Tobacco-Specific Carcinogen Enhances Colon Cancer Cell Migration Through α7-Nicotinic Acetylcholine Receptor

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Objective: To study the mechanism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-enhanced migration of colon cancer cells.

Background Data: Long-term cigarette smoking increases the risk of colorectal cancer mortality. Tobacco-specific carcinogen, NNK, was reported to increase DNA synthesis of colon cancer cells. Since metastasis is the major cause of cancer death, the influence of NNK on the migration of colon cancer cells remains to be determined.

Methods: Receptor for NNK in colon cancer cells was identified by polymerase chain reaction (PCR) and real-time PCR. The influence of NNK on migration of colon cancer cells was evaluated by transwell and wound-healing assay. Receptor-mediated migration was studied by both inhibitor and small interfering RNA.

Results: α7 nicotinic acetylcholine receptor, α7-nAChR, was identified in 2 colon cancer cell lines, HT29 and DLD-1. NNK enhanced HT29 cell migration in both transwell and wound-healing assays. NNK also enhanced DLD-1 cell migration in dose dependent manner. We used inhibitor and siRNA to demonstrate that α7-nAChR mediated NNK-enhanced colon cancer cell migration and downregulation of E-cadherin were involved in NNK-enhanced migration of colon cancer cells. Furthermore, Snail and ZEB1, 2 major transcription repressors of E-cadherin in colon cancers, were induced by NNK treatment.

Conclusions: Tobacco specific carcinogen, NNK, enhanced colon cancer metastasis through α7-nAChR and E-cadherin—one of the hallmarks of epithelial mesenchymal transition—and its transcription repressors. Therefore, smoking should be avoided in the patients with colorectal cancer.

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Smoking is a serious worldwide health problem responsible for more than 30% of cancer deaths per year in the United States.[1] It increases the risk of various cancers such as lung, oral, and gastrointestinal cancers, including colorectal cancer.[2] The link between cigarette smoking and cancers results from a powerful alliance of 2 factors—nicotine and carcinogens.[2] Several tobacco-specific carcinogens are derived from nicotine. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed by the nitrosation of nicotine and has been identified as the most potent carcinogen in cigarette smoking.[3] Previous studies suggested that NNK caused the induction of cell proliferation in normal human bronchial epithelial cells[4] and immortalized pancreatic ductal cells.[5,6] Additionally, exposure of immortalized human breast epithelial cells to NNK enhanced the progression of cellular carcinogenesis.[7]

NNK induces carcinogenesis either by reacting with DNA and forming covalently bound products known as DNA adducts, which is responsible for its genotoxic effects,[2] or by binding to nicotinic acetylcholine receptor (nAChR) to exert its biologic function.[8] nAChR can be detected on muscle and neuronal cells. Recently, neuronal nAChRs have also been found on non-neuronal cells, such as lung and colon cancer cells.[9–13] Neuronal nAChRs consist of a homomeric pentamer (α7, α9, α10) or a heteromeric pentamer with 2 different types of subunits (α and β). Among neuronal nAChRs, α7-nAChR is of interest in lung cancer therapy[11] because NNK is a high-affinity agonist for α7-nAChR.[8] NNK not only promoted lung cancer cell survival and proliferation through α7-nAChR,[9,10] but also induced lung cancer cells migration through α7-nAChR.[11,12]

Colorectal cancer is the third most common cancer in both men and women in the United States and is the second leading cause of deaths from cancer.[14] Epidemiologic studies indicate that cigarette smoking is positively related to the formation of colorectal adenomatous polyps, which are a precursor of colorectal cancer and a risk factor for recurrent colorectal polyps.[15,16] Positive associations between colorectal cancer risk and cigarette smoking were also reported.[17–19] In addition, long-term cigarette smoking increased the risk of colorectal cancer mortality in both men and women.[20,21] Cigarette smoke extract promoted colon cancer growth, through activation of cell proliferation and angiogenesis.[22] Additionally, NNK was reported to stimulate colon cancer growth via α7-nAChR.[13]

Clinically, colorectal cancer can be surgically cured when the cancer is confined to a primary site. Unfortunately, about 20% of colorectal cancer patients are initially diagnosed with distant metastasis,[14] the leading cause of death in cancer patients.[23] However, the effect of NNK exposure on colon cancer cell metastasis remains to be determined. Here, we demonstrated that NNK enhanced the migration of HT29 and DLD-1 colon cancer cells through α7-nAChR and the downregulation of adhesion molecule, E-cadherin, and the upregulation of its transcription repressors, Snail and ZEB1.

MATERIALS AND METHODS

Chemicals

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was purchased from ChemSyn Laboratories (Lenexa, KS) and α7-nAChR antagonist, methyllycaconitine, was purchased from Sigma-Aldrich (St Louis, MO).

Cell Culture

The cell line HT29 (HTB-38; American Type Culture Collection [ATCC], Rockville, MD) was isolated from Dukes' stage B human colon adenocarcinoma. The cell line DLD-1 (CCL-221; ATCC) was isolated from Dukes' stage C human colon adenocarcinoma. The cells were grown in RPMI 1640 supplemented with

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10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified incubator (37°C, 5% CO_2). The cells were either subcultured or used before they reached 80% confluence.

RT-Polymerase Chain Reaction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from colon cancer cells using the Trizol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). The cDNA was amplified from 2 μg of total RNA in a final volume of 20 μL using M-MLV (Moloney Murine leukemia virus) reverse transcription at 37°C for 90 minutes. The PCR primer sequences of α7-nAChR and the quantitative PCR primer sequences of α7-nAChR,[24] E-cadherin, Snail,[25] ZEB1[25] and β-actin are summarized in Table 1. The PCR conditions for the detection of mRNA expression were as follows: the template cDNA was first denatured at 95°C for 5 minutes. During 40 cycles of amplification, the denaturation step was at 95°C for 30 seconds, the annealing step at 58°C~60°C for 30 seconds and the extension step at 72°C for 30 seconds. The final extension step was at 72°C for 7 minutes. The annealing temperature was 58°C for α7-nAChR and 60°C for β-actin. The quantitative Real-Time (RT)-PCR reaction was carried out using a commercial SYBR Green reaction mix. Thermal cycling was performed in ABI StepOne (Applied Biosystems) and the quantitative PCR condition was: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

α7-nAChR Small Interfering RNA (siRNA) and Plasmid Construct

We ablated α7-nAChR expression in DLD-1 colon cancer cells with siRNA. The target sequences for the human *CHRNA7* mRNA (NM_000746) gene was 5′-GGACAGATCACTATTTACA-3′. The luciferase gene was used as a negative control target and the sequence of scrambled siRNA was 5′-CGTACGCGGAATACTTCGA-3′. After BLAST analysis to verify that there was no significant sequence homology with other human genes, the selected sequence was inserted into a BglII/HindIII-cut pSUPERIOR vector to generate the pSUPER-α7si nAChR and pSUPER-scramble vectors. All constructs were confirmed by DNA sequence analysis. The transfection protocol has been described previously.[26,27] Briefly, 1.5×10^5 cells were washed twice with phosphate buffered saline and mixed with 0.5 μg plasmid. We applied 1 pulse for a duration of 20 milliseconds under a fixed voltage of 1.4 kV on a pipette-type microporator MP-100 (Digital Bio, Seoul, Korea).

Proliferation Assay

Cell proliferation was estimated by MTT assay. HT29 and DLD-1 cells (1×10^4) were seeded into a 24-well plate and incubated overnight for attachment. They were then incubated with varying concentrations of NNK. At different time interval, the medium was aspirated. The remaining cells were further incubated with 0.25 mg/mL of MTT for 1 hour. MTT was extracted with dimethyl sulfoxide (DMSO), and the color change in the extract was measured at 550 nm by spectrophotometer (ELISA reader).

TABLE 1. Primer Sequence

Name	5′-Sequence-3′
α7-nAChR (PCR)	F: CGCAACCACTCCCGTCTACT
	R: GCAGTGCTGCACATCAAAGG
α7-nAChR	F: GCTGCTCGTGGCTGAGATC
	R: TGGCGAAGTACTGGGCTATCA
E-cadherin	F: CGGGAATGCAGTTGAGGTC
	R: AGGATGGTGTAAGCGATGGC
Snail	F: CGCGCTCTTTCCTCGTCAG
	R: TCCCAGATGAGCATTGGCAG
ZEB1	F: AGCAGTGAAAGAGAAGGGAATGC
	R: GGTCCTCCTCAGGTGCCTCAG
β-actin	F: AGCGCGGCTACAGCTTCA
	R: GGCCATCTCTTGCTCGAAGT

Migration Assay

In vitro cell migration was performed by the BD Falcon cell culture insert (BD Biosciences). 1×10^5 DLD-1 or HT29 cells suspended in 500 μL of serum free RPMI 1640 were seeded into the upper compartments of each chamber in the presence of DMSO or varying concentrations of NNK with or without receptor inhibitors, whereas the lower compartments were filled with 1 mL of RPMI 1640 with 10% fetal calf serum. After incubation for 24 hours at 37°C in 5% CO_2, the nonmigrating cells were removed from the upper surface of the membrane by scrubbing. Cells on the reverse side were stained with 0.1% crystal violet, and migrating cells were counted under a microscope at 100 × magnification.

Wound-Healing Assay

HT29 cells were seeded into a 6-well plate and allowed to grow to 80% confluence in RPMI 1640 and then the cell monolayers were wounded by a 10-μL pipette tip. The wounded monolayers were washed 4 times with phosphate buffered saline and incubated in serum free RPMI1640 containing DMSO or 10 μM NNK for 24 hours.

Statistical Analysis

All experiments were repeated a minimum of 3 times. All data collected from RT-PCR, cell proliferation, and migration assays were expressed as mean ± SD. The data presented in some figures are from a representative experiment, which was quantitatively similar in the replicate experiments. Statistical significance was determined with Student *t* test (2-tailed) comparison between 2 groups of data set. Asterisks shown in the figures indicate significant differences of experimental groups compared with the corresponding control condition ($P < 0.05$, mentioned in Figure legends).

RESULTS

The Expression Pattern of α7-nAChR in the Colon Cancer Cells and Human Colon Cancer Tissues

NNK can exhibit its biologic function by binding to the nAChRs[2] so the expression levels of nAChRs in HT29 and DLD-1 cells were checked by semi-quantitative RT-PCR and quantitative real-time PCR. The expected PCR product of α7-nAChR was obtained as 350 bp. α7-nAChR was identified in both DLD-1 and HT29 colon cancer cells (Fig. 1A), and the quantitative RT PCR revealed that the expression level of α7-nAChR was higher in DLD-1 (Fig. 1B). Additionally, we also tested 5 pairs of cancer tissue and adjacent normal tissue from patients with metastatic colorectal cancer and α7-nAChRs were detected in 4 cancer tissues (data not shown). These results suggested that α7-nAChR expression may play a role in the pathogenesis of colon cancer.

The Proliferation Effect of NNK on Colon Cancer Cells

To test the effect of NNK induced proliferation in colon cancer, the MTT assay was performed in DLD-1 and HT29 cells. Cells (1×10^4) were seeded in 24-wells plate and cultured with medium containing varying concentrations of NNK for 72 hours. As shown in Figure 2, treatment of NNK revealed no significant effect on the proliferation of DLD-1 (Fig. 2A) and HT29 (Fig. 2B) cells. Although NNK was reported to increase DNA synthesis in colon

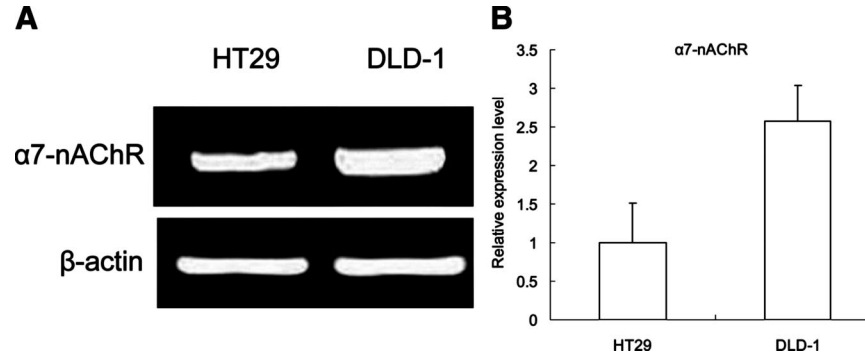

FIGURE 1. Expression pattern of α7-nAChR in colon cancer cells. Total RNA was extracted from HT29 and DLD-1 cells and analyzed by RT-PCR and quantitative PCR. β actin was used as an internal control. A, α7-nAChR was identified in both HT29 and DLD-1 cell lines. B, Quantitative analysis revealed DLD-1 cells with a higher expression level of α7-nAChR than HT29.

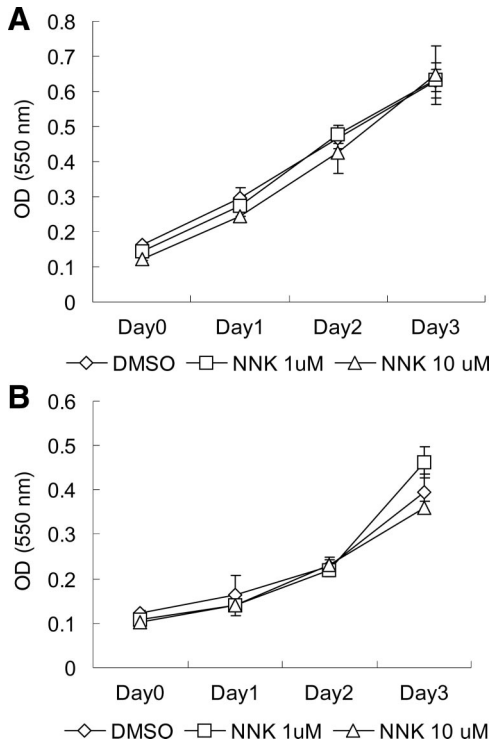

FIGURE 2. No influence on colon cancer cell proliferation by NNK. 1×10^4 DLD-1 and HT29 cells were cultured with complete medium containing varying concentrations of NNK in the 24-well plate. MTT assay was used to evaluate the proliferation. A, NNK on DLD-1 cell proliferation. B, NNK on HT29 cell proliferation.

cancer cells,[13,28] our results suggest that NNK in concentrations of 1 and 10 μM did not influence the proliferation of colon cancer cells.

NNK-Enhanced Migration Ability of Colon Cancer Cells

Presently, metastatic disease is still a major problem in the management of colon cancer. To know whether NNK influences the metastasis of colon cancer cells, the migration assay was performed by the transwell system (BD Biosciences) and wound-healing assay. DLD-1 and HT29 cells (1×10^5) were seeded in the upper chamber of filter inserts with serum free medium containing varying concentrations of NNK or DMSO as control. The migration ability was increased by NNK treatment in a dose-dependent manner in DLD-1 (Fig. 3A). The number of migrated cells increased dramatically in HT29 with NNK treatment compared with DMSO treatment (Fig. 3B). In addition, we also used a different assay system to confirm the enhancement of migration by NNK. The wound-healing assay was applied to study the effect of NNK on colon cancer cell migration. HT29 cells were cultured to reach a 80% confluent monolayer in 6-well plates and scratched with a 10-μL pipette tip. After incubation with DMSO or 10 μM NNK for 24 hours, the migration ability of HT29 was enhanced by NNK treatment (Fig. 3C).

α7-nAChR Mediated NNK-Enhanced Colon Cancer Migration

To verify the role of α7-nAChR in NNK-enhanced cell migration, methyllycaconitine, an antagonist of α7-nAChR, was applied in the migration assay. With the transwell system, 1×10^5 DLD-1 cells were seeded in the upper chamber of filter inserts containing serum free medium with different chemicals. For combined treatment, the cells were pretreated with 10 μM methyllycaconitine for 1 hour. After 24 hours, 10 μM NNK treatment significantly increased the number of migrated cells in comparison with DMSO treatment and the enhanced migration effect was reversed by methyllycaconitine. However, methyllycaconitine itself did not significantly influence the DLD-1 cells migration (Fig. 4A). To further evaluate the role of α7-nAChR in NNK-enhanced colon cancer cells migration, the siRNA technique was used to suppress the expression of α7-nAChR (Fig. 4B). NNK increased the migration ability of α7-nAChR scramble DLD-1 cells but the effect was not observed in the α7-AChR siRNA DLD-1 cells (Fig. 4C). According to these results, NNK-enhanced DLD-1 cell migration through α7-nAChR.

NNK-Enhanced Colon Cancer Migration by Downregulation of E-Cadherin and Upregulation of Snail and ZEB1

As epithelial to mesenchymal transition (EMT) is a major pathologic event in cancer metastasis and loss of E-cadherin is one of the hallmarks of EMT, we further evaluated the expression of E-cadherin in colon cancer cells after NNK treatment. As shown in Figure 5, NNK suppressed the expression of E-cadherin in DLD-1 cells (Fig. 5A) and HT29 cells (Fig. 5B). The downregulation of E-cadherin in DLD-1 cells by NNK was also reversed by methyllycaconitine (Fig. 5C). These results suggested that NNK-enhanced colon cancer cell migration by the downregulation of E-cadherin, in which α7-nAChR was involved. Because Snail and ZEB1 are 2 major transcriptional repressors of E-cadherin in colon cancer cells, we also studied the expression level of Snail and ZEB1 in DLD-1 cells. NNK increased the expression level of Snail (Fig. 5D) and ZEB1 (Fig. 5E) in DLD-1 cells which were reversed by methyllycaconitine. These results suggested that α7-nAChR was involved in NNK-suppressed E-cadherin expression and NNK-induced Snail

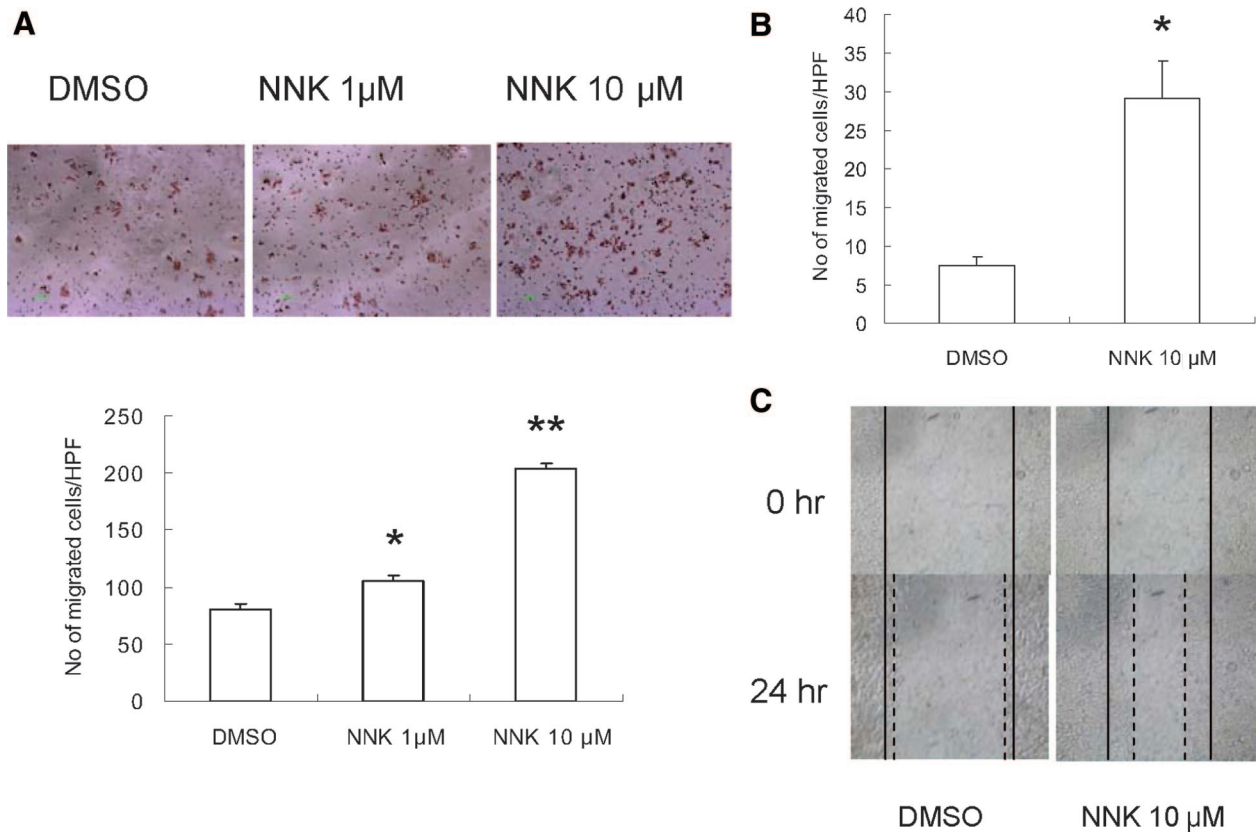

FIGURE 3. NNK-enhanced colon cancer cell migration. The Transwell system and wound-healing assay were used to evaluate migration ability. A, NNK-enhanced DLD-1 cell migration in a dose-dependent manner. B, The Transwell system demonstrated HT29 cell migration was enhanced by NNK. C, After 24 hours treatment of DMSO or 10 μM NNK, wounded gap of HT29 cells decreased 25% and 50% respectively. *NNK 1 μM versus DMSO: $P < 0.05$, **NNK 10 μM versus DMSO: $P < 0.05$.

and ZEB1 expression. For further confirmation, α7-nAChR siRNA DLD-1 cells were used. Treatment of NNK decreased E-cadherin expression (Fig. 6A) and increased Snail (Fig. 6B) and ZEB1 (Fig. 6C) expression in α7-nAChR scramble DLD-1 cells, but no significant changes were noted in the α7-nAChR siRNA DLD-1 cells. These results demonstrated that NNK enhanced the migration of colon cancer cells through α7-nAChR.

DISCUSSION

Colorectal cancer is the second leading cause of cancer deaths in United States[14] and the third leading cause in Taiwan. Approximately 20% of patients have distant metastases at the time of presentation.[14] Metastatic disease is the major cause of death in patients with colorectal cancer. Epidemiologic studies demonstrate that long-term cigarette smoking increases the risk of colorectal cancer mortality.[20,21] However, whether smoking induces colorectal cancer metastasis and the mechanism by which it does so remains elusive.

Smoking could induce cancer cell migration through α7-nAChR.[12,29,30] Thus, in the present study, the expression of α7-nAChR in HT29 and DLD-1 cells was investigated by both RT-PCR and real-time PCR and α7-nAChR was identified in the colon cancer cells. α7-nAChR was also identified in human colon cancer tissues and its expression level was found to be higher than in normal colon tissues.

NNK has been identified as the most potent carcinogen in cigarette smoking. Earlier studies demonstrated that the DNA synthesis is enhanced in HT-29 cell with a short-period treatment (4–5 hours) by NNK or nicotine.[28,31] However, our results did not see the enhancement effect in HT-29 cells after NNK treatment (Fig. 2B). There are some possible reasons to explain different results. First, our study used the MTT assay to detect the cell proliferation rate after NNK treatment instead of thymidine incorporation. As the sensitivity of thymidine incorporation is higher than MTT assay. That may be the reason why we did not see the NNK-enhanced effect in our result. Second, the NNK exposure time in earlier reports was 4 to 5 hours, but the time interval to detect the cell survival was 1, 2, and 3 days after NNK treatment in our study. However, the dynamic interaction of NNK to nicotinic receptor is still unclear. We did not know whether the interaction between NNK and nicotinic receptor or the stability of compounds (NNK) caused the different results or not. This may need more experiments to prove it.

Metastatic disease is the leading cause of death in patients with colorectal cancer. In the past, increased migration and invasion by NNK were only found in lung cancer cells.[12] In this study, we demonstrated that NNK also enhanced colon cancer cell migration in vitro by both the transwell system and wound-healing assay. The process of metastasis is thought to be the migration of individual cells that detach from a primary site, traveling through the circulation and seeding in distant organs.[32] The detachment of malignant tumor cells is in part characterized by the ability of a tumor cell to overcome cell-cell adhesion and to invade surrounding tissue[33]—this process has been termed EMT. Adhesion between vertebrate

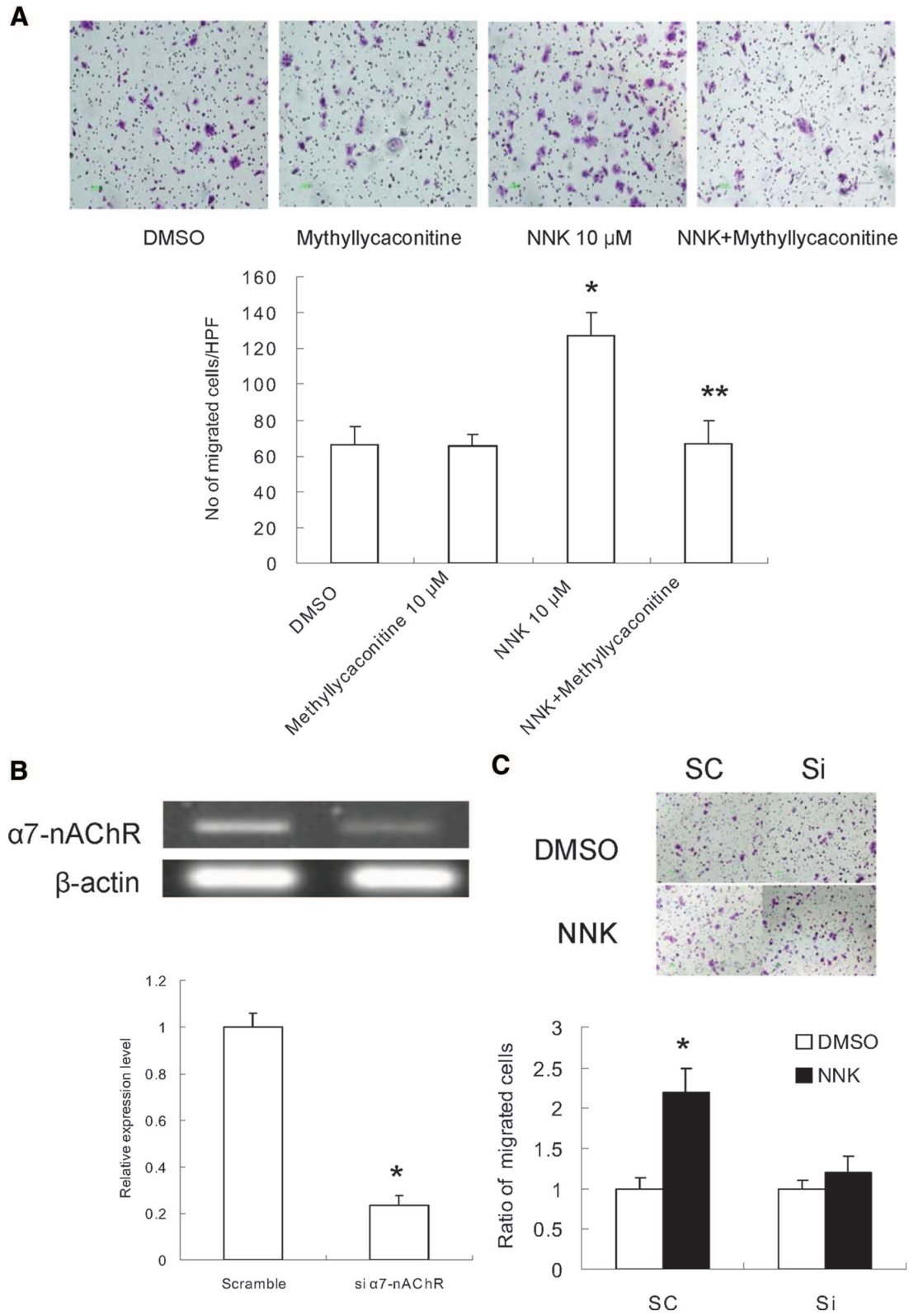

FIGURE 4. α7-nAChR mediated NNK-enhanced migration. α7-nAChR antagonist, Methyllycaconitine, and α7-nAChR siRNA were used to verify the role of α7-nAChR. A, NNK enhanced the migration ability of DLD-1 cells which was reversed by Methyllycaconitine, but Methyllycaconitine by itself did not influence the migration ability of DLD-1 cells. B, α7-nAChR siRNA suppressed 80% expression of α7-nAChR in DLD-1 cells. C, NNK-enhanced migration ability of DLD-1 cells and the effect was blocked by α7-nAChR siRNA.

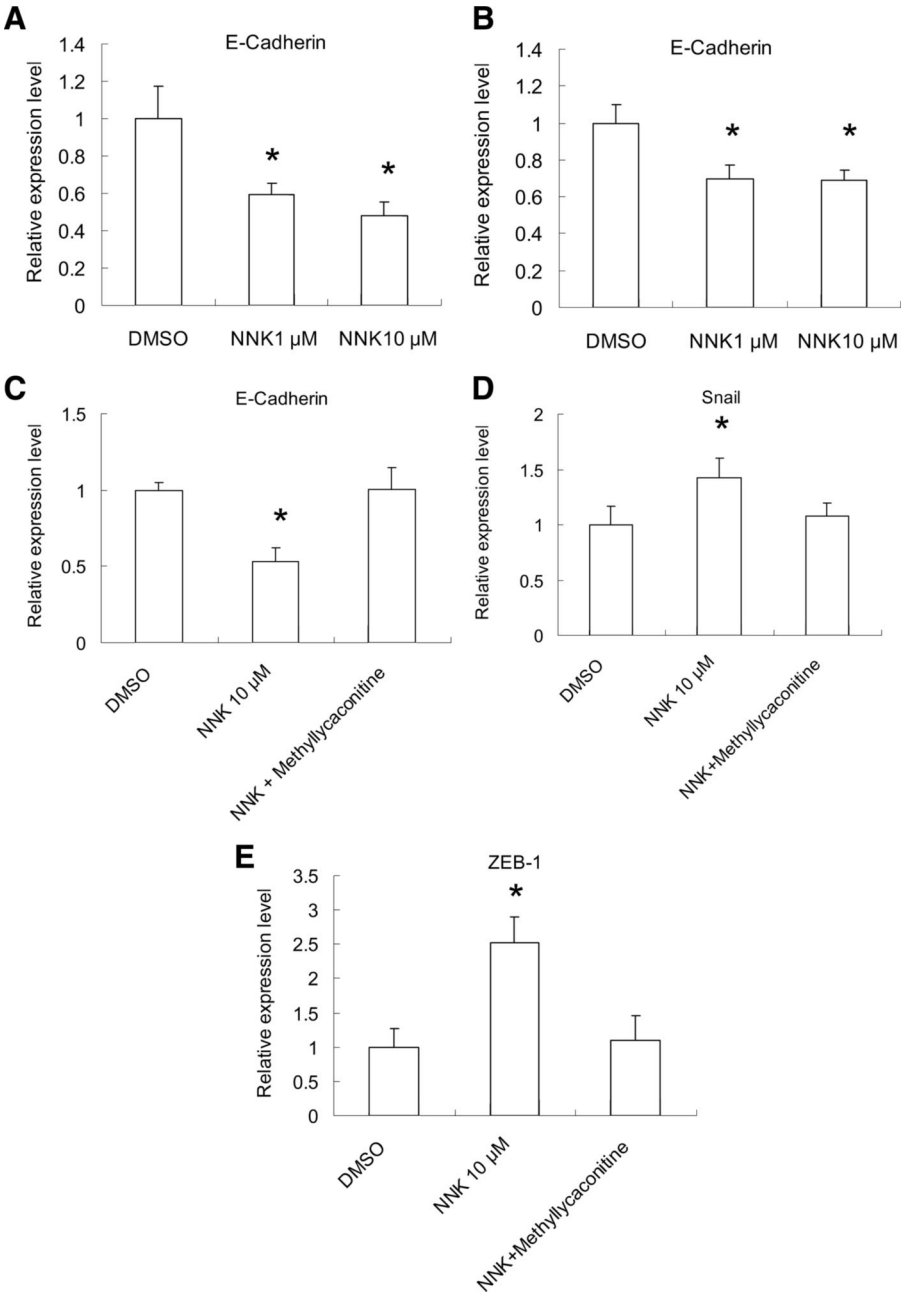

FIGURE 5. α7-nAChR antagonist Methyllycaconitine, reversed NNK-induced expression change of E-cadherin, Snail, and ZEB1. A, NNK suppressed E-cadherin expression in DLD-1 cells in a dose-dependent manner. B, NNK also suppressed E-cadherin expression in HT29 cells. C, NNK suppressed expression of E-cadherin in DLD-1 cells, which was reversed by α7-nAChR antagonist. D, Snail, a transcriptional repressor of E-cadherin, was induced by NNK and the effect was reversed by α7-nAChR antagonist. E, ZEB1, other transcriptional repressor of E-cadherin, was also induced by NNK, which was reversed by α7-nAChR antagonist.

cells is maintained by 3 types of adhesion junctions: adherens junctions, tight junctions, and desmosomes. Cadherins are the principal components of adherens junction in cell-cell contact in most solid tissue.[34] Among them, E-cadherin is the key player in cell polarity and organization in the epithelium. Since loss of E-cadherin was found to be the rate limiting step of pancreatic β-cell progression from adenoma to carcinoma and the formation of tumor metastasis,[35] loss of E-cadherin has become one of the hallmarks of EMT. In the present study, we demonstrated that NNK suppressed the expression level of E-cadherin in a dose-dependent manner, which might play a role in NNK-enhanced colon cancer cell migration.

To clarify the role of α7-nAChR in NNK-enhanced colon cancer cell migration, we used both a receptor antagonist and α7-nAChR siRNA, which blocked the NNK-enhanced migration of colon cancer cells and NNK-suppressed E-cadherin expression. These results confirmed the role of α7-nAChR.

Because the expression of E-cadherin was suppressed by 2 transcriptional repressors, Snail and ZEB1 in colon cancer,[36] we also studied the expression level of these 2 factors in colon cancer cells after NNK treatment. Again, these 2 factors were increased by NNK and the increased expression was blocked by α7-nAChR antagonist and siRNA. These results demonstrated that Snail and ZEB1 might play a role in NNK-suppressed expression of E-cadherin, which further influenced colon cancer cell migration.

In conclusion, our data for the first time demonstrated that NNK-enhanced colon cancer cell migration without influencing proliferation, mediated through the α7 nAChR. The enhanced

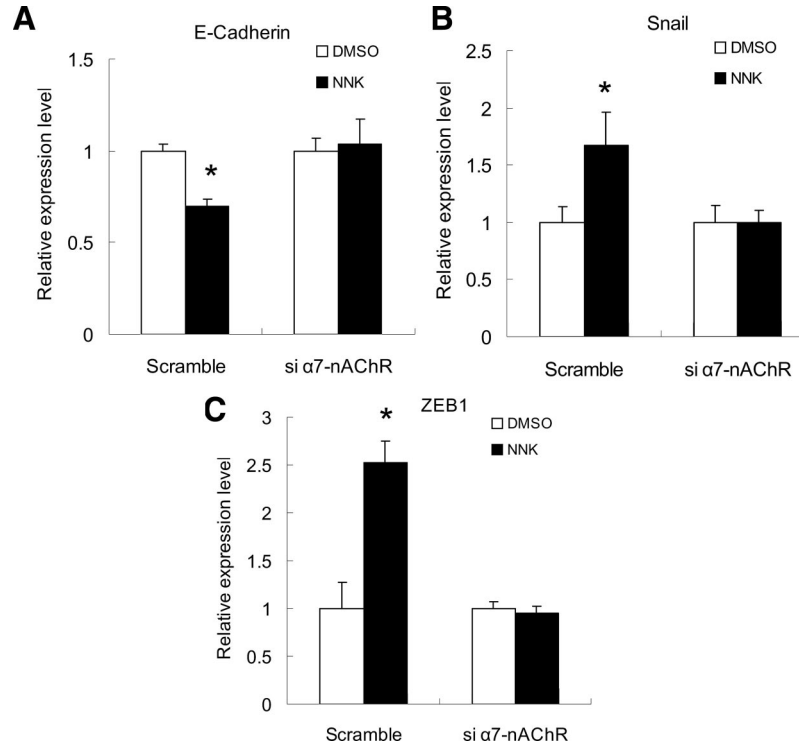

FIGURE 6. α7-nAChR siRNA blocked NNK-induced expression change of E-cadherin, Snail, and ZEB1. A, NNK suppressed E-cadherin expression in DLD-1 cells was blocked by α7-nAChR siRNA. B, Snail, a transcriptional repressor of E-cadherin, was induced by NNK and α7-nAChR siRNA blocked NNK-enhanced Snail expression. C, ZEB1, other transcriptional repressor of E-cadherin, was also induced by NNK in DLD-1 cells and the induction was blocked by α7-nAChR siRNA.

migration was caused by the downregulation of E-cadherin expression and upregulation of its transcription repressors, Snail and ZEB1.

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