A Novel FoxM1-Caveolin Signaling Pathway Promotes Pancreatic Cancer Invasion and Metastasis

Chen Huang1,2,4, Zhengjun Qiu1,2, Liwei Wang1,3, Zhihai Peng1,2, Zhiliang Jia4, Craig D. Logsdon5, Xiangdong Le4, Daoyan Wei4, Suyun Huang6, and Keping Xie4

Abstract

Caveolin-1 (Cav-1), a principal structural component of caveolar membrane domains, contributes to cancer development but its precise functional roles and regulation remain unclear. In this study, we determined the oncogenic function of Cav-1 in preclinical models of pancreatic cancer and in human tissue specimens. Cav-1 expression levels correlated with metastatic potential and epithelial–mesenchymal transition (EMT) in both mouse and human pancreatic cancer cells. Elevated levels in cells promoted EMT, migration, invasion, and metastasis in animal models, whereas RNA interference (RNAi)-mediated knockdown inhibited these processes. We determined that levels of Cav-1 and the Forkhead transcription factor FoxM1 correlated directly in pancreatic cancer cells and tumor tissues. Enforced expression of FoxM1 increased Cav-1 levels, whereas RNAi-mediated knockdown of FoxM1 had the opposite effect. FoxM1 directly bound to the promoter region of Cav-1 gene and positively transactivated its activity. Collectively, our findings defined Cav-1 as an important downstream oncogenic target of FoxM1, suggesting that dysregulated signaling of this novel FoxM1-Cav-1 pathway promotes pancreatic cancer development and progression. Cancer Res; 72(3); 655–65. ©2011 AACR.

Introduction

Pancreatic cancer represents one of the leading causes of cancer deaths in industrialized countries with an estimated 43,140 new cases and 36,800 deaths occurring annually in the United States (1). At presentation, the disease has, in most cases, already spread locally and to distant organs that preclude a resection, and only about 5% to 25% of cases constitute candidates for surgical resection. Among patients undergoing a potentially curative resection, the long-term outcome remains unsatisfactory due to early recurrence and metastatic disease (2). Effective systemic therapy capable of reversing the aggressive biology of this disease is currently not available. Therefore, there is an urgent need for a better understanding of the molecular mechanisms underlying pancreatic cancer pathogenesis (3, 4).

Caveolin-1 (Cav-1) is the major structural protein in caveolae; 50- to 100-nm omega-shaped invaginations of the plasma membrane. Caveolae are involved in signal transduction, wherein Cav-1 acts as a scaffold to organize multiple molecular complexes regulating a variety of cellular events (5). Recent studies have suggested that Cav-1 may play important roles in cancer development and progression (6–10). Paradoxically, Cav-1 seems to impact both positively and negatively on cancer biology (6), although recent studies suggest a positive association between Cav-1 expression and pancreatic cancer poor prognosis (11–13). Specifically, tumor cells in pancreatic cancer show significantly higher Cav-1 expression relative to tumor stroma, and Cav-1 knockdown significantly induces cell apoptosis and enhances the radiosensitivity of pancreatic cancer cells (14, 15). Furthermore, Cav-1 expression could be detected in the fibroblasts of the desmoplastic pancreatic cancer stroma but not in stromal cells of the normal pancreas. Importantly, coexpression of Cav-1 and fatty acid synthase significantly correlates with clinical features and survival status of patients with pancreatic cancer showing that Cav-1 and other factors may functionally cooperate in the process of pancreatic tumorigenesis (16). All of this experimental and clinical evidence supports that Cav-1 is an oncogene in pancreatic cancer and may be good candidate prognostic markers and targets for therapeutic intervention. Interestingly, researchers have shown that Cav-1 expression can be upregulated during epithelial–mesenchymal transition (EMT), and furthermore, once expressed, Cav-1 can influence cancer cell motility (17). Moreover, Cav-1 overexpression increased motility and invasion of human hepatocellular carcinoma cells.
metastasis. However, the precise function and underlying mechanisms of Cav-1 in pancreatic cancer EMT and metastasis remain unclear and how Cav-1 expression is regulated is unknown.

FoxM1, a transcription factor of the Forkhead box (Fox) protein superfamily, is a key regulator of both G1–S and G2–M phases of the cell cycle and mitotic spindle integrity (19). Recent studies have strongly suggested that FoxM1 is oncogenic and plays important roles in angiogenesis, invasion, and metastasis (20, 21). Interestingly, FoxM1 may regulate EMT through downregulation of E-cadherin while upregulation of vimentin showing the important roles of Cav-1 in EMT and metastasis of human cancer (18). However, the precise function of FoxM1 in Cav-1 expression and function. We are unknown. In this study, we sought to determine the role of Cav-1 in pancreatic cancer EMT and metastasis and regulatory function of FoxM1 in Cav-1 expression and function. We discovered that the novel FoxM1-Cav-1 signaling pathway critically regulates pancreatic cancer EMT, invasion, and metastasis.

Materials and Methods

Cell lines and culture conditions

The human pancreatic adenocarcinoma cell lines AsPC-1, CaPan-1, MiaPaca-2, and PANC-1 were purchased from the American Type Culture Collection; MDA Panc-28, and MDA Panc-48 were gifts from Dr. Paul J. Chiao (MD Anderson Cancer Center, Houston, TX); and COLO357 human pancreatic ductal epithelial (HPDE) cell line (provided by Dr. Tsao, Ontario Cancer Institute, Toronto, ON, Canada) was maintained in keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen). The immortalized normal pancreatic cell lines (Panc02-H7) were described previously (23–25). All of these cell lines were maintained in plastic flasks as adherent monolayers in Eagle’s minimal essential medium supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, t-glutamine, and vitamin solution (Flow Laboratories). The immortalized normal human pancreatic ductal epithelial (HPDE) cell line (provided by Dr. Tsao, Ontario Cancer Institute, Toronto, ON, Canada) was maintained in keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen).

Western blot analysis

Standard Western blotting was carried out using whole-cell protein lysates and primary antibodies against FoxM1 (Santa Cruz Biotechnology), Cav-1, E-cadherin and vimentin (BD Biosciences), β-catenin and N-cadherin (Cell Signaling Technology); and a secondary antibody (anti-rabbit IgG or anti-mouse IgG; Santa Cruz Biotechnology). Equal protein sample loading was monitored using an anti-GAPDH antibody (Santa Cruz Biotechnology).

Promoter reporter and dual luciferase assay

The Cav-1 promoter luciferase reporter p3LUC-caveolin-1 (pLuc-Cav) was described previously (28). Pancreatic cancer cells were transfected with the indicated pLuc-Cav, siRNAs, or specific gene expression plasmids. The Cav-1 promoter activity was normalized by cotransfection with a β-actin/renilla luciferase reporter containing a full-length renilla luciferase gene (31). Luciferase activity in the cells was quantified using a dual luciferase assay system (Promega) 24 hours after transfection.

Human tissue samples and immunohistochemical analysis

Cav-1 and FoxM1 expression was analyzed using human pancreatic cancer and normal tissue microarray (US Biomax). The use of the tissue samples was approved by the Institutional Review Board of MD Anderson Cancer Center. Standard immunohistochemical procedures were carried out using anti-FoxM1 antibody (Santa Cruz Biotechnology) and anti-Cav-1 antibody (BD Biosciences). The staining results were scored by 2 investigators blinded to the clinical data as described previously (32). For negative controls, the primary antibody was omitted and replaced by negative IgG (Supplementary Fig. S1).

Statistics

The significance of the data from patient specimens was determined by the Pearson correlation coefficient. The significance of the in vitro data and in vivo data was determined by Student t test (2-tailed), Mann–Whitney test (2-tailed), or one-way ANOVA. P < 0.05 was considered significant.

Reverse transcription-PCR, chromatin immunoprecipitation assay, cell scratch wound assay, cell migration assay, cell invasion assay, cell immunofluorescence, animals, subcutaneous tumor growth, and experimental liver metastases

For experimental liver metastases, refer to the work of Marvin and colleagues (33). Details are described in the Supplementary Materials and Methods.

Results

Cav-1 overexpression and its direct association with pancreatic cancer pathologic features

We first investigated the expression of Cav-1 protein in the 70 primary pancreatic tumor and 10 normal pancreatic tissue
specimens in a tissue microarray. We observed Cav-1–positive staining in the membrane and cytoplasm of the tumor cells and tumor stroma with Cav-1–negative or weak Cav-1–positive staining in adjacent normal pancreatic cells and normal pancreatic cells (Fig. 1A and Supplementary Fig. S2). Increasing Cav-1 expression correlated with decreased level of tumor differentiation and significant difference between well (grade I) versus poorly differentiated (grade III) tumors (Fig. 1B and Supplementary Fig. S3). In addition, Cav-1 expression was positively correlated with disease stage showing that Cav-1 is upregulated in late-stage tumors. This association was significant between stage I versus stage IV tumors (Fig. 1C and Supplementary Fig. S3). Moreover, Cav-1 expression in lymph node metastasis or distant metastasis specimens was significantly higher than that in nonmetastasis specimens (Fig. 1D and Supplementary Fig. S3). These findings strongly indicated that Cav-1 expression plays a critical role in pancreatic cancer development and progression and is a valuable biomarker for this disease.

**Association of Cav-1 expression with metastatic potential and EMT phenotype**

Cav-1 is markedly upregulated in most pancreatic cancer cell lines as compared with a transformed HPDE cell line (Fig. 2A). Then, we carried out cell immunofluorescence and Western blot analyses for Cav-1 in the poorly metastatic human and mouse pancreatic cancer cell lines (COLO357, Panc02) as well as highly metastatic human and mouse pancreatic cancer cell lines (L3.7, Panc02-H7). The levels of Cav-1 expression directly correlated with metastatic ability (Fig. 2B and D and Supplementary Fig. S4A). We further observed the cell morphology under invert phase-contrast microscope. The poorly metastatic cells (COLO357, Panc02) exhibited a typical epithelial morphology, whereas the highly metastatic cells (L3.7, Panc02-H7) exhibited a typical mesenchymal morphology (Fig. 2C). The unique morphology changes were consistent with the expression of EMT markers, that is, the expression of epithelial marker (E-cadherin, β-catenin) in the poorly metastatic cells (COLO357, Panc02).
was higher than that in the highly metastatic cells (L3.7, Panc02-H7; Fig. 2D). In contrast, the expression of mesenchymal marker (vimentin, N-cadherin) in the poorly metastatic cells (COLO357, Panc02) was lower than that in the highly metastatic cells (L3.7, Panc02-H7; Fig. 2D). These results strongly indicated that Cav-1 expression directly correlated with EMT phenotype and metastatic potential of pancreatic cancer cells.

**Altered Cav-1 expression directly affects epithelial or mesenchymal phenotype of pancreatic cancer cells**

To determine the effect of altered Cav-1 expression on epithelial or mesenchymal phenotype of pancreatic cancer cells, we transfected the Cav-1 expression vector pcDNA3.1-Cav-1 or the control vector pcDNA3.1 into COLO357 cells, which typically express low levels of Cav-1 and have epithelial phenotype. We found that elevated expression of Cav-1 in
COLO357 cells (Fig. 3A and C, left; and Supplementary Fig. S4B) significantly increased vimentin but decreased E-cadherin expression (Fig. 3C, left) and caused typical morphology changes of EMT (Fig. 3B, top). In contrast, we transfected Cav-1 siRNA or the control siRNA into L3.7 cells, which typically express high levels of Cav-1 and have mesenchymal phenotype. This transfection significantly inhibited Cav-1 protein expression in L3.7 cells (Fig. 3A and C, right; and Supplementary Fig. S4C) and Cav-1 knockdown led to typical morphology changes of MET of L3.7 cells (Fig. 3B, bottom) and a concomitant decrease in vimentin but increase in E-cadherin expression (Fig. 3C, right). These results clearly indicated that altered Cav-1 expression affected epithelial or mesenchymal phenotype of pancreatic cancer cells.

**Altered Cav-1 expression affected migration and invasion in vitro and growth and metastases in vivo of pancreatic cancer cells**

To determine the effect of altered Cav-1 expression on migration of pancreatic cancer cells, COLO357 and L3.7 cells were transfected with pcDNA3.1-Cav-1 and Cav-1 siRNA for 48 hours, respectively. The transfected cells were wounded by scratching and maintained at 37°C for additional 12 hours. The overexpression of Cav-1 strongly promoted the flattening and spreading of COLO357 cells [Fig. 4A (i)], whereas knockdown of Cav-1 attenuated the flattening and spreading of L3.7 cells [Fig. 4B (i)]. The results of cell migration assay also indicated that overexpression of Cav-1 promoted the migration ability of COLO357 cells [Fig. 4A (ii) and Supplementary Fig. S5A], whereas knockdown of expression of Cav-1 attenuated the migration ability of L3.7 cells [Fig. 4B (ii) and Supplementary Fig. S5B]. Similarly, overexpression of Cav-1 promoted the invasiveness of COLO357 cells [Fig. 4A (iii) and Supplementary Fig. S6A], whereas knockdown of expression of Cav-1 attenuated the invasiveness of L3.7 cells [Fig. 4B (iii) and Supplementary Fig. S6B]. Consistent with the impact of altered Cav-1 expression on migration and invasion of pancreatic cancer cells in vitro, pcDNA3.1-Cav-1 transfection significantly promoted pancreatic tumor growth [Fig. 5A (i), (ii), and (v)] and increased liver metastases of COLO357 cells [Fig. 5A (iii), (iv), and (vi)] and Supplementary Fig. S7A], whereas Cav-1 siRNA transfection significantly inhibited pancreatic tumor growth [Fig. 5B (i), (ii), and (v)] and abrogated liver metastases of L3.7 cells [Fig. 5B (iii), (iv), and (vi) and Supplementary Fig. S7B] in nude mice. Thus, our data clearly established that Cav-1 is oncogenic and promotes invasion and metastasis of pancreatic cancer.

**Close relationship between altered expression of FoxM1 and Cav-1 in pancreatic cancer**

To explore the mechanisms underlying Cav-1 overexpression, we initially analyzed both FoxM1 and Cav-1 expression in pancreatic cancer tissues and cell lines. Pancreatic cancer tissues expressed both FoxM1 and Cav-1 (Fig. 6A) and their direct correlation was found statistically significant ($r = 0.574; P < 0.001$; Fig. 6B). Consistently, the expression of Cav-1 directly
correlated with the expression of FoxM1 in pancreatic cancer cell lines (Fig. 6C).

To provide casual evidence for the direct correlation between the expression FoxM1 and Cav-1, we determined the impacts of altered FoxM1 expression on Cav-1 expression in human pancreatic cancer cell lines that have either low (COLO357 and AsPC-1) or high (L3.7 and PA-TU-8902) levels of FoxM1 expression. We found that increased expression of FoxM1 in COLO357 and AsPC-1 cells [Fig. 6D (i)] led to significantly increased Cav-1 mRNA and protein [Fig. 6D (i)]. Conversely, knockdown of FoxM1 expression by cotransfecting FoxM1-siRNA into L3.7 and PA-TU-8902 cells suppressed Cav-1 promoter activities [Fig. 6E (ii)].
Collectively, our findings strongly indicated that FoxM1 regulates Cav-1 expression most likely at the transcriptional level.

**Direct interaction of FoxM1 with Cav-1 promoter**

Finally, we analyzed the sequence of the Cav-1 promoter for the presence of potential FoxM1-binding elements by using the FoxM1 consensus sequence 5'-A(C/T)AAA(C/T)AA-3' (34), 5'-AGATTGAGTA-3' (35), and 5'-TAATCA-3' (31). We identified 3 putative FoxM1-binding elements (referred to as DNA sequences #1, #2, and #3) in the Cav-1 promoter region (Fig. 7A). To provide direct proof that FoxM1 is recruited to the endogenous Cav-1 promoter during transcription in vivo, we conducted chromatin immunoprecipitation assays using chromatin prepared from COLO357, AsPC-1, L3.7, and PA-TU-8902 cells and 3 primer sets framing the 345-bp (−1,771 to −1,427; #1 site), 108-bp (−1,260 to −1,153; #2 site), and 181-bp (−1,174 to −994; #3 site) regions of the Cav-1 promoter (Fig. 7A). The 181-bp DNA fragment was amplified from the precipitates by anti-FoxM1 antibodies but not by control IgG in COLO357, AsPC-1, L3.7, and PA-TU-8902 cells (Fig. 7B, right), suggesting that endogenous FoxM1 bound to the region between −1,174 and −994 bp of the Cav-1 promoter in pancreatic cancer cells. However, we did not observe any detectable binding of FoxM1 to the site #1 and #2 (Fig. 7B, left and middle). These results were further confirmed using COLO357 and AsPC-1 cells with engineered overexpression of FoxM1, which led to increased FoxM1 recruitment to Cav-1 promoter (Fig. 7C), and using L3.7 and PA-TU-8902 with FoxM1 knockdown, which led to decreased FoxM1 recruitment to Cav-1 promoter (Fig. 7D). The altered FoxM1 recruitments were consistent with changes of Cav-1 promoter activities (Fig. 7E and F). Collectively, these findings showed that FoxM1 bound primarily at position −1,055 to −1,050 bp of Cav-1 promoter and positively regulates Cav-1 transcription.
Discussion

In this study, we determined the critical roles of Cav-1 and FoxM1 in pancreatic cancer pathogenesis and their underlying mechanisms. We found FoxM1 transcriptionally activated Cav-1 gene, constituting a novel signaling pathway that directly impact EMT, invasion, and metastasis of pancreatic cancer cells and its alterations inform the clinicopathologic behaviors of pancreatic cancer. Collectively, our novel clinical and mechanistic evidence strongly suggested that dysregulated FoxM1 expression causes abnormal Cav-1 expression and critically contributes to pancreatic cancer pathogenesis and aggressive biology.

Cav-1 is an essential constituent of caveolae and interacts with a variety of cellular proteins and regulates cell signaling events. However, the potential roles of Cav-1 in cancer...
development and progression are highly inconsistent, functioning from as a tumor suppressor to as an oncogene (6, 36). First possible reason could be due to tumor types. For example, Cav-1 seems to be tumor suppressor in breast cancer and ovarian carcinoma (37, 38), whereas Cav-1 acts more as an oncogene in gastrointestinal cancer, including pancreatic cancer (11–13, 39). Second possible reason is the tumor stage. For example, loss of Cav-1 is necessary and sufficient to promote fibroblasts cell transformation in the early stages of cancer progression, supporting the notion that it functions as a tumor suppressor (40). However, increased expression of Cav-1 correlates with advanced pathologic stage, the presence of metastasis, and poor cancer prognosis of non–small cell lung cancer (41) and esophageal carcinoma (9). Third possible reason could be due to research approaches. For example, most studies using human pancreatic tumor specimens have found a Cav-1 overexpression and its direct correlation with a poor prognosis (11–13). However, biology studies suggested otherwise (42). In our current study, we provided many lines of evidence to show Cav-1 as an oncogene. First of all, elevated Cav-1 expression directly correlated with poorer tumor differentiation and advanced disease stage, particularly with lymph node and distant metastasis. Thus, we provide the first clinical evidence that Cav-1 promotes pancreatic cancer metastasis.

Our notion is further supported by our novel findings using our unique cell line systems, that is, mouse pancreatic cancer line Panc02 and Panc02-H7 and human pancreatic cancer cell lines COL0357 and L3.7, which derive from biologically isogenic cell lines but have distinct metastatic properties. Specifically, Cav-1 protein levels positively correlated with metastatic potential of pancreatic cancer cells. Elevated expression of Cav-1 promoted metastasis of poorly metastatic pancreatic cancer cells, whereas knockdown of Cav-1 attenuated migration and invasion abilities of highly metastatic pancreatic cancer cells. Our findings were consistent with recent studies, which have suggested a regulatory role for Cav-1 in metastasis of hepatocellular carcinoma and lung adenocarcinoma cells (10, 43).

Cancer metastasis is a complex process and its cellular and molecular basis remains to be determined (44, 45). Emerging evidences suggest that EMT plays a key role in metastasis (46, 47). In this study, we presented 2 cell systems with cell morphology, molecular biomarkers, and biology behaviors being consistent with EMT or MET. We used these
systems to show the critical role of Cav-1 in pancreatic cancer EMT and progression. Specifically, the poorly metastatic human and mouse pancreatic cancer cell lines (COLO357 and Panc02) exhibit a typical epithelial morphology and high expression of epithelial markers (E-cadherin and β-catenin), whereas the highly metastatic human and mouse pancreatic cancer cell lines (L3.7 and Panc02-H7) exhibit a typical mesenchymal morphology and have high expression of mesenchymal marker (vimentin and N-cadherin). Moreover, we observed that elevated Cav-1 expression could contribute to the acquisition of EMT phenotype by upregulation of mesenchymal cell marker vimentin and downregulation of epithelial cell marker E-cadherin, whereas decreased Cav-1 expression could contribute to the acquisition of MET phenotype by downregulation of mesenchymal cell marker vimentin and upregulation of epithelial cell marker E-cadherin. Our new findings were consistent with recent studies, which have shown one correlation between Cav-1 overexpression and downregulation of E-cadherin but upregulation of vimentin in human hepatocellular carcinoma cells (18) and another correlation between cancer cell EMT and Cav-1 upregulation (17). We showed for the first the critical role of Cav-1 in pancreatic cancer EMT.

Given the important role of Cav-1 in pancreatic cancer EMT and metastasis, we further determined the underlying mechanisms responsible for Cav-1 overexpression in malignancies, which are currently unclear (48). Our current study showed that Cav-1 was a novel downstream target of FoxM1, an important transcription factor that has been implicated in the development and progression of many types of cancer (19–21). Our specific findings include (i) the expression levels of Cav-1 directly correlated with the expression levels of FoxM1 both in pancreatic cancer cell lines and in pancreatic cancer tissues; (ii) overexpression of FoxM1 led to increased expression of Cav-1 whereas knockdown of FoxM1 did the opposite; and (iii) FoxM1 directly bound to the promoter regions of Cav-1 gene and transactivated Cav-1 gene transcription. Therefore, our findings provided clinical and mechanistic evidence supporting the existence of a novel FoxM1-Cav-1 signaling pathway and its critical contribution to pancreatic cancer growth and metastasis. In addition, there are reports indicating the regulatory roles of Sp1 and Stat3 in Cav-1 expression (49, 50). It would be interesting and significant to further determine the cross-talks between those transcription factors and FoxM1 in regulating Cav-1 expression and function, how these cross-talks are altered during pancreatic carcinogenesis and contribute to pancreatic cancer progression and metastasis, and future potential of designing effective therapies that target FoxM1-Cav-1 signaling.

In summary, this study provided critical insight into the role of the Cav-1 in pancreatic cancer progression and metastasis; the critical role of upstream FoxM1 transcription factor in Cav-1 expression; and critical role of this novel FoxM1-Cav-1 signaling in EMT and MET phenotypes and malignancy of pancreatic cancer. Collectively, our study not only provides a novel molecular mechanism for pancreatic cancer progression and metastasis but also underscores this aberrant FoxM1-Cav-1 signaling as a promising new molecular target for designing novel therapeutic modalities to control pancreatic cancer progression and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Don Norwood for editorial comments.

Grant Support

The work was supported in part by grants R01-CA129995, R01-CA149954, and R01CA152399 (to K. Xie), and R01-CA116528 and R01-CA157933 (to S. Huang) from the National Cancer Institute, NIH and grant No.81101844 (to C. Huang) from the National Natural Science Foundation of China.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 19, 2011; revised November 9, 2011; accepted November 28, 2011; published OnlineFirst December 22, 2011.

References

FoxM1–Caveolin-1 Signal in Pancreatic Cancer


