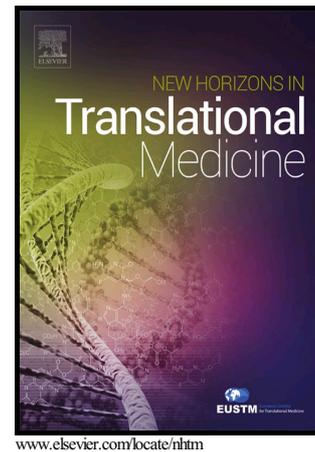# Author's Accepted Manuscript

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Nicotine mediated activation of Pak1/NFkB cascade in Pancreatic cancer cells – A pilot study.

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Abstract

Background:

Tobacco smoking is a major established risk factor for pancreatic cancer (PC), increasing the incidence up to six fold depending on the duration and intensity of smoking. Nicotine is a key toxin in tobacco and cigarette, which may contribute to development of pancreatitis and PC. Our previous studies revealed an aberrant expression of Pak1 in PC as compared to normal pancreas and its association with cancer progression, tumor angiogenesis, drug resistance and metastasis. Here, we explore a potential link between Pak1 expression and smoking-mediated PC pathogenesis and the use of Pak1 inhibitors to curtail this association.

Methods:

Mia Pa Ca 2 cell line was obtained from NCCS, Pune and grown in the presence and absence of 0.5 μM (0.112 μg/ml) nicotine hemisulphate salt (5h) and further nicotine exposed cells were treated with Pak1 inhibitor, IPA-3 (1h). Protein, mRNA and kinase activity of Pak1 were evaluated. Using human pancreatic cancer tissue, mRNA from smokers (n=10) and non –smokers (n=10) were assessed for Pak1 expression.

Results:

Nicotine significantly enhanced the expression and kinase activity of Pak1, with subsequent activation of NF-κB signalling cascade in cooperation with other pathways, this effect was blocked by IPA-3. Also, it was observed that pharmacological blockage or silencing of α7-nAChR abrogated nicotine mediated activation of Pak1/NF-κB. Additionally, we demonstrated up-regulated Pak1 mRNA expression in tissue sample from smokers compared to non-smokers.

Conclusion:

Our findings suggest probable mechanism of action of nicotine through Pak1 signalling on PC pathogenesis and this could be targeted using Pak1 inhibitors for PC treatment.

Key words: Smoking, Nicotine, Pancreatic cancer, Pak1, inhibitor, IPA-3

1 Introduction

Pancreatic cancer (PC) is the 11th most common incident cancer in men and 9th most common among women [1]. Worldwide, the incidence of all types of PC (85% of which are adenocarcinomas) ranges from 1 to 10 cases per 100,000 people, is generally higher in developed countries and among men, and has remained stable for the past 30 years relative to the incidence of other common solid tumors [2, 3]. In India this year, PC is predicted to develop in 13,019 people, and 11,806 people are expected to die from it (Globocan 2012).

The causes of PC remain unknown. Several environmental factors have been implicated, but evidence of a causative role exists only for tobacco use. Cigarette smoking is a consistent risk factor for pancreatic cancer [4–7]. History of diabetes, obesity, and family history of pancreatic cancer are also risk factors [8–11]. Cigarette

smoking may be responsible for approximately 20% of pancreatic cancer cases [12, 13]. A recent meta-analysis indicated that current cigarette smokers, compared with never smokers, have about a 2.5 fold risk of pancreatic cancer and that the risk increases incrementally with the number of cigarettes smoked and the number of years of smoking [12, 13].

Nicotine is the major addictive constituent of cigarette that induces modifications in the functional properties of cancer cells such as enhancing the rate of proliferation and angiogenesis [14-16] at concentrations that are normally found in the blood-stream of smokers (10^{-8} M to 10^{-7} M) [14, 16]. Importantly, several studies showed high levels of nicotine and cotinine, which is a metabolic derivative of nicotine, in pancreatic tissues of cigarette smoking exposed animals [14, 17, 18].

Our previous studies have illustrated a strong correlation between the Pak1 expression and the development of PC. p21-activated kinase 1 (Pak1), - a serine/threonine kinase, is a well-known regulator of cytoskeletal remodelling, cell motility, cell proliferation, and cell survival. Recent reports suggest that Pak1 by itself can have an oncogenic role in a wide variety of cancers. Our previous investigations have demonstrated a differential expression Pak1, in pancreatic adenocarcinomas as compared to the normal pancreas [19]. Moreover, using the Pak1-knockdown and overexpression in the PC cell models, we have shown that Pak1 potentiates pancreatic tumor cell growth and metastasis by triggering EMT especially fibronectin [19]. Recently, our lab investigations have also demonstrated that Pak1 confers a resistance to anti-cancer agent gemcitabine in PC cells, hence rendering the current therapeutic regimens ineffective [20].

Usually, PC patients have a mortality rate of nearly 100% and long-term exposure to cigarette-smoke is one of the several factors that contributes to this high rate [14, 21]. Therefore, a better understanding of the tobacco-smoking-mediated PC pathogenesis would lead to the identification of potential molecular targets and is likely to improve the prospect of designing effective therapies to combat this lethal malignancy.

In this study we explored the potential association of nicotine and Pak1 in PC pathology and to analyse the expression pattern of Pak1 in smokers and non-smoker PC patients and the prospective action of IPA-3, a Pak1 inhibitor on this association.

2 Materials and Methods

2.1 Chemicals

Nicotine hemisulphate salt (Sigma, Saint Louis, MO, USA), Pak1 inhibitor - IPA-3 (CAS# 3622, Tocris Bioscience, Bristol, UK), site-selective α7-nAChR antagonist, α-bungarotoxin (B1601, Invitrogen, Carlsbad, California, USA), Pak1, phospho-p65, and phospho-Pak1 antibody (Cell Signaling Technology, Danver, MA, USA), Vinculin (Sigma, Saint Louis, MO, USA), p65, Control siRNA, α7-nAChR siRNA (Santa Cruz Biotechnology, Dallas, Texas, USA) were purchased.

2.2 Cell lines & Tissues

The PC cell line Mia Pa Ca 2 was purchased from ATCC through NCCS, Pune, India. The cells were maintained in the log phase of cell growth by being cultured in RPMI 1640 or DMEM/F-12 supplemented with FBS (10%, v/v), 1X antibiotic-antimycotic solution from Himedia (India) at 37°C in humidified CO_2 incubator. Human Pancreatic Tissue samples (n=20) from pancreatic cancer patients were collected with the informed written consent was obtained from each patient and the study has been approved by the Institutional Review Board and Human Ethics Committee. The pancreatic cancer patients were categorised into smokers (n=10) and non-smokers (n=10) based on their habits and life style.

2.3 Western blotting

Western blotting was performed using standard methods. Cells treated with 0.5µM nicotine for 5h and/or 20µM IPA-3 for 1h were washed with cold PBS and lysed using RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM Na_2EDTA, 1% Nonidet P-40, 1% sodium deoxycholate]. Lysates were centrifuged at 14,000 rpm for 15 min at 4°C. Pancreatic tissues (100mg) were minced by using a homogenizer, protein concentrations were determined by Bio-Rad DCTM protein assay (BioRad, Hercules, CA). Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham, UK). Immuno-blotting was performed as per antibody manufacturer's details.

2.4 *In vitro* Kinase Assay

Kinase assays were performed as described previously [19]. Briefly, activity of PAK1 was measured using the known substrate, MBP. Estimation was done based on radioactive labelling, in 1X Kinase Buffer (500mM HEPES, 20mM $MnCl_2$, 100mM $MgCl_2$, 2mM DTT) with γ-^{32}P-ATP.

2.5 Real Time PCR

RNA was isolated from cell lines and tissue samples using the TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) and quantified using the NANODROP. A total of 1 µg of RNA from each sample was used to generate single-strand cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was done using the TaqMan master mix and probes. Corresponding Human β-Actin primer set was used as internal control. Relative Gene expression (RQ) was calculated using the ΔCt method.

2.6 siRNA Transfection

Cells at 40% density were transfected with siRNA 24h post-seeding, using Oligofectamine (Invitrogen) according to manufacturer's instructions. 48h post-transfection, cells were treated with nicotine/ smoke condensate for 24 h. RNA and protein were collected for analysis.

2.7 Preparation of Smoke condensate

Smoke condensate were prepared as described previously [22].The smoke particulate matter was dissolved in DMSO at 40 mg/ml, aliquoted into small vials and stored frozen at –80°C. On the day of the experiment, each vial of condensate solution was opened and diluted in the serum-free cell culture medium to desired concentration, vortexed vigorously and used for treatment of cells. Control cells were treated with medium containing an equivalent amount of DMSO. Repeated freezing and thawing of the condensate solution was avoided as much as possible.

2.8 Statistical analysis

Data are expressed as the mean ± S.E.M and analysed by Mann Whitney test using Graph Pad Prism 5 Software (GraphPad Software, San Diego, CA). Each experiment was repeated thrice. The P values less than 0.05 were considered to denote statistical significance.

3 Results

3.1 Nicotine enhances Pak1 kinase activity in PC cells

PC cells were treated with varying concentration of nicotine ranging from 0.5×10^{-9} to 0.5×10^{-6} M, showed an increase in the phosphorylation of Pak1 at 0.5μM (Supplementary data). Hence, the subsequent studies were performed at 0.5μM concentration of nicotine. PC cells were treated with 0.5μM nicotine for 5h and then treated with Pak inhibitor IPA-3 for 1h, lysates were collected and subjected for kinase activity. The kinase assay revealed a significant enhancement of kinase activity as evident from the band intensity of phosphorylated MBP (Myelin Basic Protein) which is a substrate of Pak1. On treatment with IPA-3, the nicotine induced enhanced Pak1 kinase activity was attenuated (Figure 1A).

3.2 Nicotine up-regulates the expression of Pak1 and phosphorylation of Pak1 and p65

The nicotine/IPA-3 treated cell lysate were subjected to protein expression studies. The immunoblot data suggests that nicotine augment the expression and phosphorylation status of Pak1 in PC cells. Previous study from our group has shown that Pak1 interacts with NF-κB p65 subunit [19] and Frost et al., [23] showed that Pak1 phosphorylates p65 subunit, forced us to look NF-κB p65 subunit, downstream of Pak1. As expected, there was an increase in phosphorylation of p65 subunit without change in total p65 expression. Also we had observed a reduction in phosphorylation of Pak1 and p65 subunit on combined treatment with nicotine and IPA-3 indicating the potential role of Pak1 signalling in the nicotine induce pancreatic cancer progression (Figure 1B).

3.3 Pharmacological blockage or silencing of α7-nAChR abrogates Nicotine mediated activation of Pak1/NF-κB

Previous work suggests that nicotine effects in pancreatic cancer could be mediated by the activation of α7-nAChR [24] and the expression of these receptors has been demonstrated in Mia Pa Ca 2 cells [25]. These reports triggered us to investigate whether both pharmacological blockade and silencing of α7-nAChR abrogate nicotine effects on Pak1 and NF-κB. Using α7-nAChR selective inhibitor α-bungarotoxin (α-BTX) and siRNA targeting α7-nAChR, we analysed the

phosphorylation status of Pak1 and NF-κB. It was evident from the result (Figure 2 A & B) that both pharmacological blockage (α-BTX) and silencing of α7-nAChR could abrogate the activation of Pak1/NF-κB mediated by nicotine.

3.4 Pak1 mRNA expression is elevated in PC tissue of smokers

The PC tissue samples were obtained from 20 PC patients. The patients were grouped into smokers and non-smokers based on their habits and life style, each group comprising 10 patients. The mRNA from tissue was isolated and analysed for expression status using qPCR. The data showed a significant up-regulation ($P<0.001$) of Pak1 in smokers compared to non-smokers (Figure 2D). Cigarette smoke does not contain only nicotine, the finding that Pak1 was higher in smokers does not necessarily support the hypothesis that nicotine was involved. To indirectly address this point we performed experiments on Mia Pa Ca 2 cells exposed to smoke condensate after silencing α7-nAChR. To our surprise, we found that silencing of α7-nAChR followed by exposure to smoke condensate did not alter the activation of Pak1/NF-κB cascade (Figure 3). This connotes the prominent association of smoking and Pak1, which by *per se* an oncogene in PC.

4 Discussion

In previous study, we demonstrated the functional expression of Pak1 in human PC cell lines and tissues [19]. In this study, we provided evidences that the etiologic factor smoking (nicotine) enhance the expression of Pak1 in PC cells. Additionally, there was significant increase in the phosphorylated expression of Pak1 and p65 subunit in the PC cells after nicotine stimulation. These effects were blocked by treating with Pak1 inhibitor IPA-3, indicating that nicotine might affect cell function *via* Pak1/NF-κB pathway.

Pak1 is a critical regulator of multiple biological functions and a known oncogene in various types of cancers. Pak1 activation and its downstream signalling is implicated in wide array of cancers especially those of the gastrointestinal tract. Fang and Svoboda [26] reported in human gingival fibroblast cells that nicotine modulates Pak1 activity and its link with oral carcinogenesis. As nicotine is a major chemical component in tobacco/ cigarette and also well known for its carcinogenicity, here we intended to study whether nicotine could activate Pak1, a well establish oncogene in

PC [19]. Studies have revealed that Pak1 activation can persuade NF-κB activation which could elicit the downstream activation of multiple pathways. In this study, we found a significant increase in phosphorylation expression status of NF-κB p65 subunit in PC cells after nicotine administration. Enhanced phosphorylation of p65 subunit is closely related to NF-κB activity and changes in activation factors (19,20). In our study, we demonstrated treatment with IPA-3 could suppress this effect, suggesting that the nicotine induced NF-κB activation is through Pak1 signalling.

Our results using α7-nAChR siRNA and α-BTX revealed the significance of α7-nAChR, in nicotine mediated activation of Pak1 and also reiterate the previous findings that nAChR regulates and activates multiple cell signalling pathways that are over expressed in PC. Studies using smoke condensate on α7-nAChR silenced cells given us the evidence that there are multiple other regulatory pathways such as ERK/MAPK/Src [24,] that are deregulated by the components or metabolites of cigarette smoke which could elevate and activate Pak1. The individual role of each components of smoke condensate or nicotine metabolite has to be studied in future for better understanding of the mechanism of signalling initiated by these agents towards carcinogenesis.

Another important finding of this study is the elevated mRNA expression of Pak1 in PC patients who are addicted to smoking or having the habit of smoking, suggesting that smoking causes Pak1 expression activation, triggering Pak1 signalling one of the key modulator in PC pathogenesis. This data also suggest that Pak1 activation status could be used as a prognostic marker to identify the suitable regiment for PC treatment.

This report is the first to examine the key role of Pak1 signalling in human PC cells in response to nicotine, suggesting that Pak1/NF-κB pathway triggered by nicotine might take part and aggravates smoking associated PC progression and metastasis. Although we gained some interesting results, these results were based on the response of PC cells. Further investigation using normal pancreatic cell lines and in animal model is required to determine whether these findings reflect the processes that occur in normal and *in vivo*.

Acknowledgements

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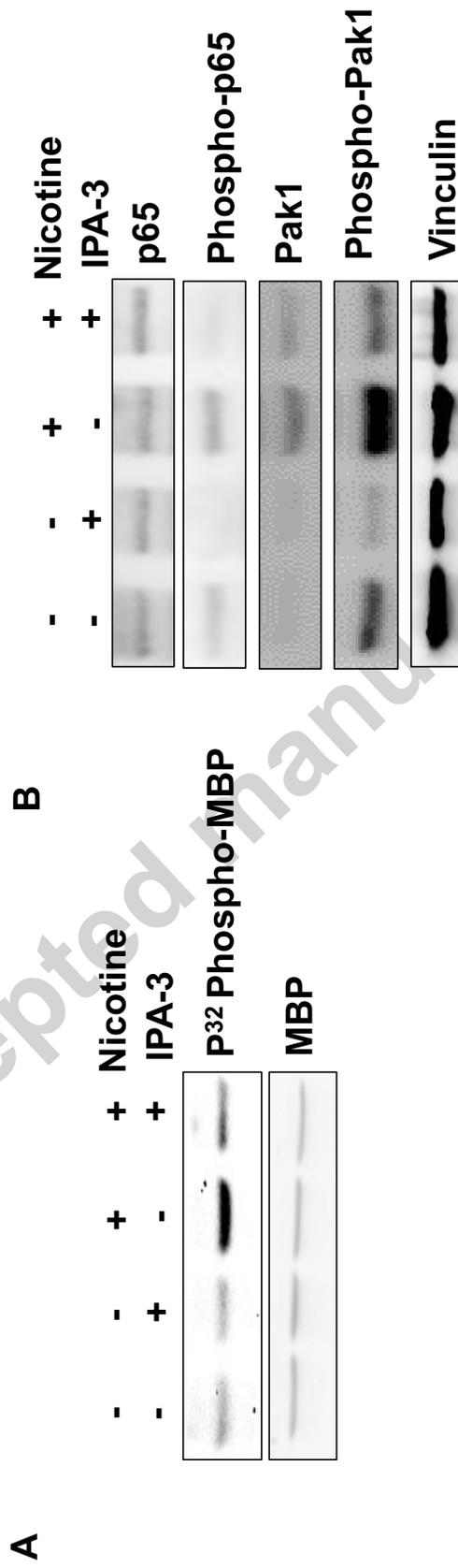
FIGURE LEGEND

Figure 1: **A**, Kinase assay showing the phosphorylated status of MBP as observed in autoradiogram and total MBP showing equal loading, stained with Ponceau, in the presence or absence of 0.5μM nicotine or 20μM IPA-3. **B**, Western blotting the expression pattern of p65, phospho-p65, Pak1, phospho-Pak1 and Vinculin after exposing the Mia Pa Ca 2 cells with nicotine (0.5μM) for 5h and then treating with IPA-3 for 1h.

Figure 2: **A** & **B,** Expression of Phospho-Pak1 (pPak1) and phospho-p65 under α7-nAChR siRNA and α-bungarotoxin (α-BTX) treatment. **C,** Graph representing the mRNA level expression of α7-nAChR after siRNA treatment. **D**, Graph showing relative expression of Pak1 expression in PC patients (n=20) who were grouped as smokers (n=10) and non-smokers (n=10). Data is expressed as Mean ± SEM. The significance was calculated using Mann Whitney test and *** denotes $P<0.001$.

Figure 3: Expression of Pak1, p65 and its phosphorylation status in the presence and absence of smoke condensate after α7-nAChR silencing.
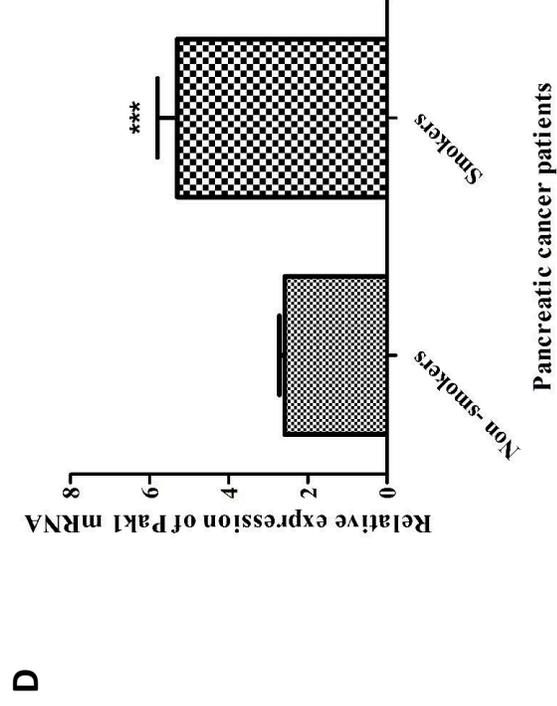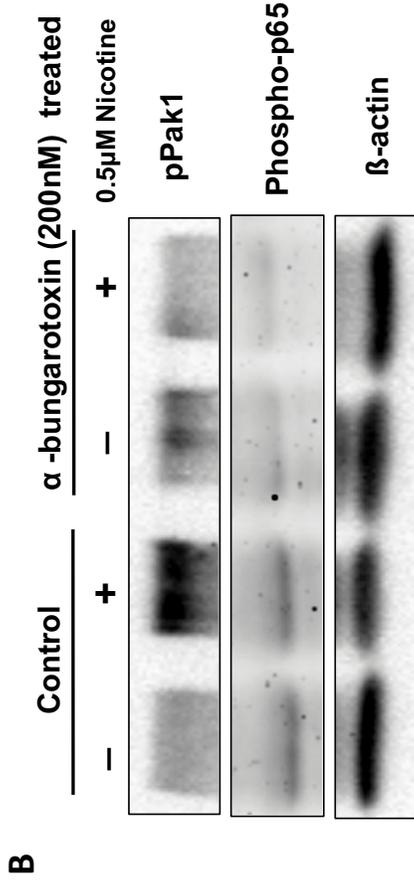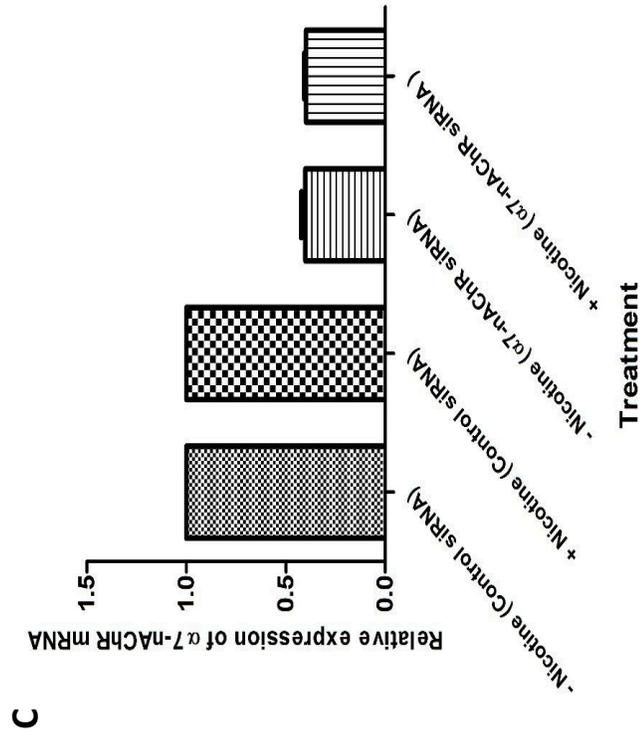
Figure 1

Figure 2

Figure 3