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# Nicotine induces aberrant hypermethylation of tumor suppressor genes in pancreatic epithelial ductal cells

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ABSTRACT

Tobacco smoking is an independent risk factor for the initiation of pancreatic cancer (PC). Hypermethylation of tumor suppressor genes has been demonstrated to be associated with smoking. This study aimed to find the relationship between nicotine exposure and hypermethylation of tumor suppressor genes in normal pancreatic epithelial cells. Human pancreatic epithelial cells ware cultured exposing to nicotine and the methylation status of tumor suppressor genes were detected. Proenkephalin (PENK) was chosen as the target gene and methylation level of PENK promoter region was measured. Expression of DNA methyltransferase (DNMT), nicotine acetylcholine receptor (α7nAChR) and signaling pathway downstream were analyzed. Nicotine induces overexpression of DNMT3A and 3B, and methylated-inactivation of PENK gene in normal pancreatic epithelial cells. An activation of α7nAChR and MAPK signaling pathway has been detected in the nicotine-treated group. Demethylated drug, antagonist of α7nAChR and inhibitor of p38 MAPK is verified to attenuate the overexpression of DNMTs stimulated by nicotine as well as inhibit aberrant hypermethylation-related silence of PENK gene. Nicotine stimulation can induce aberrant hypermethylation of tumor suppressor genes by α7nAChR and MAPK signaling pathway-mediated up-regulation of DNMTs in pancreatic epithelial cells, thus we can provide epigenetic evidence of the mechanisms by which smoking causes pancreatic cancer and find new therapeutic target.



## 1. Introduction

Pancreatic cancer (PC) remains one of the fatal malignancies for lack of early detection and its unresponsiveness to routine chemotherapeutics. It has been universally accepted that tobacco smoking is an independent risk factor for the initiation and progression of PC [1,2], and smokers have 2-fold risk of developing PC than nonsmokers [3,4]. Nicotine, as widely studied as the addictive component before, has been demonstrated to have a function of genotoxicity as well as carcinogenesis in recent years [5,6]. In general, the mechanism of nicotine-induced PC remains unclear.

Aberrant methylation of DNA is considered to be one of the most consistent epigenetic alterations in cancer and hypermethylation of tumor suppressor genes promoter region is a common alteration, observed in PC [7,8]. This has been suggested to have potential association with smoking behavior. Cigarette smoke leads to high level DNA methyltransferase (DNMT) activity [9] and some reports demonstrated that components of cigarette smoke could enhance the expression of DNMT [10].

The nicotine acetylcholine receptors (nAChRs) have been confirmed to play a key role in nicotine-induced pathogenesis in different types of cancers, and α7nAChR is the main subunit mediating this effect [1,11]. Recent study has demonstrated that nicotine induced activation of Erk1/2 by acting on α7nAChR, and lead to proliferation of human bladder cancer cells [12]. Several reports have shown an activation of the Ras/Raf/MEK/ERK pathway downstream of α7nAChR in cancer cell lines [13], and found the link between MAPK kinase pathway, aberrant DNA methylation and DNMTs regulation.

By knowing the potential link between nicotine exposures, aberrant methylation of tumor suppressor genes, nicotinic acetylcholine receptor and MAPK signal pathway, normal pancreatic ductal epithelial cell lines were cultured to study the nicotine-

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induced changes of DNA methylation patterns, and to explore the mechanisms for the first time.

## 2. Materials and methods

### 2.1. Cell culture

Human pancreatic epithelial duct cell line hTERT-HPNE cells and PC cell lines (Panc-1, AsPc-1) were purchased from ATCC. All cells were maintained in an atmosphere of 5% $CO_2$, 37 °C. HPNE cells were stimulated with nicotine (Sigma) in the presence or absence of other drugs (5-aza-2′-deoxycytidine, 5-Aza or Mecamylamine, MAA). For signaling inhibitor studies, HPNE cells were treated with ERK inhibitor U0126 (1 μM) and p38 MAPK inhibitor SB203580 (1 μM).

### 2.2. Cell counting kit-8 (CCK-8) assay and EdU test

The CCK-8 detection kit (Dojindo, Japan) was used to measure cell viability according to the manufacturer's instructions followed by absorbance measurements at 450 nm. We investigated the newly synthesized DNA of replicating cells after treating by 10 nM and 100 nM nicotine for 72 h using EdU DNA cell proliferation kit according to the manufacturer's instructions (Guangzhou RiboBio, Guangzhou, China).

### 2.3. Flow cytometric immunophenotyping

Cells treated with different concentrations of nicotine or other agents were incubated for 72 h at 37 °C temperature in a 6-well plate ($10^5$ cells/well). Following by incubation with Annexin V-FITC/PI solution as described according to the protocol (BD Biosciences, San Jose, CA). The cells were analyzed by flow cytometry system.

### 2.4. DNA extraction, bisulfite modification, MSP

DNA of each sample was extracted using DNA Extraction Kit (QIAGEN, GmbH, Hilden, Germany) and then subjected to bisulfite conversion step according to the instructions of EpiTect Plus Bisulfite Kits (QIAGEN, GmbH, Hilden, Germany). Methylation Specific PCR (MSP) was conducted using EpiTect MSP Kit (QIAGEN, GmbH, Hilden, Germany). Primers for MSP is listed in Table S1.

### 2.5. RNA extraction and real-time PCR

Total RNAs were isolated using Trizol reagent according to the manufacturer's instructions. 1 μg of total RNA of each sample was subjected to reverse transcription using Superscript III First-Strand Synthesis System (Invitrogen, Grand Island). Quantitative reverse transcriptase PCR (Q-PCR) was conducted using an ABI 7500 system with SYBR green (Invitrogen, Grand Island). The primers are presented in Table S2.

### 2.6. Western blot and antibodies

Proteins of each sample were prepared using RIPA (Sigma) solution. An equal amount of protein was loaded into 10% SDS-PAGE and transferred onto NC membranes. The primary antibodies were as follows: anti-DNMT3A (cat: HPA026588, Sigma Aldrich), anti-DNMT3B (cat: HPA001595, Sigma Aldrich), anti-DNMT1 (cat: ab13537 Abcam), anti-β-actin (cat:3700 Cell Signaling Technology), anti-Prenkephalin (PA5-53021, Thermo fisher), anti- α7nAChR (cat: ab13537 Abcam), anti-MAPK signaling pathway antibody were all purchased from Cell Signaling Technology.

### 2.7. Quantitive detection of methylation level

DNA methylation of CpG dinucleotides were measured by a mass spectrometry-based method (Epityper, Sequenom). 2000bp upstream from the transcriptional start site was chosen as the predicted promoter region and the relative ratios of methylated DNA were then calculated based on the control, which was defined as 100% DNA methylation.

### 2.8. DNMT activity

DNMT activity test were conducted using a DNMT activity inhibition assay kit (Epigentek, Farmingdale, NY). The nuclear protein was isolated using the Nuclear Protein Extraction Kit (Thermo Scientific, Pittsburgh, PA). The relative DNMT activities were calculated based on the ratio of the treatment group to the control group.

### 2.9. DNMTs siRNA interference and transfection

The DNMT3A and 3B-specific siRNA were chosen from the GenBank sequences and synthesized by Sangon Biotech (Shanghai). The DNMT3A siRNA se-quences were 5′-CGGCUCUUCUUUGAGUUCUTT-3′ and 5′-AGAACUCAAAGAAGAGCCGTT -3′, and the DNMT3B siRNA sequences were 5′-GAUGAUUGAUGCCAUCAAATT -3′ and 5′-UUUGAUGGCAUCAAUCAUCTT -3′. The Opti-MEM (Gibco) and Lipofectamine 3000 Transfection Kits (Invitrogen) were used for siRNA transfections according to the instructions book.

### 2.10. Statistical analysis

Data was expressed as mean ± SD and then analyzed using SPSS. The one-way ANOVA was used for data analysis between two groups. Differences associated with $P < 0.05$ were considered statistically significant.

## 3. Results

Nicotine promotes the proliferation and inhibits the apoptosis of HPNE cells.

hTERT-HPNE cells were cultured exposing to different concentrations of nicotine (10 nM-10μM). The effect of nicotine on proliferation was detected by CCK-8 and EdU. As shown in Fig. S1A, nicotine can significantly accelerate the proliferation of HPNE cells especially in 10 nM and 100 nM groups, compared to the negative control. After exposed to nicotine (10 nM, 100 nM) for 72 h, the percentage of EdU-positive cells was proved to be increased relative to the negative control ($p < 0.01$) (Fig. S1B). The level of apoptosis was assessed by flow cytometry and the result showed that nicotine can reduce the rate of HPNE cells apoptosis. 100 nM nicotine-treated group turned out to have the lowest rate of apoptosis (Fig. S1C).

Nicotine can change the methylation patterns of tumor suppressor genes in HPNE cells.

Promoter methylation status of ten genes (P16, PENK, FoxE1, NPTX2, E-cadherin, UCLH1, SPARC, THBS1, LHX, hMLH), which were all confirmed to be hypermethylated in PC according to earlier articles (Table S3), were detected by MSP (Fig. 1 A). After stimulated by nicotine (100 nM) for 72 h, 3 genes (P16, PENK and UCLH1) showed different methylation patterns compared to the control group. PENK and UCLH1, which are both completely unmethylated in normal pancreatic cell line, showed aberrant methylation in the nicotine-treated group.

P16, PENK, FoxE1, NPTX2, UCLH1 and SPARC genes showed promoter hypermethylation in both PC cell lines (AsPc-1, PanC-1);