, et al. The expression of phospho-AKT, phospho-mTOR, and PTEN in extrahepatic cholangiocarcinoma. *Clin Cancer Res*. 2009; 15:660–7. [PubMed: 19147772]
41. Pineau P, Marchio A, Nagamori S, et al. Homozygous deletion scanning in hepatobiliary tumor cell lines reveals alternative pathways for liver carcinogenesis. *Hepatology*. 2003; 37:852–61. [PubMed: 12668978]
42. Meng F, Henson R, Wehbe-Janek H, et al. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*. 2007; 133:647–58. [PubMed: 17681183]

Abbreviations

COX-2	cyclooxygenase-2
EGR1	early growth response-1
EGFR	epidermal growth factor receptor
HiBEC	human intrahepatic biliary epithelial cell
mPGES-1	microsomal prostaglandin E synthase-1
Mcl-1	myeloid cell leukemia-1
mTOR	mammalian target of rapamycin
NFκB	Nuclear Factor-κB
PGE₂	prostaglandin E ₂
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog deleted on chromosome 10
TLR4	Toll-like receptor4
5'-UTR	5'-untranslated region

Figure 1. mPGES-1 expression in human cholangiocarcinoma cells and its effect on tumor cell growth in vitro

A. (*Upper panel*) Immunohistochemical staining for mPGES-1 in human cholangiocarcinoma tissues (X100, scale bar 200 μm). Positive mPGES-1 staining was observed in human cholangiocarcinoma cells (indicated by arrows). The insert shows high power view of a malignant gland (top) and the adjacent two benign peribiliary glands (bottom). Note the negative or trace mPGES1 staining in the nonneoplastic peribiliary glands (surrounded by asterisks). (*Lower panel*) Western blotting of mPGES-1 (approximately 17KDa) from three cholangiocarcinoma cell lines (SG231, HuCCT1 and CCLP1) and normal human intrahepatic biliary epithelial cells (HiBECs).

B. Immunofluorescence staining and Western blotting for mPGES-1 in CCLP1 cells stably transfected with the mPGES-1 overexpression vector or RNAi vector. *Upper panels:* Immunofluorescence staining of mPGES-1 in four stable cell lines (TRITC staining with DAPI counterstaining) and the corresponding GFP fluorescence (original magnification ×100; scale bar 10μm). *Lower panels:* Western blotting of mPGES-1 in four stable cell lines showing that the level of mPGES-1 is increased in mPGES-1 overexpressed cell line (44KD, GFP-mPGES-1 fusion protein) and decreased in mPGES-1 knockdown cell line (17KD).

C. The synthesis of PGE₂ in CCLP1 cells stably transfected with the mPGES-1 overexpression vector or RNAi vector. Cell culture supernatants were collected to measure PGE₂ level by using the PGE₂ enzyme immunoassay system (Amersham Biosciences). The data are presented as mean ± SEM (p<0.01 compared to the corresponding vector control cells).

D. Cell counting assay *in vitro*. The samples were assayed in triplicates. Each point represents the mean value from three independent samples (* p<0.05; ** p<0.01).

E. Soft-agar colony-formation assay. Representative photographs and bar graphs of colony formation from three independent experiments.

Figure 2. The effect of mPGES-1 on cholangiocarcinogenesis *in vivo*

A. Tumor growth parameters. 1×10^8 cells suspended in 100 μ l PBS from four different stably cell lines were injected subcutaneously at armpit in SCID mice individually. **a.** Photography of xenograft tumors from SCID mice. **b.** The weight of xenograft tumors. The data represent means + SEM from 6 SCID mice. **c.** The onset time (days) of xenograft tumors. The data represent means + SEM from 6 SCID mice.

B. Histological and immunohistochemical analysis of the tumors from SCID mice. **a.** representative H&E stain and Ki-67, PCNA and PTEN immunostains (original magnification $\times 100$, scale bars 10 μ m). **b.** semi-quantification of Ki67 and PCNA positive cells from four different groups. The data represent mean $\pm$ SEM (n=6). **c.** Western blotting analysis for mPGES-1 and PTEN using xenograft tumor tissues (6 samples for each of the four different groups).

Figure 3. The effect of mPGES-1 on cell cycle progression, cell migration/invasion and injury repair

A. Cell cycle analysis by flow cytometry. Cell cycle analysis was performed on the cells from those four different stable cell lines. The data are presented as mean $\pm$ SEM.

B. BrdU immunofluorescence. Representative photographs of BrdU positive cells from four different groups are shown as left panel (scale bars 10 μ m). Quantitative analysis of BrdU positive cells are shown as right panel. The data are presented as mean $\pm$ SEM.

C. Transwell assay. Representative photographs of invaded cells from four independent groups are shown as upper panel (scale bar 10 μ m). Quantitative analysis of invaded cells is shown as lower panel. The data are presented as mean $\pm$ SEM from three different experiments.

D. Wound healing assay. Representative photographs from those four different groups were taken at 0, 8 and 24 hours after scratch (scale bar 500 μ m). The average wound diameters for each of the four groups at different time points are shown at the lower panel (** $p < 0.01$).

Figure 4. mPGES-1 inhibits the expression of PTEN

A. Immunofluorescence staining of PTEN and phosphorylated PTEN. Four different CCLP1 stable cell lines were stained for evaluation of the effect of mPGES-1 on PTEN and p-PTEN (Ser380). a. Cellular level of PTEN (red). b. Cellular level of p-PTEN (Ser380) (red). (original magnification X100, scale bars 10 μ m.)

B. Western blotting of PTEN and p-PTEN. The effect of mPGES-1 on PTEN or pPTEN was confirmed by western blotting with either PTEN or pPTEN antibodies. β -actin was used as the loading control.

C. RT-PCR analysis of PTEN. The effect of mPGES-1 on PTEN was further confirmed by RT-PCR. β -actin was used as the internal control.

D. The effect of PTEN on mPGES-1-induced cell growth. mPGES-1 overexpressed cells were transfected with PTEN expression vector and the cell growth was analyzed by WST-1 assay.

E. The effect of PTEN depletion on mPGES-1 knockdown-induced inhibition of cell growth. CCLP1 cells with mPGES1 knockdown were transfected with PTEN siRNA and the cell growth was analyzed by WST-1 assay.

Figure 5. mPGES-1 inhibits EGR1-induced PTEN expression and prevents EGR1 binding to PTEN

A. Western blot and RT-PCR analyses for PTEN in CCLP1 cells transfected with indicated plasmids.

B. Immunoprecipitation and Western blotting analysis to determine the interaction between PTEN and EGR1 in CCLP1 cells transfected with indicated plasmids.

C. Immunoprecipitation and Western blotting analysis to determine the interaction between p-PTEN and EGR1 in CCLP1 cells transfected with indicated plasmids.

Figure 6. The effect of mPGES-1 on EGR1 sumoylation and binding to 5'-UTR of PTEN promoter

A, B & C. Immunoprecipitation and immunoblotting. Equal amount of cell lysate from CCLP1 cells transfected with mPGES-1 overexpression plasmid, mPGES-1 siRNA plasmid or control plasmids were subjected to immunoprecipitation with SUMO1 antibody followed by EGR1 western blotting, or vice versa.

D. DNA pull down assay. Equal amount of cell lysate was pull down with biotinylated EGR1 DNA probe followed by immunoblotting with anti-EGR1 antibody.

E. DNA pull down assay. Equal amount of cell lysate was first pull down with biotinylated EGR1 DNA probe. The precipitates were eluted from the first pull down for either immunoprecipitation with anti-SUMO1 antibody and immunoblot with anti-EGR1 antibody or immunoprecipitation with anti-EGR1 antibody and immunoblot with anti-SUMO1 antibody.

Figure 7. mPGES-1 activates PI3K-AKT-mTOR pathway
A. Western blotting with indicated antibodies in CCLP1 stable cell lines. β -actin was used as loading control.
B. Western blotting with indicated antibodies in CCLP1 cells transiently transfected with mPGES-1 expression or RNAi plasmid for 48 hours. β -actin was used as loading control.
C. Schematic diagram illustrating the mechanisms for mPGES-1-mediated cholangiocarcinogenesis.