

Nicotine-Derived Nitrosamines Promote Renal Cell Carcinoma Cell Migration Via Binding to Nicotinic Acetylcholine Receptors

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ABSTRACT

Several risk factors such as smoking, obesity, diabetes, and hypertension are related to the incidence of kidney cancer. Renal cell carcinoma (RCC) originates from the renal tubular epithelium and is the major form of kidney cancer. Notably, the relative risk of RCC in smokers compared to non-smokers is 1.38 while revealing a dose-dependent risk reduction in smoking cessation. Carcinogenic substances in tobacco, specifically nicotine-derived nitrosamines like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosoornicotine (NNN), were known to induce the formation of DNA adducts, mutating genes that potentially initiate cancers. In addition to carcinogenesis, nicotine-derived nitrosamines may also contribute to modify the malignant features, which accelerates the cancer progression. This study was aimed to evaluate the effects of NNK in the modulation RCC cell proliferation and motility, besides, the possible underlying mechanisms were also studied. Our findings showed differential expression of nicotinic acetylcholine receptor (nAChR) mRNA subunits between normal human renal tubular cell (HK-2) and RCC cells (Achn), suggesting that RCC cells may have the ability in respond to the stimuli of NNK. While NNK didn't significantly show an influence in RCC cell viability, it enhanced cell motility and migration ability. Utilizing specific inhibitors, it was inferred that NNK stimulates RCC cell migration via nAChR activation and subsequent calcium channel opening. Our research underscores the necessity of smoking cessation in RCC management, even as further studies on diverse RCC cell lines and real-world data are required.

Introduction

Renal Cell Carcinoma (RCC for short) is one type of kidney cancer. It is a fatal cancer that starts from the epithelium of renal tubules in our kidneys[1]. According to a survey conducted in 2008 in Taiwan, 887 people were diagnosed with kidney cancer, and 91.77% of them were RCC. A total of 526 people died from kidney cancer in that year. Therefore, it is urgent to find a solution for the management of RCC.

It has been noted that several factors potentially lead to the progression of cancer, such as smoking, obesity, diabetes, and hypertension are all potential risk factors [2]. Among those factors, specifically, smoking may play a major driver from external sources [3]. It has been known that smoking has adverse impacts on our body, harming multiple organs. Approximately of 5-6 million people die each year due to tobacco use, while middle-aged men and women account for 31% and 6% of the total diagnosed population [4].

Although smoking is a major factor that leads to RCC, the associated risk in reality is not explicitly high, however still noteworthy [2]. According to a meta-analysis, the relative risk for RCC for smokers compared to people who never smoke was 1.38. In addition, the risk of RCC is significantly dose-dependent. If one quits smoking for a decade or longer, the risks of being diagnosed with RCC become lower than one who just quit for 1-10 years [5]. Hence, the research shows the benefit of quitting the habit of smoking.

Harmful chemicals contained in cigarettes, nicotine-derived nitrosamines like 4-(methylnitrosamino)-1-(3pyridyl)-1-butanone (NNK) and N¹-nitrosonornicotine (NNN) are known to form DNA adducts and lead to mutations in vital genes such as Rb, p53, and K-Ras in smokers, which may initiate some types of cancer [6, 7]. Apart from this, smoking has a positive correlation with cancer metastasis, which is the major cause of cancer-related death. In addition to carcinogenesis, NNK and NNN may also participate in promoting malignant behaviors in cancer cells, such as proliferation and migration ability [8, 9].

In my study, we examined the differential expression levels of different types of nicotinic acetylcholine receptors (nAChR) in a normal human renal tubular cell line (HK-2) and RCC cell line (Achn). To further understand and evaluate the process, we further applied specific pharmacological inhibitors to observe the effects of NNK on the proliferation and migration ability in RCC cells.

Materials & Methods

Cells

Two human kidney cell lines were used in this study, including a normal tubular cell line (HK-2) and a RCC cell line (Achn). Both cells were grown at 37°C in a humidified incubator which was injected with 5% of CO₂. The HK-2 cell was maintained in DMEM/F-12 with 5% Fetal Bovine Serum (FBS) while the Achn cell was maintained in Minimum Essential Medium with 10% FBS. In addition, all chemicals (NNK, hexamethonium bromide, and nifedipine) were obtained from Sigma-Aldrich.

Cell Growth Assay

We placed 2×10^3 cells per well within the 96 plates and let them incubate overnight. The day after, we then changed the medium to serum-free medium and let them starve for 24hr. After that, we added different concentrations (0, 1, 10, 100 μ M) of NNK to check cell growth at indicated time points by MTT assay.

Wound-Healing Assay

For this test, we seed 3×10^4 cells on each side of a special insert [ibidi, $400 \pm 50 \mu$ m (Ibidi, Martius, Germany)] and let it incubate overnight. Later, we removed the culture insert to create a scratch wound and washed the cells twice with PBS. We then replaced the medium containing 0.5 % FBS w/o 10 μ M of NNK to monitor cell migration. After 17 hours, we photographed in order to measure the coverage area of cells to determine how the cells moved towards the "wound". For pharmacological inhibitors, 10 μ M of hexamethonium bromide and 10 μ M nifedipine were treated 30 mins before adding NNK.

Boyden Chamber Assay

We seeded 2.5×10^4 cells in a tool called Boyden chamber. The chamber has a permeable membrane containing 8.0 μ m diameter pore (Becton Dickinson). NNK was added either inside or outside the chamber to monitor the cells' motility over 24 hours, which mimicked the regulation of cell motility by either promoting cell movement or chemotaxis.

RNA Extraction, Reverse Transcription, and Real-Time PCR

We grew cells in a dish until they were 80% full. We used Trizol reagent to extract RNA from the cells. After getting the RNA, we measured RNA concentration by Nanodrop. Next, we performed reverse transcription to turn the 1µg RNA into complementary DNA by GoScript™ Reverse Transcriptase. Thereafter, we used 1µl of cDNA to mix with either specific nAChRs and GAPDH primers (housekeeping gene) and 2xTaq PCR MasterMix to perform quantification in either regular or real-time PCR by PCR thermocycler. The regular PCR product was separated by electrophoresis in 1% agarose gel in 100V for 15mins.

Results & Discussions

The nAChR has been explored in relation to various cancers, but its expression in the human kidney and RCC cells hasn't been fully defined. In the rat kidney study, it has been reported that the mRNA of $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\alpha 10$, $\beta 1$, $\beta 2$, and $\beta 4$ subunits of nAChR were constitutively expressed in the kidney [10]. Herein, we had firstly examined the expression of subunits of nAChR mRNA in the human proximal tubular cell line, HK-2. As shown in Fig.1A, only $\alpha 5$, $\alpha 7$, $\alpha 10$, $\beta 1$, and $\beta 4$ subunits were expressed in the HK-2 cell. In order to check whether differential expression levels in cancerous cells, we had performed the quantification PCR to compare the expression levels of the mentioned subunits between HK-2 cell and RCC cell line, Achn. Among these, Achn cell showed higher mRNA expression of $\alpha 5$, $\alpha 7$, $\alpha 10$ and $\beta 1$ subunits in comparison to HK-2 cell (Fig.1B). This data suggested that RCC cells may exhibit higher potential in response to nicotine and its derivatives, such as NNK.

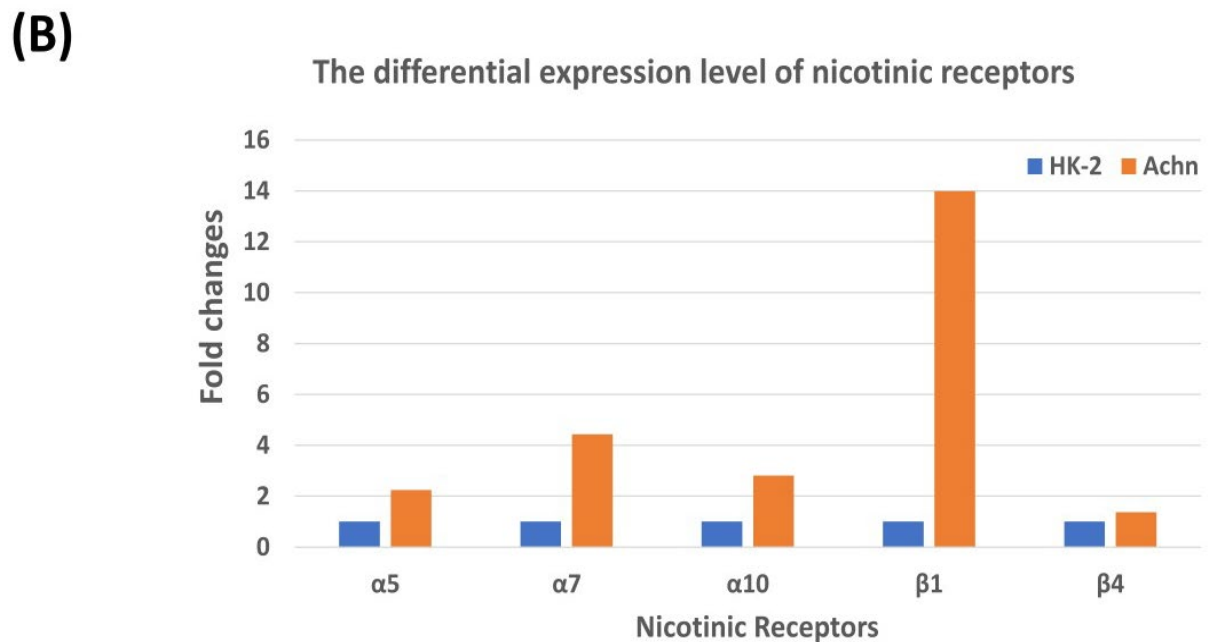
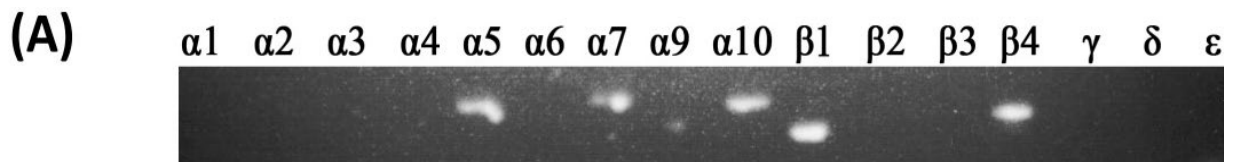


Figure 1. Variation in nicotinic acetylcholine receptor levels in HK-2 and Achn cells. (A) We took RNA from HK-2 cells and used RT-PCR to see how different nAChRs are expressed. The PCR focused on nAChR types $\alpha 5$, $\alpha 7$, $\alpha 10$, $\beta 1$, and $\beta 4$. (B) By quantification real-time PCR analysis, Achn cell showed more than two-fold increase in $\alpha 5, \alpha 7$, $\alpha 10$, and $\beta 1$ than HK-2 cell.

Therefore, we had performed certain tests to conduct the biological activities of NNK in regulating tumorigenic behaviors in RCC. In the cell viability, treatment with various concentrations of NNK had less effects on the Achn cell viability, besides, no significant difference in cell proliferation had been observed (Fig.2). In the Boyden Chamber assay, we had observed significantly increased cell invasion ability pass through the membrane, indicating the increase of cell motility. Of note, in comparison with treatment NNK together with Achn cell and treatment in the lower chamber, treatment with NNK together enhances the degree of motility (Fig.3). These data imply NNK may represent as a stimulator for RCC motility rather than as a chemoattractant substance.

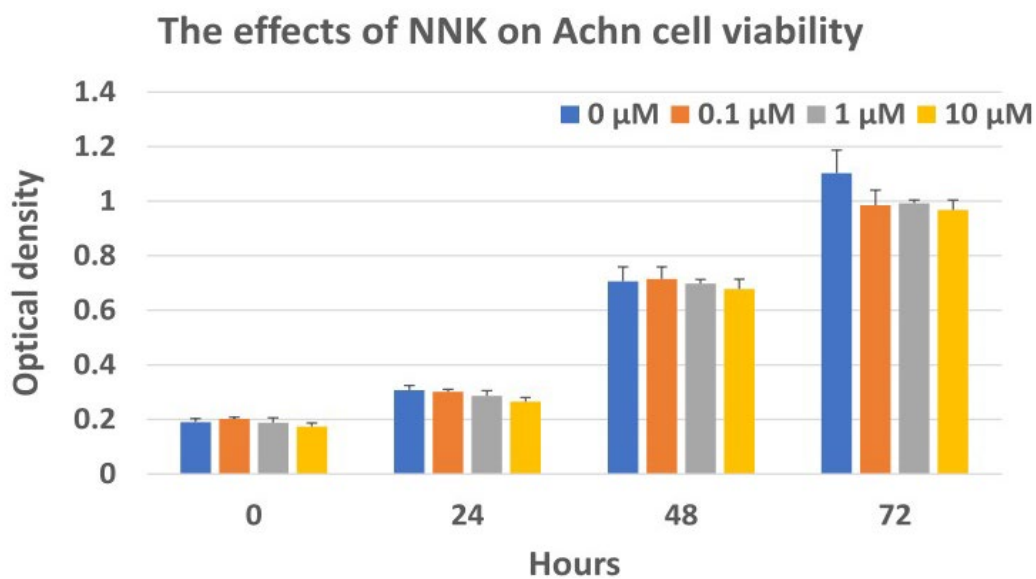


Figure 2. The effects of NNK on Achn cell viability. We treated 2×10^3 Achn cells with varying amounts of NNK over 24, 48, and 72 hours. We then checked the cell viability using the MTT assay.

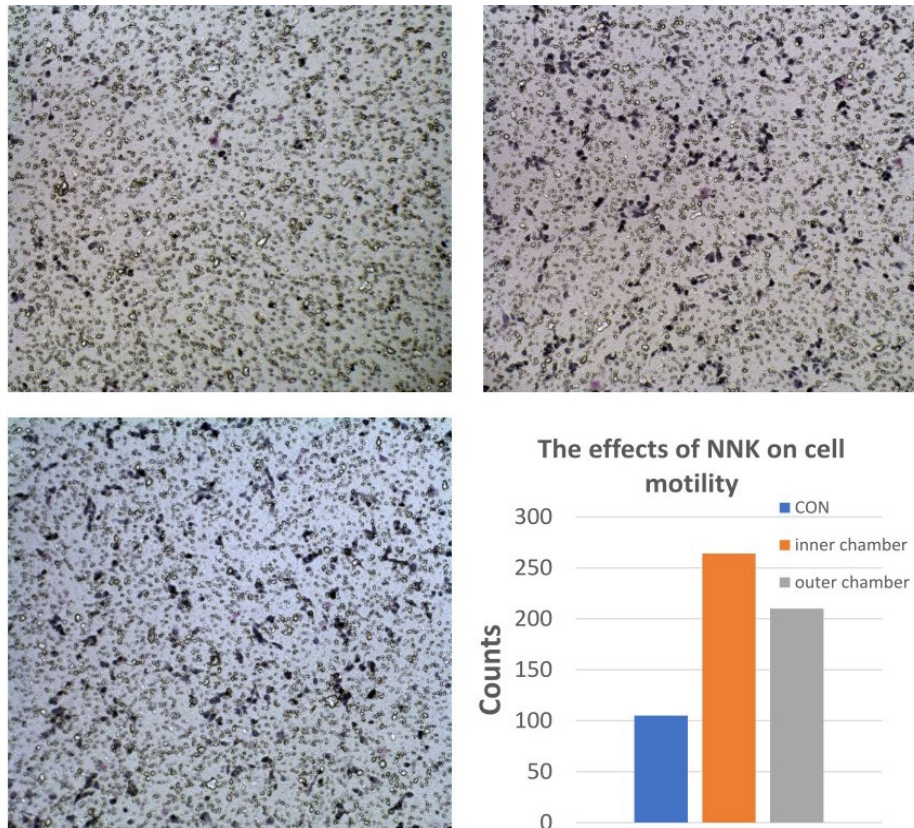


Figure 3. The effects of NNK on cell motility in Boyden chamber assay. 1×10^5 Achn cells were placed on Boyden chamber membranes. Upper left is the control group, Lower left had $10 \mu\text{M}$ NNK in the upper chamber (NNK inside chamber). Upper right had $10 \mu\text{M}$ NNK in the bottom chamber (NNK outside chamber). After 24 hours, cells were set in place and colored with crystal violet and counts.

Since the NNK can promote the cell motility, we had set up the wound closure assay to evaluate the migratory of Achn cells in response to NNK. As shown in Fig.4, treatment with NNK had also increased the migratory as evidenced by the recovery of wound coverage area. It is known that the nAChRs are acetylcholine gated ion channels are highly calcium ions permeable and allow releases of calcium ions from intracellular stores to the cytosol of cells [11]. To ascertain the underlying mechanisms, we applied specific inhibitors to examine the influence of NNK in modulating Achn cell migration ability. Treatment with either general nAChR antagonist (hexamethonium bromide) or calcium channel blocker (nifedipine) could suppress NNK-induced cell migration (Fgi.5). This evidence had supposed that NNK-mediated Achn cell migration through the engagement of nAChR, and open of downstream calcium channel to lead cell migration.

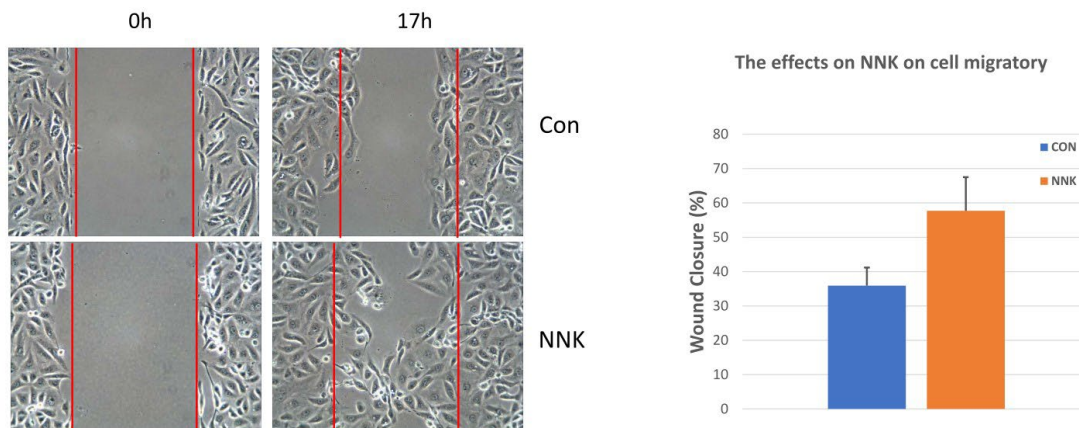


Figure 4. The effects of NNK on Achn cell migratory. We placed 1.5×10^4 A498 cells into each slot of a special 24-well plate that had been "wounded" or scratched. After letting them sit for 24 hours, a single layer of cells formed. Once the cells were properly attached, we took out the inserts and let the cells grow in a 0.5% FBS solution mixed with either 10 μ M NNK or DMSO. Over 17 hours, we watched and took pictures of the cells moving towards the scratched area.

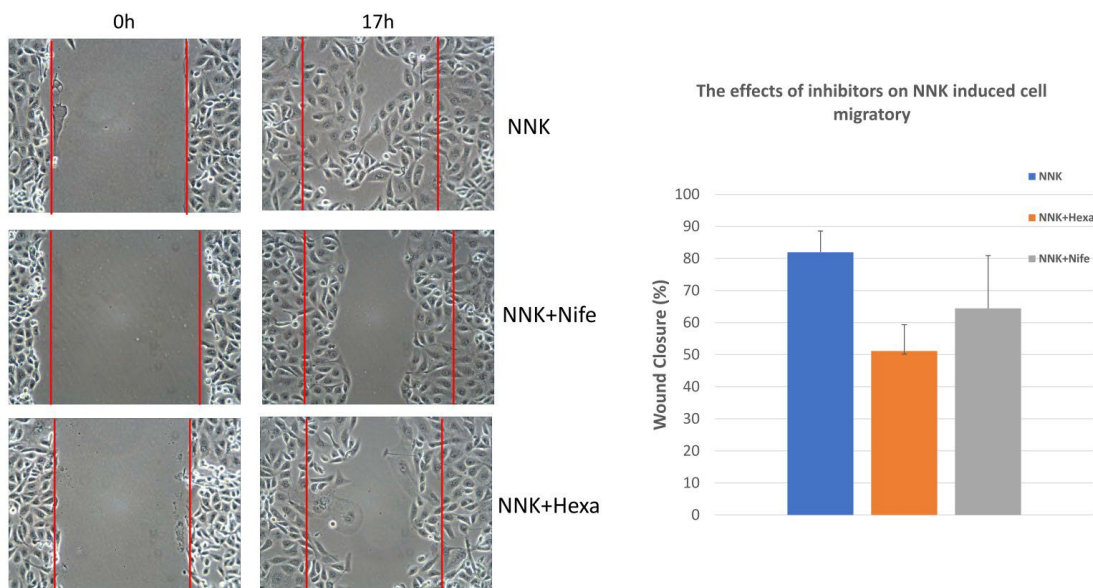


Figure 4. The effects of specific inhibitors on NNK induced Achn cell migratory. We placed 1.5×10^4 Achn cells into each "wounded" slot of the culture plate and let them sit overnight. Once the cells were properly attached, we removed the inserts and treated the cells with a mix containing 1 μ M of Nicotine, 10 μ M Nifedipine, and Hexamethonium bromide. We monitored and captured photos of the cells heading towards the scratched area after 17 hours.

Although our data showed that NNK can promote RCC cell line migratory, there are several limitations that need to be addressed. Firstly, only one RCC cell line is used in this study, we may need to examine more cell lines or primary tumor cells to increase the strength of our findings. Besides, we only performed in vitro

study to prove our hypothesis, the real-world demographic data from the population health surveys may help to clarify these observations.

Based on the performance of this project, we set set-up a series to monitor the cancer cell malignant behaviors, which might be applied to different compounds in regulating cancer cells. Tumor cells from primary tumors will get certain malignant features in the progression of cancer, such as proliferation for the increase of tumor mass, and migration for the invasion and metastasis. Our data suggested that NNK may exhibit as an enhancer in promoting cancer malignant features, quitting smoking is an important action in the management of cancer patients.

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