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EXPRESSION OF A PRO-METASTATIC SPLICE VARIANT OF OSTEOPONTIN, OPNC, IN HUMAN PANCREATIC DUCTAL ADENOCARCINOMA

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Abstract

Introduction—Osteopontin (OPN) is a secreted phosphoprotein that confers on cancer cells a migratory phenotype. We showed recently that nicotine, a major risk factor in pancreatic ductal adenocarcinoma (PDA), increases OPN expression in PDA cells. An OPN splice variant, OPNc, supports anchorage independence and maybe the most potent OPN isoform to convey metastatic behavior. In this study, we tested the effect of nicotine on OPNc expression, and analyzed the correlation between total OPN/OPNc levels and patients' smoking history.

Methods—Real time PCR and UV-light-illumination of ethidium-bromide staining were used to examine the mRNA expression in tissue and in PDA cells treated with or without nicotine (3–300 nM). OPN and OPNc were localized by immunohistochemistry, and ELISA was used to analyze OPN serum levels.

Results—Nicotine treatment of PDA cells selectively induced denovo expression of OPNc. OPNc was found in 87% of invasive PDA lesions, of which 73% were smokers. The levels of OPNc correlated well with higher expression levels of total OPN in the tissue and serum from patients with invasive PDA.

Conclusions—Our data suggest that smoking and nicotine may contribute to PDA metastatic potential through promoting OPNc expression. Although the direct role of OPNc in PDA progression is not defined, OPNc may have value as a diagnostic and prognostic marker, especially in invasive PDA.

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Keywords

pancreatic cancer; nicotine; osteopontin

INTRODUCTION

The incidence of pancreatic ductal adenocarcinoma (PDA) was estimated at 37,170 in 2007 (1). There is only a 5% overall 5-year survival rate, and more than 85% of the tumors are diagnosed after the tumor has infiltrated into adjacent vital organs or when distant metastases are present. There is, therefore, an urgent need for better understanding of the basic molecular mechanisms that contribute to the aggressive nature of PDA and for the design of more effective therapeutic strategies.

Cigarette smoking is among the most notable risk factors, lending upwards of a twofold increase to the risk of developing PDA (2). It is estimated that cigarette smoking may be responsible for 25 to 30% of all PDA cases (3). Nicotine, a major component of tobacco and cigarette smoke, is an addictive agent and has been characterized as a drug of abuse by the U.S. Surgeon General (4). Pancreatic cancer have been linked to nicotine exposure through cigarette smoking in many human studies (5,6). In addition, animal studies have demonstrated that nicotine or its metabolites can induce pathological and functional changes in the pancreas (7). It is unknown, however, how these functional or morphological changes could contribute to the progression of pancreatic cancer. A possible mechanism is nicotine-mediated induction of stress response genes, which are important in cancer metastasis (8).

One such stress response gene is osteopontin (OPN), a secreted noncollagenous, sialic acid-rich phosphoprotein, which functions as both an extracellular matrix component and a cytokine (9,10). OPN can support migration and protect against programmed cell death after binding to certain integrin receptors or a CD44 variant on the cell surface (11). The biological functions of metastasis-associated gene products are extensively regulated on the post-transcriptional and post-translational levels (8). Consistently, OPN secreted from various cells has diverse structural characteristics (12,13) and tumor-derived OPN forms are smaller than osteopontin secreted by nontransformed cells (14). Recent studies have shown that an OPN splice variant (OPNc) is expressed in invasive, but not in noninvasive, breast and liver tumor cell lines (10, 15). It has been suggested that this isoform may support tumor progression by conveying anchorage independence and inducing the expression of oxidoreductases (16). Although high levels of OPN have been reported in PDA tissue (17), the possible regulation of this OPN metastatic isoform by PDA carcinogens, such as nicotine, has not been studied.

We have recently shown that nicotine induces the expression of OPN in PDA cell lines (18). In this study, we tested the effect of nicotine on OPNc expression in 3 PDA cell lines. We also correlated the patients' smoking history with the expression levels of serum and tissue OPN, and tissue OPNc in premalignant (intra papillary mucinous neoplasms, IPMN) and in invasive PDA.

MATERIALS AND METHODS

Cell Culture

The human PDA cell line MiaPaca was purchased from the American Type Culture Collection (Manassas, VA). BxPC-3 and HS766T cells were kindly provided by Dr. Scott Kern, Johns Hopkins University School of Medicine, Baltimore, MD. Cells were counted and cultured at 1×10^4 cells to near confluence in 96-well plates and maintained in DMEM supplemented with 10% fetal bovine serum in a humid atmosphere of 5% CO₂/95% air. Cells were treated with

nicotine (3 - 300 nM) for 3 and 24 h, and were evaluated for the expression of OPN mRNA by real time PCR. OPNc expression was evaluated by UV light illumination of ethidium bromide staining of PCR products.

RNA Extraction and Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from PDA cells or pancreata using Trizol reagent (Life Technologies, Gaithersburg, MD). RNAs were quantified and input amounts were optimized for each amplicon. OPN and GAPDH (internal control) primers and probes were designed with the help of Primer Express Software (Applied Biosystems; Foster City, CA). cDNA was prepared, diluted, and subjected to real-time PCR using the TaqMan technology (7500 Sequence Detector; Applied Biosystems). The relative mRNA levels were presented as unit values of $2^{[C_T(\text{GAPDH}) - C_T(\text{OPN})]}$, where C_T is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

Semi-quantitative PCR

RNAs from cells and tissues were quantified, DNase-digested, and cDNAs were prepared using ImProm-IITM Reverse Transcription System (Promega), then subjected to semi-quantitative PCR using master mix (Promega). The primers used were: OPNc human forward 5'-TCAGGAAAAGCAGAATGCTG-3', reverse 5'-GTCAATGGAGTCCTGGCTGT-3'. Upstream and downstream primers that could anneal with the 3'-untranslated region of human GAPDH were included in the PCR reaction as an internal standard forward 5'-TGAAGGTCCGAGTCAACGGATTTGGT-3', reverse 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The linear range of amplification for each set of primers was determined to ensure that we used a number of cycles in the linear range. PCR products were electrophoresed on 2 % agarose gels and band intensities were quantified using Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290).

Protein isolation and Western blot analysis

Cell lysates were analyzed as described elsewhere. Anti- total OPN and anti- β -actin antibodies were purchased from Santa Cruz and Sigma, respectively.

Human Tissue Acquisition and Analysis

Histologically confirmed human invasive PDA (n=40, 29 smokers and 11 non smokers), intra papillary mucinous neoplasms, IPMN (n=6, 2 smokers, 4 non-smoker) were obtained from patients who underwent surgical resection at the Thomas Jefferson University Hospital between 2005 and 2008. All patients signed an appropriate consent for tissue acquisition and study. The study was approved by the Institutional Review Board of Thomas Jefferson University. Tissue samples were stored in *RNA Later* for RNA analysis. The mean age of the patients at the time of diagnosis was 64 years (range 48-87); 23 were men and 23 were women. Patients' smoking history was extracted from the clinical notes and correlated with OPN, and OPNc expression levels.

Immunohistochemistry

Paraffin blocks were sectioned at 5 μ m stained with a monoclonal antibody against OPN (2A1, Santa Cruz) (1:100). A vectastain universal elite ABC kit and 3,3'-diaminobenzidine tetrahydrochloride chromogenic substrate (Vector Laboratories Inc.) was used to visualize tissue staining. Serial sections were stained with an affinity-purified anti-OPNc chicken IgY (produced by Gallus Immunotech from the peptide ac-SEEKQNAVSC), at 1:160 dilution, as described previously (16). Antibody specificity was validated with nonimmune isotype serum. Negative control sections, where the primary or secondary antibodies were omitted were also prepared.

ELISA

Serum samples were obtained by venous puncture from invasive PDA patients who were smokers (n=29) and non-smokers (n=11), and from IPMN patients who were smokers (n=2) and non-smokers (n=4). OPN protein levels were measured using an ELISA kit (R&D; USA) according to the manufacturer's instructions. Spectrophotometric evaluation of OPN levels was made by Synergy HT multi-detection Microplate reader (BioTeck, Winooski, VT).

Statistical analysis

All experiments were performed 4 to 6 times. Data were analyzed for statistical significance by ANOVA with post-hoc student *t* test analysis. Data are presented as mean $\pm$ SEM. Continuous, normally distributed variables were analyzed by Student-t-test. Spearman's rank correlation test was performed to analyze the correlation between OPN, OPNc and nAChR mRNAs expression. Fisher's exact test or chi-square (χ^2) test were also used to analyze the distribution of OPN-strongly positive cases and OPNc expression. Analyses were performed with the assistance of a computer program (JMP 5 Software SAS Campus Drive, Cary, NC). Differences were considered significant at $P \leq 0.05$.

RESULTS

Expression of Total OPN and OPNc in PDA cells

Analysis of total OPN levels by real time PCR revealed that PDA cells express variable basal levels of OPN. MiaPaca cells expressed undetectable levels of OPN mRNA transcripts when compared with BxPC-3 cells, which displayed 1.6 times higher levels of OPN mRNA transcripts than HS766T cells (Fig 1A). To confirm that the protein expression levels match the basal OPN mRNA levels, we analyzed the basal OPN protein expression by western immunoblotting. As seen in Fig 1B, there was a considerable amount of OPN protein, especially in BxPC-3 and HS766T cells. Two bands of OPN protein at ~65 kDa and ~25 kDa could be recognized. UV-light-illumination of ethidium-bromide staining of PCR products after agarose gel electrophoresis showed a 155 bp band for OPNc that was detectable only in BxPC-3 cells (Fig 1C), but not in HS766T or MiaPaca cells. These data suggest a correlation between high expression levels of OPN and presence of OPNc in PDA cells.

Effect of nicotine on OPN and OPNc expression

Addition of nicotine (3-300nM) to PDA cells for 3 and 24 h induced a differential increase in total OPN mRNA expression. In MiaPaca cells (Fig 2A), nicotine induced a dose-dependent significant increase of OPN mRNA at 3 h. In HS766T cells (Fig 2B), nicotine induced a dose- and time-dependent significant increase in OPN mRNA. In BxPC-3 cells, nicotine treatment was associated with a non-significant trend toward increased OPN mRNA expression (Fig 2C). These data suggest that high basal levels of OPN mRNA might regulate the synthesis of additional OPN in response to nicotine.

Nicotine had no effect on OPNc expression in MiaPaca cells, which lacked the OPNc isoform (data not shown). Interestingly, nicotine (30nM) induced denovo expression of OPNc isoform in HS766T cells after 24h (Fig3). Nicotine (3 nM) at 3 and 24 h increased OPNc mRNA expression in BxPC-3 cells, which constitutively expressed OPNc (Fig 3). These data suggest that high nicotine concentrations promote the expression of OPNc isoform in certain PDA cell lines.

Expression of total OPN mRNA in tissue samples of smokers and non-smokers

Total OPN levels in IPMN (n=6, 2 smokers, 4 non-smoker) and invasive PDA (n=40, 29 smokers and 11 non-smokers) were analyzed by real time PCR. RT-PCR relative quantification

(RQ) values of OPN/GAPDH of >1 indicated high total OPN and was labeled (+++), a value of $0.5 - 1$ was labeled (++), of $0.1-0.5$ was labeled (+), and of <0.1 was labeled (-) (Fig4A). In PDA samples taken from smokers, more than 70% of the cases expressed high (+++) OPN mRNA levels, compared to 35% of the non-smokers. IPMN lesions, the majority of which were from non-smokers, expressed minimal amounts of OPN (-). These data suggest that in PDA, the pancreas might be an active source of OPN and that OPN among the invasive PDA lesions is expressed significantly more in smokers' samples ($p<0.05$).

Expression of OPNc mRNA in tissue samples of smokers and non-smokers

OPNc was analyzed by RT-PCR using specific primers and GAPDH as an internal control. OPNc band at 155 bp was found in all (100%) invasive PDA specimens that contained high (+++) OPN mRNA ($n=25$, 21 smokers and 4 non-smokers). In the premalignant lesions, OPNc was present in 50% of the smokers, while no OPNc could be detected in the non-smokers. A representative of these findings is seen in Fig 4B. OPNc band intensities were labeled (+++) for high intensity, (++) for moderate intensity, (+) for low intensity, and (-) for minimal intensity (Fig 4C). There was a significant ($p<0.005$) correlation between OPNc band intensities with total OPN levels (Fig 4D). These data suggest that increased OPN expression in smokers is associated with the expression of OPNc isoform.

Immunohistochemical expression of OPN and OPNc in tissue sections of smokers and non-smokers

As shown in Figure 5A, in invasive PDA lesions from smokers, intense immunoreactivities for both OPN and OPNc are seen in the malignant ducts colocalized to the membrane and cytoplasm of the tumor cells, and in the fibroblasts of the desmoplastic stroma. In the invasive lesions from non-smokers, intense OPN staining still could be detected in the malignant ducts and surrounding stromal cells. However, evident reduction of OPNc immunoreactivity could be seen in the invasive lesions from non-smokers (Fig 5B). In the IPMN lesions OPN immunoreactivity was clearly seen in the transformed ducts (Fig 5C). No OPNc immunoreactivity could be detected in IPMN lesions from smokers and non-smokers (Data not shown). These data indicate that an OPN/OPNc generating system is constitutively present in the malignant ductal cells as well as in the stroma. The presence of low expression levels of OPNc in the invasive lesions of non-smokers suggests that it might be regulated by nicotine.

OPN levels in the serum of smokers and non-smokers

To evaluate whether increased tissue OPN and OPNc in invasive PDA from smokers is accompanied by increased circulating levels of OPN, an ELISA was carried out in serum samples that matched the tissue we used (Fig 6A). There was a ~ 1.5 -fold increase in OPN serum levels in patients with invasive PDA when compared to premalignant lesions from non-smokers ($p<0.05$). There was no significant difference between serum OPN in invasive PDA from smokers when compared to non smokers. There was a significant ($p<0.05$) correlation between high OPN serum levels (+++) and high OPNc tissue levels (+++) (Fig 6B). However, most of the samples that expressed no OPNc (-), especially in the premalignant lesions, still expressed moderate (++) amounts of serum OPN. This suggests that serum OPN does not correlate with tissue levels of OPN and OPNc. This might be due to serum OPN having different sources. Nonetheless, our data suggest that OPN might not be an accurate marker for tumor invasiveness.

DISCUSSION

In this study, we investigated the potential molecular basis of the role of nicotine as a major risk factor in PDA. We show that an OPN isoform, OPNc, which has been shown to support anchorage independence and metastatic behavior (15,16), is expressed in high levels in invasive

PDA, especially in smokers. We demonstrate that the expression levels of OPNc correlate significantly with total OPN levels and the invasive status of PDA. Evidence for the involvement of OPN in tumor progression, such as growth, metastasis and angiogenesis, has accumulated in several types of cancer (19). Until now, however, there have been no reports analyzing the relationship between the expressions of OPN and OPNc with patient's smoking history as an indication for their regulation by nicotine. Thus, the present study is the first to investigate the correlation between OPN and OPNc in invasive and premalignant PDA and patients' smoking history.

Our in vitro data show that nicotine increased OPN mRNA expression, an increase that might be regulated by the basal levels of OPN, since nicotine could not significantly induce additional OPN transcription in BxPC-3 cells (Fig 2C), which constitutively express high levels of OPN. Interestingly, nicotine induced denovo expression of OPNc in HS766T cells (Fig 3). Nicotine also increased OPNc expression in BxPC-3 cells. This is the first report to demonstrate a relationship between nicotine and the OPNc isoform. We showed previously nicotine upregulates OPN promoter activity (18). Additional studies are now required to delineate the details of this relationship and whether nicotine-OPN promoter activation is related or separated from OPN alternative splicing with the resultant expression of OPNc. Furthermore, the effect of the nicotine-mediated increase of OPNc on PDA cell behavior and function is the subject of our currently ongoing studies in the laboratory.

Numerous studies have correlated high levels of OPN expression with tumor progression and metastasis in many cancers, including pancreatic cancer (9,17). OPN promotes cell survival and facilitates metastatic cell behavior through activation of the PI-3 kinase/AKT-NF- κ B pathways (20) and matrix metalloproteinase-2 (21). OPN also induces the expression of vascular endothelial growth factor (22) and promotes integrin-mediated endothelial cell migration (23). In tumor microenvironment macrophages, OPN downregulates the activity of inducible nitric oxide synthase, leading to protection of tumor cells from the macrophage nitric oxide-mediated cytotoxicity (24). Alternative splicing has been reported as one mechanism, by which cancer cells alter the structure and function of OPN, leading to increased support of anchorage-independence (11). Our analyses reported here have found that OPN and OPNc are expressed in the majority (~87%) of invasive PDA cases, out of which 73% were smokers, but not in premalignant lesions (IPMNs). The levels of OPNc correlate with smoking history in invasive PDA. Immunohistochemical analysis of the different lesions confirmed our mRNA data (Fig 5). This makes OPNc a candidate marker for the invasive potential of PDA, which could give rise to novel diagnostic approaches. It remains to be determined whether OPN/OPNc levels correlate with pathologic stage, survival or recurrence. Furthermore, additional studies are required to determine whether similar findings could be obtained from Electronic Ultra Sound Fine Needle Aspiration (EUS FNA) samples.

Nevertheless, the presence of OPNc and high OPN levels in the non-smokers with invasive lesions opens the door for further questions about the likelihood that other factors might contribute to elevating OPN/OPNc levels. For example, second hand smoke exposure could very well have an impact on OPN/OPNc expression levels in the non-smokers. Additional studies addressing these possibilities are currently ongoing in our laboratory.

Several studies have suggested OPN to be a candidate serum marker for PDA. However, more recent studies have shown that OPN is over expressed in other non-malignant conditions (25). In our studies, although we show a significant correlation between serum OPN and tissue OPNc, most of the samples that expressed no OPNc expressed moderate amounts of serum OPN (Fig 6B). This suggests that there are different sources of serum OPN that might affect its value as a diagnostic/prognostic marker for invasive PDA.

Our study demonstrates that nicotine elicits a pro metastatic response in PDA cells by stimulation of OPNc production. History of cigarette smoking in invasive PDA patients correlated well with increased tissue expression levels of OPNc. Although the exact role of OPNc in PDA remains to be defined, the existence of OPNc as a downstream effector of nicotine that is capable of mediating its carcinogenic effects in PDA cells is novel and could provide a unique potential target to control pancreatic cancer aggressiveness, especially in the cigarette smoking population.

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Figure 1.

Expression of OPN in PDA cells. **A.** Real time PCR analysis of OPN mRNA transcripts relative to GAPDH in 3 PDA cell lines shows that BxPC-3 cells express the highest levels of OPN mRNA transcripts followed by HS766T cells. MiaPaca cells contained minimal levels of OPN. **B.** Representative Western immunoblot showing the expression of variable levels of OPN protein in PDA cells that is seen at two bands at ~65 and ~25 kDa. **C.** Representative agarose gel with the PCR product of PDA cells showing expression of OPNc only in BxPC-3 cells (155- and - 109-bp bands correspond to the amplified OPN and GAPDH, respectively).

Figure 2.

Nicotine induces OPN accumulation in cultured PDA cells. MiaPaca (**A**), HS766T (**B**), and BxPC-3 (**C**) cells were treated with nicotine (3-300 nM) for 3 and 24 h. Significant induction of OPN mRNA expression is seen in A and B with the maximum induction after 24 hr at 3 and 30 nM of nicotine. BxPC-3 cells did not show any significant increase in OPN. Values are expressed as mean $\pm$ SEM of three experiments. * $p < 0.05$, # $p < 0.005$ vs. control levels, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by student *t*-test.

Figure 3.

PCR analysis of OPNc mRNA transcripts revealed the presence of no OPN mRNA in control HS766T untreated cells and small amounts in control untreated BxPC-3 cells. Time response of OPN mRNA in nicotine dose course shows denovo expression of OPNc in HS766T cells at 24 h at nicotine concentration of 30 nM. Visible upregulation of OPN mRNA levels in BxPC-3 cells at 3h and 24 h at nicotine concentration of 3 nM (155- and - 109-bp bands correspond to the amplified OPN and GAPDH, respectively).

Figure 4.

A. Analysis of RT-PCR relative quantification (RQ) values of OPN/GAPDH of >1 indicated high total OPN and was labeled (+++), a value of $0.5 - 1$ was labeled (++), of $0.1-0.5$ was labeled (+), and of <0.1 was labeled (-). There is significant correlation ($p<0.05$) between high OPN mRNA levels and smoking history in invasive PDA. IPMN lesions from smokers and non-smokers expressed minimal levels (-) of OPN.

B. Representative of PCR analysis of OPNc mRNA transcripts revealed the presence of considerable amount of OPNc mRNA in invasive PDA, especially in smokers. IPMN lesions from smokers and non-smokers expressed minimal amounts of OPNc (155- and - 109-bp bands correspond to the amplified OPNc and GAPDH, respectively). The OPNc mRNA contents are expressed as ODs corrected for GAPDH.

C. Representative of PCR analysis of OPNc mRNA. Average densitometry values of the samples were multiplied to obtain the arbitrary levels. (+++) = strong band intensities, (++)= moderate band intensities, (+) = mild band intensities, (-) = no OPNc.

D. Significant correlation ($p < 0.005$) between tissue OPN and OPNc mRNAs. High OPNc (+++) was found in 100% of the invasive PDA samples that expressed high OPN (+++). No OPNc was found in IPMN lesions that expressed very low levels of OPN.

Fig 5.

Representative immunohistochemical staining for OPN and OPNc in invasive PDA and premalignant lesions. Paraffin embedded pancreatic serial sections were stained with OPN and OPNc antibodies **A:** In invasive PDA from smokers, OPN and OPNc are colocalized to the membrane and cytoplasm of pancreatic ductal epithelial and stromal cells. **B:** In invasive lesions from non smokers, less OPNc could be detected in the malignant ducts and surrounding stroma. **C.** IPMN lesions from smokers shows OPN immunoreactivity in the transformed ducts. Negative control (-ve C) samples where the primary antibody was omitted did not show non-specific reaction. X 200 original magnification.

Figure 6.

A. OPN protein in the serum was measured using human-specific ELISA kit. Levels of OPN showed significant difference only in IPMN lesions from non-smokers. * $p < 0.05$ vs. invasive PDA from non-smokers. **B.** Significant correlation ($p < 0.05$) between OPNc mRNA and serum OPN. High OPNc (+++) was found in ~70% of the invasive PDA samples that expressed high serum OPN (+++). No OPNc was found in IPMN lesions that expressed moderate (++) levels of serum OPN.