

Expression and regulation of nicotine receptor and osteopontin isoforms in human pancreatic ductal adenocarcinoma

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Summary. Osteopontin (OPN) is a secreted phosphoprotein that confers on cancer cells a migratory phenotype. We have recently shown that nicotine, a risk factor in pancreatic ductal adenocarcinoma (PDA), induces an $\alpha 7$ -nicotine acetylcholine receptor ($\alpha 7$ -nAChR)-mediated increase of OPN in PDA cells. In this study, we tested nicotine's effect on the expression of OPN splice variants (OPNa, b, c) in PDA cells. We also analyzed the correlation between patients' smoking history with OPN and $\alpha 7$ -nAChR levels. RT-PCR and UV-light-illumination of ethidium-bromide staining were used to examine the mRNA expression in tissue and PDA cells treated with or without nicotine (3-300 nM). Localization of total OPN, OPNc and $\alpha 7$ -nAChR was analyzed by immunohistochemistry, and their mRNA tissue expression levels were correlated with the patients' smoking history. PDA cells expressed varying levels of OPNa, OPNb, and $\alpha 7$ -nAChR. Nicotine treatment selectively induced denovo expression of OPNc and increased $\alpha 7$ -nAChR expression levels. In PDA tissue, OPNc was found in 87% of lesions, of which 73% were smokers. OPNc and total OPN levels were correlated in the tissue from patients with invasive PDA. Nicotine receptor was expressed in the invasive and premalignant lesions without clear correlation with smoking history. We show here for the first time that $\alpha 7$ -nAChR is expressed in PDA cells and tissues and is regulated by nicotine in PDA cells. This, together with our previous findings that $\alpha 7$ -nAChR mediates the metastatic effects of nicotine in PDA, suggest that combined targeting of $\alpha 7$ -nAChR and OPNc could be a valid novel therapeutic strategy for invasive PDA, especially in the smoking population.

Key words: Pancreatic cancer, Nicotine receptor, Cigarette smoke, Osteopontin

Introduction

With an estimated 36,800 deaths in 2010, pancreatic cancer remains the fourth leading cause of cancer death in the United States (Jemal et al., 2010). Currently, surgical resection remains the most effective treatment for pancreatic ductal adenocarcinoma (PDA) but only 10-15% of patients are candidates for this curative resection as the disease is usually unresectable at the time of diagnosis. Even after pancreatic resection, the 5-year survival rate is only approximately 20% (Warshaw et al., 1992). Therefore, there remains an urgent need for better understanding of the basic molecular mechanisms that contribute to the aggressive nature of PDA in order to develop more effective therapeutic or preventative therapies.

Cigarette smoking is among the most important avoidable risk factors, lending upwards of a threefold increased risk of developing PDA (Wang and Wang, 2005). It is estimated that cigarette smoking may be responsible for 25 to 30% of all PDA cases (Lowenfels and Maisonneuve, 2006). Nicotine, a major component of tobacco and cigarette smoke, is an addictive agent and has been characterized in 1986 as a drug of abuse by the U.S. Surgeon General. Pancreatic cancer has been linked to nicotine exposure through cigarette smoking in many human studies (Farrow and Davis, 1990; Silverman et al., 1994). In addition, animal studies have demonstrated that nicotine or its metabolites can induce pathological and functional changes in the pancreas (Chowdhury et al., 1992). It is unknown, however, how these functional or morphological changes could contribute to the progression of pancreatic cancer.

Osteopontin (OPN) is a secreted noncollagenous, sialic acid-rich phosphoprotein, which functions as both an extracellular matrix component and a cytokine (Denhardt et al., 2001; Khan et al., 2005). OPN can support migration and protect against programmed cell death after binding to certain integrin receptors or a CD44 variant on the cell surface (He et al., 2006). Originally isolated from bone, osteopontin expression has been reported in multiple tumor types including breast, ovarian, prostate, liver, and pancreatic (Rittling and Chambers, 2004). Several studies have suggested that the overexpression of OPN in human tumors is associated with advanced tumor progression, metastatic disease and decreased patient survival (Wai and Kuo, 2008). OPN knockdown impeded growth, migration and invasion of cancer cells, thereby suppressing tumorigenicity (Shevde et al., 2006).

Consistently, OPN secreted from various cells has diverse structural characteristics (Weber and Ashkar, 2000; Kon et al., 2000) and tumor-derived OPN forms are smaller than OPN secreted by nontransformed cells (Kasugai et al., 1991). Alternative splicing occurs in a region of the molecule that is upstream of the central integrin binding domain and the C-terminal CD44 binding domain. OPNb lacks exon 5 and OPNc lacks exon 4. The shortest splice variant, OPNc, may support tumor progression by conveying anchorage independence and inducing the expression of oxidoreductases.

We have recently shown an increased expression of OPN in PDA cell lines after exposure to nicotine *in vitro* (Chipitsyna et al., 2009). The nicotine-derived nitrosamine 4-(methylnitrosamino)-1-3-(3-pyridyl)-1-butanone (NNK), a potent procarcinogen found in tobacco smoke, is an agonist of nicotinic acetylcholine receptors (nAChRs) (Schuller, 2007). We showed that exposure of PDA cells to an $\alpha 7$ -nAChR antagonist, mecamylamine, showed a dose-dependent decrease in nicotine-mediated OPN production. This data suggested that nicotine-induced OPN gene expression is mediated through the nicotine receptor expressed on PDA cells.

In this study, we tested the effect of nicotine on the different OPN isoforms expression in PDA cell lines. We also incorporated clinical data to correlate the smoking history of patients with the expression of tissue OPN in non-invasive intraductal papillary mucinous neoplasms, IPMNs and in invasive PDA. Since nicotine induced OPNc and $\alpha 7$ -nAChR in PDA cells, we evaluated their expression in PDA and IPMNs and examined whether their expression was effected by patients' history of smoking.

Materials and methods

Cell culture

Four cell lines were utilized for initial analysis of OPN isoforms including: Panc 10.05, HPAF, BxPC-3,

and AsPC-1. All cells were purchased from the American Type Culture Collection (Manassas, VA). HPAF cells were kindly provided by Dr. Surinder Batra, University of Nebraska Medical Center, Omaha, NE. Cells were cultured at 1×10^4 to near confluence in 96-well plate and maintained in DMEM supplemented with 10% fetal bovine serum in a humid atmosphere of 5% CO_2 /95% air. HPAF and Panc10.05 cells were used for further analysis of the effects of nicotine. 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. OPN isoform 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, $\alpha 7$ -nAChR 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 $2^{[C_T(\text{GAPDH}) - C_T(\text{gene of interest})]}$, 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 whole 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 OPNa forward: 5'-ATCTCCTAGCCCCACAGAAT-3', reverse: 5'-CATCAGACTGGTGAGAATCATC-3'; OPNb forward: 5'-AAAATCAGTGACCAGTTCATCAG-3', reverse: 5'-ATCTCCTAGCCCCAGAGAC-3'; OPNc 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'-TGAAGGTCGGAGTCAACGGATTGGT-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).

siRNA sequences and constructs

Using GenBank™ sequence AK315461 for human OPN cDNA and computer analysis software developed by Applied Biosystems/Ambion, candidate sequences in the OPN cDNA sequence for RNAi with no homology with other known human genes were selected and used during transient transfection experiments. Human mismatch or scrambled siRNA sequences (Applied Biosystems/Ambion; Austin, TX) possessing limited homology to human genes served as a negative control. Transfection was done with TransFast (Promega, Madison, WI) in Panc 10.05 cells as directed by the manufacturer. Cells were examined for OPN expression by real time PCR.

Cell migration assay

Panc10.05 cells were plated and grown to 90% confluency in a 6-well plate (Falcon Becton Dickinson). Cells were scratched with a sterile 1000 μ l pipette tip in each well, washed with PBS to remove cell debris, then medium with or without nicotine (30 nM) was added. After 48 hours, the wounds were observed and images were taken in 20 x magnification. Similar experiments were performed with cells containing scrambled or OPN siRNA treated with or without nicotine (30 nM). The extent to which the wound had closed over 48h was calculated and expressed as a percentage of the control at 0h. The data is representative of three independent experiments.

Human tissue acquisition and analysis

Histologically confirmed human invasive PDA (n=40, 29 smokers and 11 non-smokers), benign intraductal 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. Pancreatic intraepithelial neoplasms (PanINs) were identified in sections of the malignant PDA lesions. 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. Patients' smoking history was extracted from the clinical notes and correlated with $\alpha 7$ -nAChR, OPN, and OPNc expression levels.

Immunohistochemistry

Paraffin blocks were sectioned at 5 μ m. To localize OPN, $\alpha 7$ -nAChR and OPNc, sections from the different tissues were analyzed by immunohistochemistry using monoclonal antibodies against OPN (2A1, Santa Cruz) (1:100), $\alpha 7$ -nAChR (Abcam; 1:100), and an affinity-purified anti-OPNc chicken IgY (1:60), a kind gift from Dr. Georg Weber, University of Cincinnati College of

Pharmacy, Cincinnati, OH. Sections were deparaffinized by treatment with xylene for 5 min (2x) and rehydrated by passage through descending concentrations of alcohol. Antigen retrieval was performed by microwaving the slides in 100 mM sodium citrate buffer for 15 min. Endogenous peroxide activity was quenched by incubating the slides for 5 min in 3% H_2O_2 . A vectastain universal elite ABC kit and 3,3'-diaminobenzidine tetrahydrochloride chromogenic substrate (Vector Laboratories Inc.) was used according to the manufacturer's protocol to visualize the tissue reaction. Antibody specificity was validated with nonimmune isotype serum. Negative control sections, where the primary or secondary antibodies were omitted were also prepared.

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 $\alpha 7$ -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 OPN in PDA cells

Analysis of total OPN levels by real time PCR revealed that PDA cells express variable basal levels of OPN. Panc 10.05 and AsPC-1 cells expressed low levels of OPN mRNA transcripts when compared with HPAF and BxPC-3 cells, which displayed between 6 and 14 times higher levels of OPN mRNA transcripts than Panc 10.05 and AsPC-1 cells (Fig. 1A). UV-light-illumination of ethidium-bromide staining of PCR products after agarose gel electrophoresis showed a 208 bp band for OPNa (total OPN) and a 209 bp band for OPNb that were detectable in all cell lines. The 155 bp OPNc band was detectable only in BxPC-3 cell lines (Fig. 1B). These data suggest that OPN and its isoforms are differentially expressed in PDA cells. In addition, the presence of OPNc is associated with high levels of total OPN.

Expression of $\alpha 7$ -nAChR in PDA cells

Real time PCR analysis of $\alpha 7$ -nAChR revealed that Panc 10.05 and BxPC-3 cells expressed high levels of the receptor, between five and twelve times higher than $\alpha 7$ -nAChR mRNA transcripts in HPAF and AsPC-1

cells (Fig. 1C). These data show for the first time that $\alpha 7$ -nAChR is expressed in PDA cells and suggest that there is no correlation between the basal levels of OPN isoforms and $\alpha 7$ -nAChR mRNA.

Effect of nicotine on OPN isoforms expression

Next, we used Panc 10.05 and HPAF cells to perform the nicotine treatment studies. Addition of nicotine (3-300nM) to PDA cells for 3 and 24 h induced a differential increase in total OPN mRNA expression. In Panc 10.05 cells (Fig. 2A), after 3 h, nicotine at 3 nM induced a significant ($P<0.05$) increase of OPNa mRNA with no effect on OPNb. Higher doses of nicotine and longer incubation periods did not affect OPNa, but reduced OPNb mRNA expression levels (Fig. 2A,B). In HPAF cells (Fig. 2A), nicotine induced a dose- and time-dependent significant increase in OPNa and OPNb mRNA. Interestingly, nicotine (30nM) also induced de novo expression of OPNc isoform in HPAF cells after 24h (Fig. 2A-C). These data confirm our previously reported data that nicotine stimulates OPN transcription in other PDA cell lines (Chipitsyna et al., 2009), and importantly suggest that high nicotine concentrations promote the expression of OPNc isoform in certain PDA cell lines.

Effect of nicotine on $\alpha 7$ -nAChR expression

Treatment of Panc 10.05 cells with 30 nM of nicotine induced a significant ($p<0.05$) increase in $\alpha 7$ -nAChR mRNA expression after 24h (Fig. 2C). In HPAF cells, 30nM of nicotine induced a significant time-dependent increase in $\alpha 7$ -nAChR mRNA (Fig. 2D).

Table 1. Patients (total number=46).

	n	Percentage
Gender		
PDA		
Men	18	45
Women	22	55
IPMN		
Men	2	33
Women	4	67
Age (years)		
PDA	64.7	
IPMN	66	
Smoking Status		
PDA		
Smokers	29	72
Non Smokers	11	28
IPMN		
Smokers	2	33
Non Smokers	4	67
Tumor Stage		
Smokers		
IIA	2	10
IIB	23	79
IV	3	10
Non Smokers		
IIA	1	9
IB	1	9
IIB	7	64
III	1	9
IV	1	9

n: number of patients; PDA pancreatic ductal adenocarcinoma; IPMN: intraductal papillary mucinous neoplasm; IB, tumor confined to the pancreas, > 2cm, no lymph nodes or distant metastasis; IIA, tumor growing outside the pancreas, no lymph node or distant metastasis; IIB, tumor growing outside pancreas, lymph node metastasis, no distant metastasis; III, tumor growing into adjacent large vessels or nerves, no distant metastasis; IV, distant metastasis present.

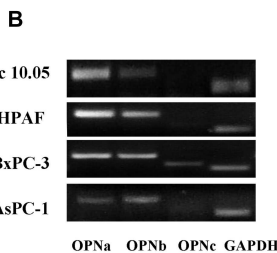
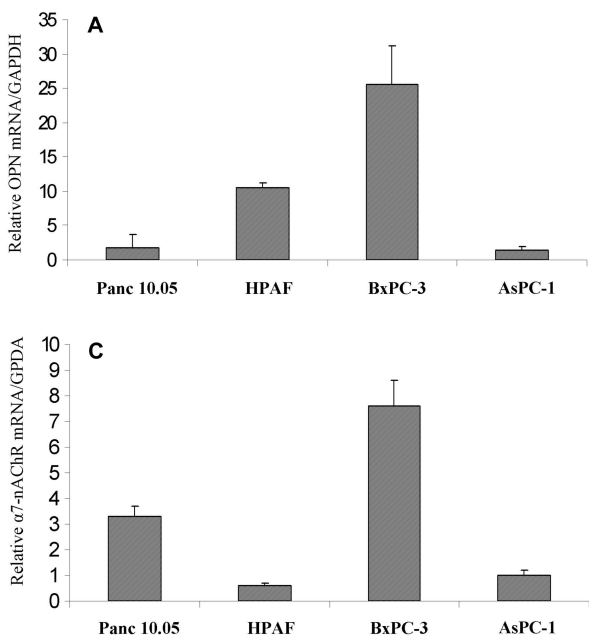


Fig. 1. Expression of OPN in PDA cells. **A.** Real time PCR analysis of OPN mRNA transcripts relative to GAPDH in 2 PDA cell lines shows that BxPC-3 and HPAF cells express higher levels of OPN mRNA transcripts compared to Panc 10.05 and AsPC-1 cells. **B.** Representative ethidium bromide agarose gel with the PCR product of PDA cells showing expression of OPNa and OPNb (208-bp and 209-bp bands, respectively) in both Panc 10.05 and HPAF, BxPC-3 and AsPC-1 cells. Only BxPC-3 cells expressed OPNc. **C.** Real time PCR analysis of $\alpha 7$ -nAChR mRNA transcripts relative to GAPDH shows higher levels in BxPC-3 and Panc 10.05 compared to HPAF and AsPC-1 cells.

Nicotine receptor in PDA

These data indicate that PDA cells respond to nicotine by increasing $\alpha 7$ -nAChR expression.

Nicotine promotes an OPN-mediated migration of PDA cells

To explore the role of nicotine in PDA cell migration and to evaluate the role of OPN in mediating these effects, we performed a wound healing assay. As seen in Fig. 3A, the addition of nicotine (30nM) induced a significant ($P<0.05$) increase in cell migration at 48h. In

figure 3B, addition of nicotine in OPN depleted cells resulted in significant ($P<0.02$) reduction of cell migration. These data show for the first time that nicotine promotes PDA migration and that OPN plays a critical role in mediating these effects.

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

Detailed patients' data are seen in Table 1, including the American Joint Committee on Cancer (AJCC)

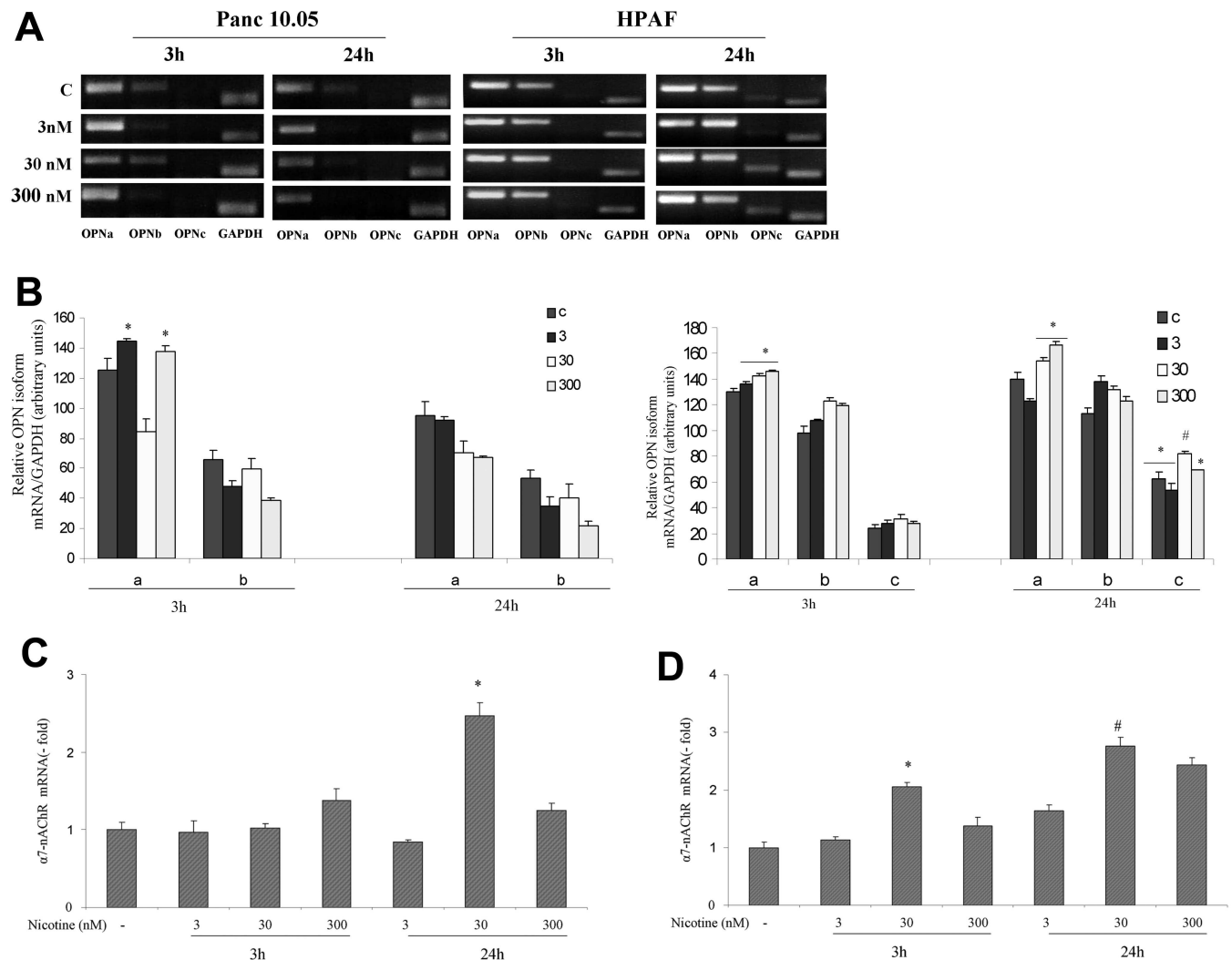


Fig. 2. A. Representative of three experiments of PCR analysis of OPNa, b and c mRNA transcripts revealed no OPNc mRNA in control Panc 10.05 and HPAF untreated cells. After 3h there is increased expression of OPNa in Panc 10.05 cells at 30nM. Visible down regulation of OPNb mRNA levels in Panc 10.05 cells at 3h and 24 h at nicotine concentration of 300 nM. Time response of OPN mRNA in nicotine dose course shows denovo expression of OPNc in HPAF cells at 24 h at nicotine concentration of 30 nM and 300nM. **B.** Quantification of band luminosities of Panc 10.05 cells. **C.** Quantification of band luminosities of HPAF cells. **C.** Nicotine induces $\alpha 7$ -nAChR accumulation in Panc 10.05 and HPAF (**D**). Cells were treated with nicotine (3-300 nM) for 3 and 24 h. Significant induction of $\alpha 7$ -nAChR mRNA expression is seen in with maximum induction after 24 hr at 30 nM of nicotine. 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.

staging. Interestingly, the majority of the smokers (90%) presented at later stages, whereas ~20% of the non-smokers presented at earlier stages (Table 1). Total OPN levels in IPMN (n=6, 2 smokers, 4 non-smokers) and 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 (-) (Table 2). In PDA samples

taken from smokers, more than 70% of the cases expressed high (+++) OPN mRNA levels, compared to 36% of the non-smokers. IPMN lesions, the majority of which were from non-smokers, expressed minimal amounts of OPN (-).

OPNc was analyzed by RT-PCR using specific primers and GAPDH as an internal control. OPNc band intensities were labeled (+++) for high intensity, (++) for moderate intensity, (+) for low intensity, and (-) for minimal intensity (Fig. 4A). There was a significant

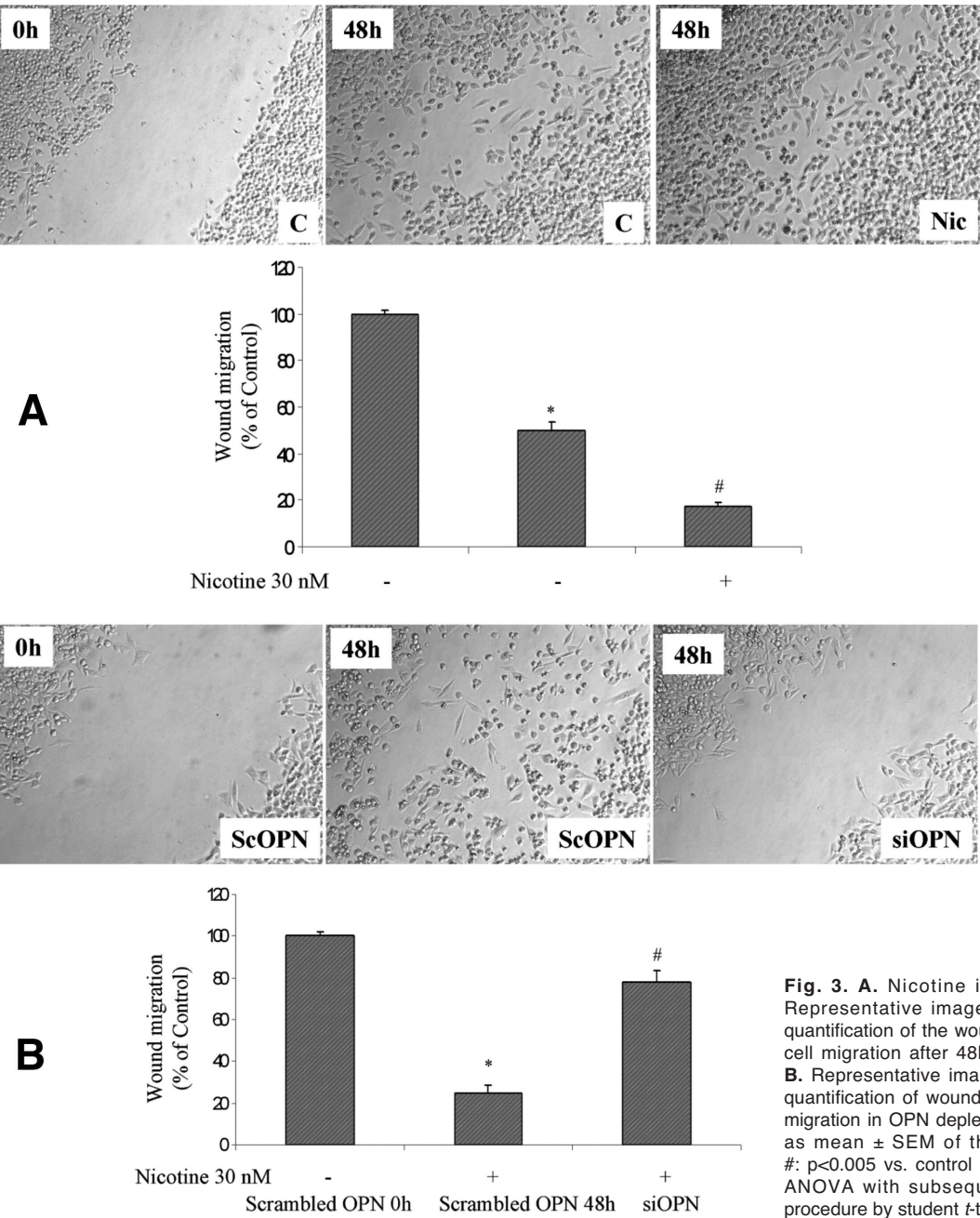


Fig. 3. A. Nicotine induces PDA cell migration. Representative image of cell migration assay and quantification of the wound distance showing increased cell migration after 48h of 30 nM nicotine treatment. **B.** Representative image of cell migration assay and quantification of wound distance showing reduced cell migration in OPN depleted cells. 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.

($p < 0.005$) correlation between OPNc band intensities with total OPN levels (Fig. 4B), where OPNc band at 155 bp was found in all (100%) 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. These data suggest that increased OPN expression in smokers is associated with the expression of OPNc isoform.

Expression of $\alpha 7$ -nAChR in tumors of smokers and non-smokers

$\alpha 7$ -nAChR levels in IPMN ($n=6$, 2 smokers, 4 non-smokers) and PDA ($n=40$, 29 smokers and 11 non-smokers) were analyzed by real time PCR. RT-PCR relative quantification (RQ) values of $\alpha 7$ -nAChR/GAPDH of >1 indicated high total $\alpha 7$ -nAChR and was labeled (+++), a value of $0.5 - 1$ was labeled (++), of $0.1-0.5$ was labeled (+), and of <0.1 was labeled (-) (Fig. 4C). In PDA samples taken from smokers, about 40% of the cases expressed high (+++) $\alpha 7$ -nAChR mRNA levels, compared to 50% of the non-smokers. IPMN lesions, the majority of which were from

non-smokers, expressed considerable amounts of $\alpha 7$ -nAChR (+++). These data suggest that $\alpha 7$ -nAChR might have importance in the premalignant lesions regardless of the patient's smoking history. The expression of $\alpha 7$ -nAChR did not correlate well with OPN and OPNc expression in PDA or in IPMN lesions (Fig. 4D).

Table 2. Analysis of RT-PCR relative quantification.

	Invasive PDA (n=40)		IPMN (n=6)	
	Smokers (n=29)	Non-smokers (n=11)	Smokers (n=2)	Non-smokers (n=4)
OPN (+++)	21(72%)	4(36%)	0	0
OPN (++)	5(17%)	3(27%)	0	0
OPN (+)	2(7%)	3(27%)	1(50%)	0
OPN (-)	1(3%)	1(9%)	1(50%)	4(100%)

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.

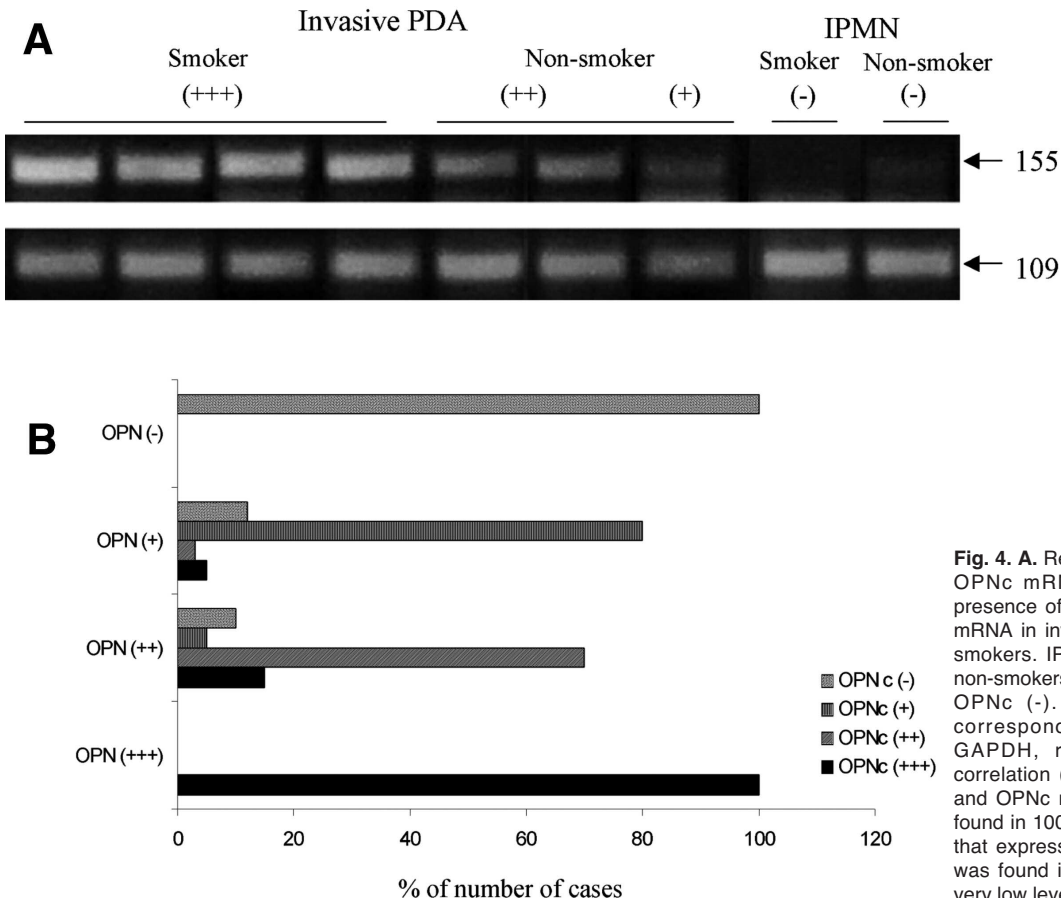


Fig. 4. A. Representative of PCR analysis of OPNc mRNA transcripts revealed the presence of considerable amount of OPNc mRNA in invasive PDA (+++), especially in smokers. IPMN lesions from smokers and non-smokers expressed minimal amounts of OPNc (-). 155- and - 109-bp bands correspond to the amplified OPNc and GAPDH, respectively. **B.** 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.

Expression of OPN and $\alpha 7$ -nAChR in the tumor adjacent tissues of smokers and non-smokers

Interestingly, whereas OPN expression in tissue adjacent to the tumor was significantly less than in the tumor area itself (Fig. 5A), the expression of $\alpha 7$ -nAChR in the tissues surrounding the tumors was significantly higher than in the tumors of smokers and non smokers (Fig. 5B), suggesting that this receptor may play a role in the carcinogenic process beyond being a receptor for nicotine.

Immunohistochemical expression of OPN, OPNc and $\alpha 7$ nAChR in tissue sections of smokers and non-smokers

In the normal pancreatic ducts, OPN was focally present and mostly on the apical surface of the ductal epithelium, while a more homogenous cytoplasmic expression of $\alpha 7$ -nAChR expression was seen (Fig. 6A). In pancreatic intraepithelial neoplasia (PanIN-3) lesions,

intense expression of OPN could be seen in the transforming cells colocalizing with low levels of cytoplasmic $\alpha 7$ -nAChR (Fig. 6B). In intraductal papillary mucinous neoplasm (IPMN) lesions, more intense staining of $\alpha 7$ -nAChR could be seen colocalizing with OPN in the transforming ducts (Fig. 6C). In PDA (Fig. 6D), OPNc staining could be recognized in the ducts and some stromal cells colocalizing with OPN. Intensified ductal epithelial staining of $\alpha 7$ -nAChR can be seen colocalizing with OPN to the membrane and cytoplasm of the tumor cells.

These data indicate that an OPN/OPNc generating system is constitutively present in the malignant ductal cells as well as in the stroma, and that such a system is colocalized with $\alpha 7$ -nAChR. The close proximity between an OPN generating system and $\alpha 7$ -nAChR suggests a potential paracrine/autocrine interaction in the malignant cells. The intense immunoreactivity of $\alpha 7$ -nAChR in the IPMN lesions and the malignant ducts

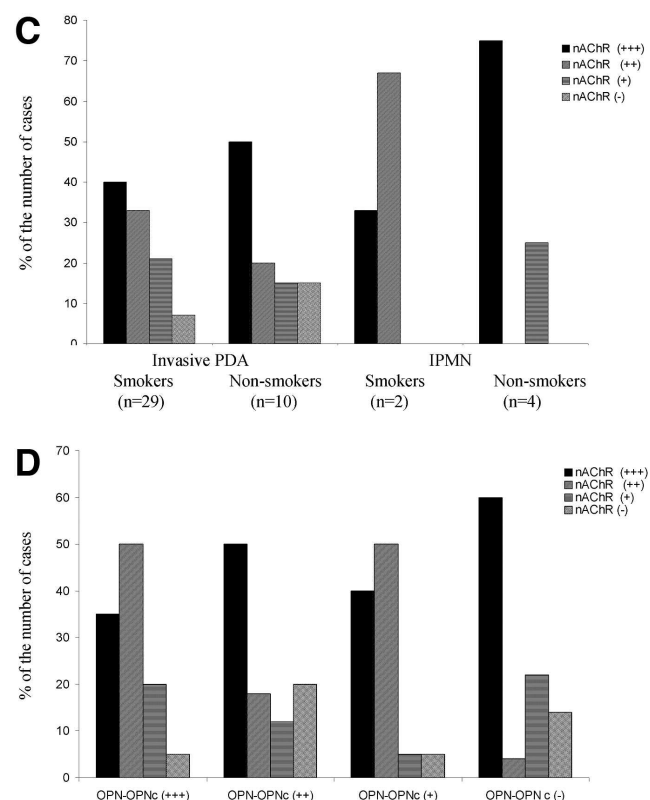


Fig. 4. C. Analysis of RT-PCR relative quantification (RQ) values of $\alpha 7$ -nAChR/GAPDH of >1 indicated high total $\alpha 7$ -nAChR and was labeled (+++), a value of 0.5-1 was labeled (++), of 0.1-0.5 was labeled (+), and of <0.1 was labeled (-). In invasive PDA there is no significant difference in $\alpha 7$ -nAChR mRNA levels between samples from smokers versus non-smokers. IPMN lesions, the majority from non-smokers, expressed high amounts of $\alpha 7$ -nAChR. **D.** Absence of correlation between $\alpha 7$ -nAChR with OPN and OPNc expression in smokers and non-smokers.

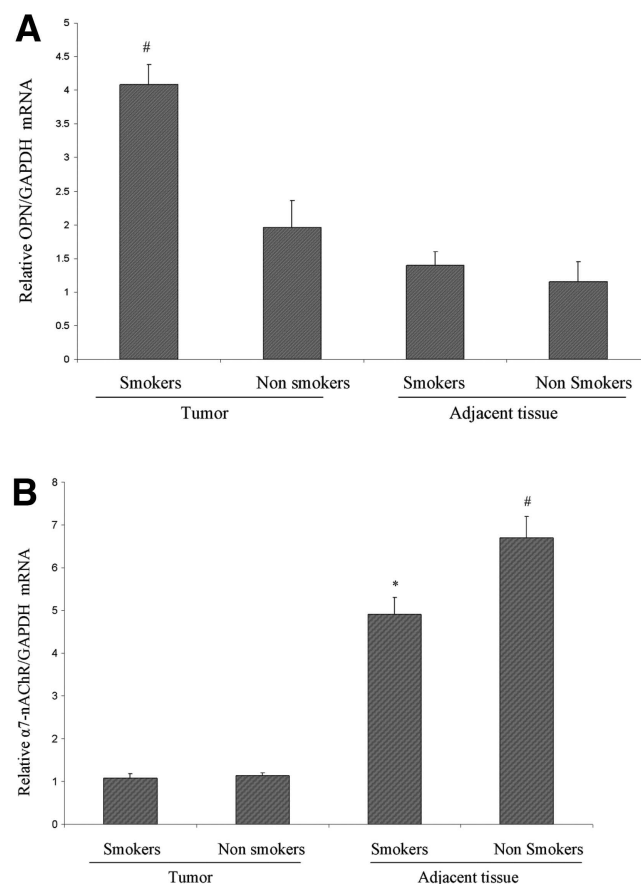


Fig. 5. Real time PCR analysis of mRNA transcripts expression of OPN (**A**) and $\alpha 7$ -nAChR (**B**) in the tumors and tumor adjacent tissues in smokers and non smokers. Values are expressed as mean $\pm$ SEM of three experiments. *: $p < 0.05$ vs. levels of adjacent levels of smokers (**A**), *: $p < 0.05$ #: $p < 0.005$ vs. tumor levels of smokers and non smokers (**B**), using one-way repeated ANOVA with subsequent all pairwise comparison procedure by student *t*-test.

Nicotine receptor in PDA

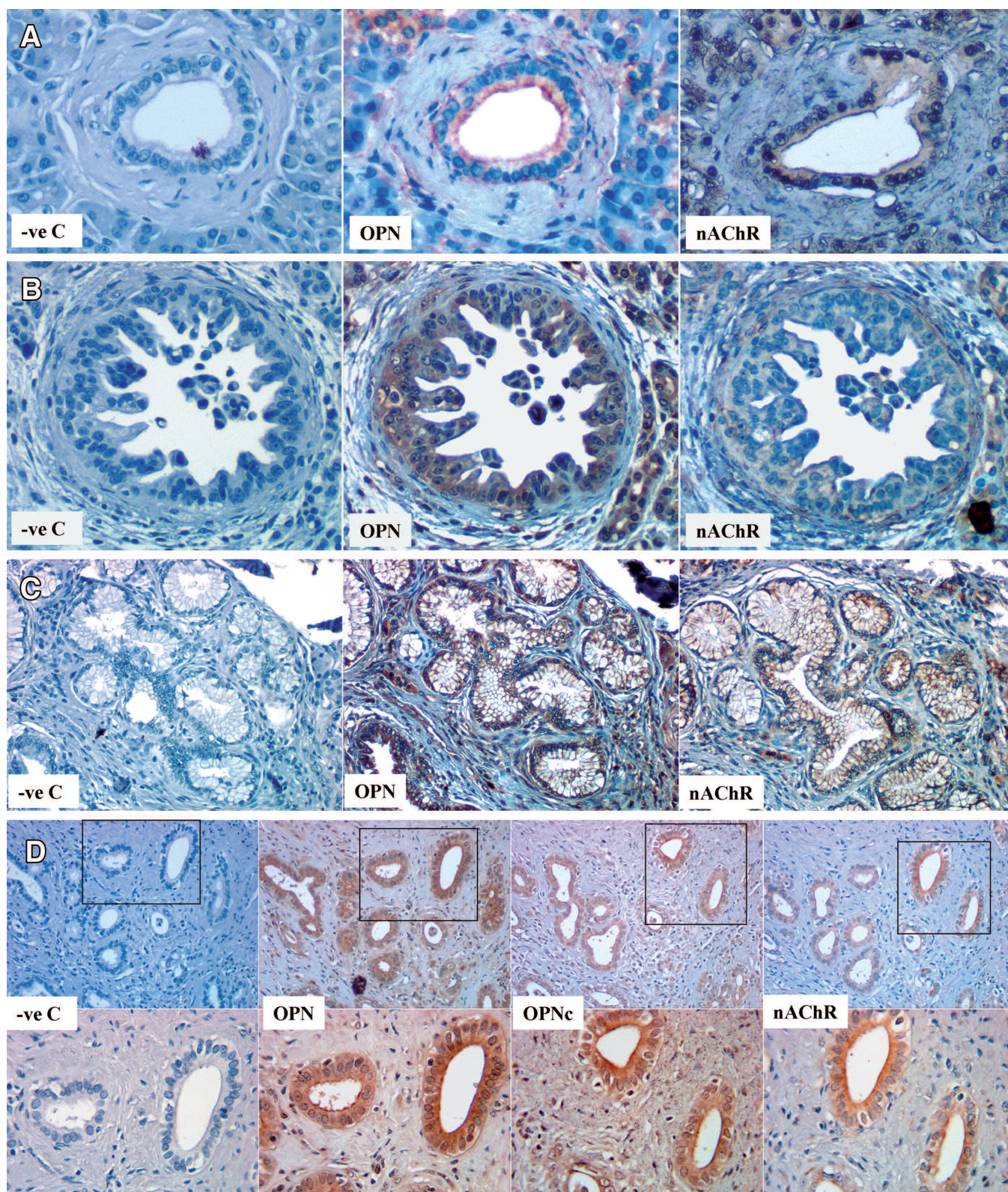


Fig. 6. **A.** Representative immunohistochemical staining for OPN and $\alpha 7$ -nAChR in non-malignant pancreatic tissue. Paraffin embedded pancreatic sections were stained with OPN and $\alpha 7$ -nAChR antibodies. In the non malignant ducts OPN is focally present and mostly on the apical surface of the ductal epithelium while $\alpha 7$ -nAChR showed a more homogenous cytoplasmic staining. **B.** In PanIN lesions OPN staining was seen in the transforming ducts, with much less staining of $\alpha 7$ -nAChR. **C.** In IPMN lesions, intense staining of OPN and $\alpha 7$ -nAChR can be seen in the transforming mucin-filled ducts. **D.** In invasive PDA, intense OPN is colocalized to the membrane and cytoplasm of the tumor cells with OPNc and $\alpha 7$ -nAChR. Negative control (-ve C) sections where the primary antibody was omitted did not show non-specific reaction. A-C, x 200; D, x 200 upper panel, x 400 inset lower panel.

suggests a role in PDA development and progression.

Discussion

The genotoxic effects of tobacco carcinogens have long been recognized, however, the contribution of tobacco components to pancreatic carcinogenesis is relatively unexplored. Nicotine, the principal tobacco alkaloid, signals through binding a nicotinic acetylcholine receptor (nAChR). We showed previously that nicotine induces the metastatic protein, OPN, through signaling pathways that involve $\alpha 7$ -nAChR (Chipitsyna et al., 2009). In the present study, we show that $\alpha 7$ -nAChR is functionally present in human PDA cells and tissue, and that it is inducible by nicotine. We also show that an OPN isoform, OPNc, which has been shown to support anchorage independence and metastatic behavior (Takafuji et al., 2007; Mirza et al., 2008), is inducible by nicotine and is highly expressed in PDA, especially in smokers. OPN involvement in tumor progression, metastasis and angiogenesis has been reported in several types of cancer (Brown et al., 1994). However, the correlation between its expression and $\alpha 7$ -nAChR with patient's smoking history as an indication for their regulation by nicotine has not been analyzed. Thus, the present study represents the first investigation of the relationship between OPN, OPNc and $\alpha 7$ -nAChR in pancreatic lesions and patients' smoking history.

Our data show that PDA cells express variable levels of OPN and its isoforms and variable basal $\alpha 7$ -nAChR levels. Our *in vitro* data show that nicotine increased OPN mRNA expression in Panc 10.05 and HPAF cells. Interestingly, nicotine induced *denovo* expression of OPNc in HPAF cells (Fig. 2). Higher doses of nicotine also increased OPNc expression in HPAF cells. Nicotine had variable effects on OPNb expression; it reduced OPNb in Panc 10.05 cells and increased its expression in HPAF cells. The biological function of OPNb and its role, if any, in cancer development and progression is not clear. Previous studies on breast cancer have shown OPNb to be present at consistently low levels in both normal and malignant tissues without clear functional relevance (Mirza et al., 2008). Further research is needed to understand the function and regulation of the different OPN isoforms in PDA.

In our previous studies, we demonstrated that nicotine increases OPN transcription through induction of its promoter activity (Chipitsyna et al., 2009). It is not clear, however, whether nicotine-OPN promoter activation is related to or separated from OPN alternative splicing, and whether nicotine has differential impact on OPN isoform expression. Nonetheless, our data show the novel finding that the induction of OPN by nicotine is essential for inducing PDA migration, since in OPN depleted cells nicotine was unable to induce cell migration (Fig. 3). It remains to be determined, however, whether nicotine itself can have a carcinogenic effect on PDA cells and whether OPN plays a role in mediating these effects. This is currently the subject of ongoing

studies in our laboratory.

Numerous studies have correlated high levels of OPN expression with tumor progression and metastasis in many cancers, including pancreatic cancer (Denhardt et al., 2001; Koopmann et al., 2006). OPN promotes cell survival and facilitates metastatic cell behavior through activation of the PI-3 kinase/AKT-NF- κ B pathways (Lin and Yang-Yen, 2001) and matrix metalloproteinase-2 (Mi et al., 2006). OPN also induces the expression of vascular endothelial growth factor (Chakraborty et al., 2008) and promotes integrin-mediated endothelial cell migration (Tuck et al., 2003). In tumor micro-environment macrophages, OPN downregulates the activity of inducible nitric oxide synthase, leading to protection of tumor cells from the macrophage nitric oxide-mediated cytotoxicity (Wai et al., 2006). 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 (He et al., 2006). Our analyses reported here have found that OPN and OPNc are expressed in the majority (~87%) of PDA cases, out of which 72% were smokers, but not in non-malignant IPMN lesions. The levels of OPNc correlated with smoking history in PDA. Immunohistochemical analysis of the different lesions showed that OPN and OPNc are expressed in the malignant ducts and in the surrounding stroma, confirming our mRNA data (Fig. 6). This makes OPNc a candidate marker for the potential of PDA, which could give rise to novel diagnostic approaches. It is yet to be explored whether OPNc levels correlate with pathologic stage, survival or recurrence. We are currently performing these studies in addition to other studies to determine whether similar findings could be obtained from endoscopic ultrasound and fine needle aspiration (EUS/FNA) samples.

Recent studies from our laboratory have shown that the nicotine-OPN induction is mediated via $\alpha 7$ -nAChR (Chipitsyna et al., 2009). In the present study, we sought to determine whether PDA cell response to nicotine depends on the amount of $\alpha 7$ -nAChR they express, and whether nicotine itself has an impact on $\alpha 7$ -nAChR expression. We show here for the first time that nicotine induces a significant increase in $\alpha 7$ -nAChR mRNA levels in the two PDA cell lines (Fig. 2C, D). Dose- and time-response studies demonstrated that $\alpha 7$ -nAChR mRNA induction requires longer exposure to nicotine, especially in Panc 10.05 cells, which express higher basal levels of $\alpha 7$ -nAChR (Fig. 1C). Analysis of $\alpha 7$ -nAChR levels in PDA and IPMN tissue, however, did not show correlation with the patient's smoking history (Fig. 4C). On the contrary, higher levels of $\alpha 7$ -nAChR mRNA were detected in the IPMN lesions of non-smokers. Previous studies have shown that $\alpha 7$ -nAChR is involved in cell proliferation and angiogenesis (Catassi et al., 2008; Arias et al., 2009) however, its role in pancreatic cancer is yet to be determined. Several factors could affect the expression of $\alpha 7$ -nAChR, among which, for example, is second hand smoking (Zhu et al., 2003),

a parameter that was not accounted for during collection of the clinical data. Nonetheless, the presence of high levels of $\alpha 7$ -nAChR in IPMN lesions is intriguing and suggestive of an important role for nicotine receptors during the transformation of IPMN into invasive cancer. Studies are now needed to elaborate on the role of this receptor during PDA development and progression.

Our study demonstrates that nicotine could enhance PDA metastatic behavior by increasing the expression of OPNc isoform. A history of cigarette smoking in patients with PDA correlated well with increased tissue expression levels of OPNc and with a trend to present at a later stage (Table 1). The role of OPNc in pancreatic carcinogenesis and malignant transformation is yet to be determined. However, the potential of OPNc acting as a downstream effector of nicotine's carcinogenic effects provides a novel therapeutic target to control pancreatic cancer metastasis. In addition, OPNc should be explored as a more sensitive marker than total OPN, which levels are indistinguishably elevated in both PDA and chronic pancreatitis (Kolb et al., 2005).

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