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α5-nAChR modulates nicotine-induced cell migration and invasion in A549 lung cancer cells

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ABSTRACT

Cigarette smoking is the most important risk factor in the development of human lung cancer. Nicotine, the major component in tobacco, not only contributes to carcinogenesis but also promotes tumor metastasis. By binding to nicotinic acetylcholine receptors (nAChRs), nicotine induces the proliferation and migration of non-small cell lung cancer. Recently studies have indicated that α5-nAChR is highly associated with lung cancer risk and nicotine dependence. Nevertheless, it is unclear whether nicotine promotes the migration and invasion through activation of α5-nAChR in lung cancer. In the present study, A549 cell was exposed to 1 μN nicotine for 8, 24 or 48 h. Wound-healing assay and transwell assay were used to evaluate the capability of A549 cell migration and cell invasion, respectively. Silencing of α5-nAChR was done by siRNA. Western blotting and PCR were used to detect α5-nAChR expression. Nicotine can induce activation of α5-nAChR in association with increased migration and invasion of human lung cancer A549 cell. Treatment of cells with α5-nAChR specific siRNA blocks nicotine-stimulated activation of α5-nAChR and suppresses A549 cell migration and invasion. Reduction of α5-nAChR resulted in upregulation of E-cadherin, consistent with E-cadherin being inhibitive of cancer cell invasion. These findings suggest that nicotine-induced migration and invasion may occur in a mechanism through activation of α5-nAChR, which can contribute to metastasis or development of human lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide and cigarette smoking accounts for almost 90% of all deaths from lung cancer (Toyooka et al., 2003). Studies present an association between cigarette smoking and lung carcinogenesis (Hashibe et al., 2002; Osada and Takahashi 2002; Porta et al., 2003). In addition to its documented risks for lung carcinogenesis, cigarette smoking has been implicated in cancer development (Eichholzer 2000). Nicotine, a major component of cigarette smoke, has been shown to be involved in the initiation, promotion, and even progression of several tumors including lung cancer. Although nicotine itself is not a carcinogen, it is the principal reinforcing component in cigarette smoking and can promote tumor growth via the induction of increased levels of proliferation, angiogenesis, migration, invasion, and epithelial-to-mesenchymal transition (Davis et al., 2009; King 1990; Singh et al., 2011). However, the mechanism by which nicotine promotes tumor metastasis remains unclear.

Metastasis is the spread of cancer from a primary tumor to distant sites of the body, which is the major cause of mortality in patients with cancer (Zhang et al., 2009). From primary tumor to secondary growth, cancer cells must invade the surrounding tissues, penetrate vessels, and travel to other sites where they arrest and resume growth (Khurgel et al., 1996). Cell migration has been considered a required process during tumor cell metastasis (Wang et al., 2009c). Therefore, mechanism of cell movement is critical to understand tumor metastasis.

Nicotinic acetylcholine receptors (nAChRs) were once thought to be restricted to neuronal cells, but recently, the expression of nAChR subunits has been shown in many nonneuronal cells, including normal human bronchial epithelial cells, human lung cancer cells (Hurst et al., 2013; Minna 2003; Schaal and Chellappan 2014). The structure of nAChRs is a homo-(α7 or α9) or heteropentamer (α2–α10; b2–b4). Several evidences suggest that nicotine exerts its cellular functions through nicotinic acetylcholine receptors (nAChRs) (Fang et al., 2009; Ma et al., 2014; Shin et al., 2004; Wang et al., 2012). While the role of different members of nAChR family may regulate converging signaling pathways, they

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often have diverse and even opposing actions (Tang et al., 2013). Recently, genome wide association studies have indicated that α5-nAChR is highly associated with lung cancer risk and nicotine dependence (Boezen 2009; Macqueen et al., 2014; Zhao et al., 2014). In our previous study, the results suggest that α5-nAChR expression was correlated with the clinical TMN stage of lung cancer. Meanwhile, the enhanced proliferation of A549 cells stimulated by nicotine is at least partly dependent on α5-nAChR expression. Nevertheless, no information has been available about whether nicotine affects migration and invasion of human lung cancer cells through regulation of α5-nAChR in vitro.

On the basis of previous studies that nicotine induced α5-nAChR expression and nicotine promoted lung cancer metastasis, the present study hypothesize that α5-nAChR may play a significant role in the invasiveness and metastasis of lung cancer stimulated by nicotine treatment. The objective of the present study was to determine the effects of α5-nAChR on the tumor suppressor gene E-cadherin, the migration and invasion of non-small cell lung cancer (NSCLC) cells induced by nicotine.

2. Materials and methods

2.1. Cell culture and nicotine treatment

The human NSCLC cell line A549 was obtained from American Type Culture Collection (ATCC). A549 cells were grown in RPMI1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. A549 cells were treated with 1.0 μM nicotine (Sigma) at different time points indicated (Cucina et al., 2012; Ma et al., 2014). The solvent control was dimethyl sulphoxide (DMSO) (0.1% v/v).

2.2. Wound healing assay

Wound-healing assay was performed to assess cell migration by determining the ability of the cells to move into an acellular space. Monolayer cells were wounded by scratching the surface on the 6-well plate as uniformly as possible with a p1000 pipette tip. The wells were rinsed three times with phosphate buffered saline (PBS) and replaced with fresh medium containing 1.0 μM Nicotine. For solvent control, medium with DMSO (0.1% v/v) was added to the wells. The cells were incubated at 37 °C for 24 h. The initial wounding and the movement of the cells in the scratched area were photographed by using an Olympus CKX41 inverted microscope equipped with a digital imaging system at 0 and 24 h. The wound width of 6 random views was measured, and the healing width was calculated by wound width at 0 h time point minus wound width at 24 h time point and normalized by solvent control.

2.3. Transwell cell invasion

The cell invasion assay was performed using a 24-well Transwell chamber (Corning, USA). Briefly, 48 h after transfection, A549 cells were seeded at a density of 1×10^5 cells to the upper chamber with an 8 μm pore size insert pre-coated with Matrigel (BD Biosciences, USA). The plate wells were filled with 400 μl RPMI1640 containing 10% FBS. After incubation for 48 h at 37 °C, cells on the upper side of the membrane were removed by clean swabs, and cells on the underside were viewed and counted. The numbers of invaded cells were counted in 10 randomly selected fields. The experiments were performed in triplicates.

2.4. CHRNA5 gene knockdown by small interfering RNA

A double strand siRNA oligonucleotide targeting CHRNA5, which encodes α5-nAChR (sense: 5′-CCCGCAAACUACAAAA-GUUTT-3′, antisense:5′-AACUUUU CUAG UUUGCCGGTG-3′), was synthesized by Shanghai Genepharma Co. Ltd. (China). A pair of negative control siRNA were also designed with sequences different from siRNA-CHRNA5 and not homologous to any sequences found in gene bank (sense: 5′-UUCUCCGAACGUGU-CACGUTT-3′, antisense: 5′- ACGUGACACGUUCG GAGA-3′). Cells were plated in 6-well plates and when cells were 30–50% confluence, siRNAs were added in final concentration of 50 nM with lipofectamin 2000 (Invitrogen) as per the manufacturer's instruction. Cells were incubated with si-CHRNA5 in serum-free conditions for 6 h at 37 °C. Serum was then added back to the culture, and cells were incubated in normal condition. Thirty-two hours after si-CHRNA5 transfection, A549 cells were incubated with 1.0 μM nicotine for 16 h (Ma et al., 2014).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Twenty-five nanogram total RNA per sample was reverse transcribed by using the Reverse Transcription Reaction Kit (TakaraCode: DRR061S) according to the manufacturer's instructions. Quantitative real-time PCR was performed analyzed on the Applied Biosystems 7300 Real-Time PCR System to determine the relative amounts of α5-nAChRand GAPDH (internal control) mRNAs expressed. The SYBR Green Supermix was used for all real-time PCR reactions. The quantitative real-time PCR parameters were 95 °C for 10 s as a pre-denature step followed by 40 PCR cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 10 min. All the samples were performed in triplicates in each experiment. The relative amount mRNA was calculated using the comparative CT method after normalization to GAPDH mRNA levels.

2.6. Western blotting assay

Protein (50 μg) from each sample was resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by western blotting. Mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody and rabbit polyclonal anti-α5-nAChR were purchased from Abcam, Inc. (Cambridge, MA). Alkaline phosphatase-coupled anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-GAPDH and anti-α5-nAChRprimary antibodies were incubated at 1:5000 and 1:500 dilution, respectively, for 2 h, and the secondary antibodies were incubated at 1:5000 dilution for 1 h. The assay was repeated twice with duplicate samples.

2.7. Immunofluorescence staining

Prior to staining, cells were fixed in 4% paraformaldehyde (Sigma) for 10 min, permeabilized in 0.1% Triton X-100 for 20 min, and blocked overnight in 1% BSA at 4 °C with two washes in PBS at room temperature between each of the previous steps. Cells were incubated with mouse monoclonal antibodies to E-Cadherin (Abcam, 1:200) overnight at 4 °C in a humidity chamber followed by incubation with goat anti mouse Alexa-488 secondary antibody (Cell Signaling, 1:500) for 1 h at room temperature. Both primary and secondary antibodies were diluted in Antibody Diluent Solution (Dako, Carpenteria, CA). Images were taken using the Zeiss LSM510Confocal Microscope.