

The role of nicotine in metastasis and anticancer drug resistance of head and neck squamous cell carcinoma

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Abstract. Epidermal growth factor (EGF) is overexpressed in many cancers and is associated with worse prognosis. EGF binds to its cell surface receptor (EGFR), which induces EGFR phosphorylation. Phosphorylated EGFR (p-EGFR) is translocated into the nucleus, which increases cancer cell activity. Nicotine, which is one of the main components of tobacco, is absorbed through pulmonary alveoli and mucosal epithelia in the head and neck region by smoking and moves into the blood. Nicotine in blood binds to nicotinic acetylcholine receptor (nAChR) in the central nervous system and serves a crucial role in tobacco addiction. Although nAChR localization is thought to be limited in the nervous system, nAChR is present in a wide variety of non-neuronal cells, including cancer cells. Recent studies suggest that nicotine contributes to the metastasis and resistance to anti-cancer drugs of various cancer cells. However, it remains unknown whether head and neck squamous cell carcinoma (HNSCC) cells can utilize nicotine-nAChR signaling to metastasize and acquire resistance to anti-cancer drugs, even though the mucosal epithelia of the head and neck region are the primary sites of exposure to tobacco smoke. To the best of our knowledge, the present study is the first to demonstrate the role of nicotine in metastasis and anti-EGFR-therapy resistance of HNSCC. The present findings demonstrated that nicotine increased proliferation, migration and invasion in HNSCC cells. It was also demonstrated that nicotine restored cetuximab-inhibited proliferation, migration and invasion of HNSCC cells. Finally, an *in vivo* experiment revealed that nicotine increased lymph node metastasis of xenografted tumors, whereas an nAChR inhibitor suppressed lymph node metastasis and p-EGFR nuclear localization of xenografted tumors. Taken together, these results demonstrated that nicotine induced nuclear accumulation of p-EGFR. These signaling pathways elevated the activities of HNSCC cells, causing lymph node metastasis and serving a role in cetuximab resistance.

Introduction

Epidermal growth factor (EGF) was isolated from mouse submaxillary glands and demonstrated to promote incisor eruption and eyelid opening in newborn rodents (1). After EGF binds to its cell surface receptor (EGFR), EGFR undergoes dimerization, which in turn induces EGFR auto-phosphorylation (2). This auto-phosphorylation elicits downstream signal transduction cascades such as the phosphoinositide 3-kinase-pyruvate dehydrogenase kinase-protein kinase B (Akt) and RAF-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase pathways, which promote cell survival and proliferation. Phosphorylated (p-) EGFR is translocated to the cytoplasm and degraded in lysosomes, which causes suppression of downstream signaling cascades in normal cells (2). However, in cancer cells, p-EGFR is accumulated in the nucleus, increasing cancer cell activity (3). EGFR is overexpressed in many cancers, including head and neck, breast, lung, colon, stomach, kidney,

prostate and ovarian cancer, and is associated with worse outcome (4-6). An anti-EGFR monoclonal antibody, cetuximab, is the first molecular target drug for head and neck squamous cell carcinoma (HNSCC) (7,8). When combined with radiotherapy and chemotherapy, cetuximab has been demonstrated to have significant survival benefits in patients with locally advanced and recurrent/metastatic HNSCC, respectively (7,8). However, resistance to cetuximab is known to develop gradually (9), and presents an important challenge for the future.

Tobacco smoking is associated with the carcinogenesis and development of cancer (10). Tobacco smoking is carcinogenic to the oral cavity, pharynx, larynx, esophagus, stomach, colon, liver, pancreas, lung, uterine cervix, ovary, kidney, renal pelvis and ureter, and bladder (10). The organs directly exposed to tobacco smoke are at high risk. Tobacco smoke is divided into a particle and gas phase (11). The particle phase includes tar and nicotine, while the gas phase includes carbon monoxide. Tar contains many carcinogenic chemicals, including benzo(a)pyrene and nitrosamine. Nicotine is responsible for tobacco addiction, and thus nicotine replacement therapy, which supplies nicotine in the form of gum or a patch, is used to help individuals with tobacco addiction quit smoking (12).

The nicotine in the tobacco smoke is absorbed across the epithelium of the lung, and the mucosal epithelia in the head and neck region, e.g., the oral cavity, nasal cavity, pharynx, nasopharynx and larynx (13). Nicotine exerts its cellular functions through nicotinic acetylcholine receptors (nAChRs). nAChRs are homomeric or heteromeric pentameric proteins consisting of $\alpha 1-10$, $\beta 1-4$, γ , δ and ϵ subunits, and are located in the central nervous system and neuromuscular junctions (14). The binding of nicotine to nAChRs in the ventral tegmental area of the midbrain induces the release of dopamine to the nucleus accumbens, which is involved in the rewarding effects of nicotine and nicotine addiction. Although nAChR localization is thought to be limited in the nervous system, nAChR is present in a wide variety of non-neuronal cells, including bronchial epithelial, urothelial, skin, endothelial and vascular smooth muscle cells (15). The mucosal epithelia of the head and neck region are the primary sites of exposure to tobacco smoke, and nAChRs have been observed in mucosal epithelial cells in these regions (16,17). Previous studies have suggested that nAChRs are also present in lung cancer, breast cancer, pancreatic cancer, colon cancer and head and neck cancer cells (15,18-21). In lung and breast cancer, nicotine has been demonstrated to contribute to tumor growth and metastasis (19,20). Nicotine also controls the expression and subcellular localization of EGFR in breast cancer cells (22). Specifically, nicotine decreases the expression of EGFR, and enhances the accumulation of p-EGFR in the nucleus, thereby increasing proliferation of breast cancer cells (22). Finally, in lung cancer cells, nicotine serves a role in the resistance against EGFR inhibitors (23,24).

These facts prompted us to explore whether HNSCC cells could utilize nicotine-nAChR signaling to metastasize

from the primary tumor to the regional lymph nodes and acquire resistance to cetuximab through EGFR activation. In the present study it was demonstrated that nicotine increased proliferation, migration, invasion, p-EGFR nuclear trans-location in HNSCC cells. It was also demonstrated that nicotine restored cetuximab-inhibited proliferation, migration and invasion of HNSCC cells. Finally, it was demonstrated that an nAChR inhibitor suppressed lymph node metastasis in a mouse model of lymph node metastasis using OSC-19 cells.

Materials and methods

Cell culture and reagents. The following human HNSCC cell lines were used in the present experiments: HSC-2, a mouth floor SCC cell line derived from a metastatic cervical lymph node; HSC-3, a tongue SCC cell line derived from a metastatic cervical lymph node; OSC-19, a tongue SCC cell line derived from the primary site; and OSC-20, a tongue SCC cell line derived from a metastatic cervical lymph node. HSC-2 and HSC-3 were obtained from the Cell Engineering Division of the RIKEN BioResource Center (Ibaraki, Japan). OSC-19 and OSC-20 were obtained from the Health Science Research Resources Bank (Osaka, Japan). Human umbilical vein endothelial cells (HUVECs) were purchased from Takara Bio, Inc. (Otsu, Japan). All cancer cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C. HUVECs were cultured in endothelial cell growth medium-2 (Takara Bio, Inc.) at 37°C. Nicotine (Sigma-Aldrich; Merck KGaA; 0.5 μ M) (19,20,22), mecamylamine hydrochloride (MCA; Sigma-Aldrich; Merck KGaA; 50 nM) (22), α -bungarotoxin (α -BTX; Abcam, Cambridge, UK; 1 μ M) (17) and cetuximab (Merck KGaA; 0.5 μ g/ml) (27) were purchased. The concentration of nicotine (0.5 μ M) used was decided based on previously published basic studies (19,20,22) and clinical investigations in which the concentrations of nicotine in the bloodstream of smokers exposed to nicotine at pharmacological concentrations were reported to be 0.09-1 μ M (25,26).

Cell proliferation. Cells were seeded at a density of 1×10^5 cells in a 6-well plate. After becoming subconfluent at 37°C, the cells were cultured for 24 h in DMEM/F-12 without FBS. They were then cultured in the presence or absence of nicotine (0.5 μ M) (19,20,22), MCA (50 nM) (22), α -BTX (1 μ M) (17) or cetuximab (0.5 μ g/ml) (27) in DMEM/F-12 supplemented with 0.5% FBS. The number of cells was counted with TC10TM automated cell counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after 5 days.

Invasion and migration assays. Invasion and migration of cells were studied as reported previously, by using Boyden chambers with or without Matrigel[®], respectively (BD Biosciences, Franklin Lakes, NJ, USA) (28). Cells in the logarithmic growth phase were detached by trypsin-EDTA, and 3×10^4 cells in serum-free DMEM/F12 were added to polycarbonate membranes (pore size, 8.0 μ m). Nicotine (0.5 μ M) was added to the lower chamber, and the system was incubated at 37°C for 24 h in 5% CO₂. Following incubation and fixation with 70% ethanol for 10 min at room temperature, the non-invading or migrating cells were removed with a

cotton swab and the remaining cells were stained with 2% crystal violet (Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. The number of stained cells on the lower side of the membrane in 4 light microscopic fields were counted, and the mean value of 3 wells was determined.

Immunoblot analysis. HUVEC, HSC-2, HSC-3, OSC-19 and OSC-20 cells in monolayer cultures were rinsed with ice-cold PBS and lysed in an ice-cold lysis buffer (50mM Tris-HCL, pH7.4, containing 150mM NaCl, 1% Triton X-100, 1% NP-40, 10mM NaF, 100mM leupeptin, 2mg/ml aprotinin and 1mM phenylmethyl sulfonyl fluoride). Protein concentration was determined using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Inc.) The cell lysates containing 10 μ g total protein in the lysis buffer were electrophoresed in 12% SDS-PAGE gels, and the proteins were then transferred to nylon membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA). The membranes were blocked with 2% non-fat dry milk in TBS at 4°C for 1h and then incubated with a 1:400 dilution of rabbit anti-human $\alpha 7$ nAChR polyclonal IgG (ab10096; Abcam) and with a 1:1,000 dilution of mouse anti-human β -actin monoclonal IgG (ab49900) overnight at 4°C. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (RPN4301) or sheep anti-mouse IgG (NA931) were used as the secondary antibody at a 1:1,000 dilution (GE Healthcare Life Sciences, Little Chalfont, UK). Bands were visualized via enhanced chemiluminescence (RPN2109; GE Healthcare Life Sciences).

Lymph node metastasis model. All animal experiments were approved by the Institutional Animal Care and Use Committee of Okayama University (Okayama, Japan; OKU-2016046). The lymph node metastasis model was prepared as described previously (29). A total of 50 male athymic mice (nu/nu; age, 5 weeks; mean body weight, 19.5 g) were obtained from CLEA Japan, Inc. (Tokyo, Japan). OSC-19 cells, 8×10^5 per mouse, were inoculated into a hind footpad. Groups of 10 mice each were peritoneally injected with either PBS, nicotine (30 μ g/mouse) (20), MCA (20 μ g/mouse) (30), nicotine and MCA, cetuximab (1 mg/mouse) (31), or cetuximab and nicotine every day. Tumor sizes and body weights were measured weekly, and the former were recorded in mm³ (length x width² / 2). Mice were sacrificed at day 42, the footpad tumor tissues and popliteal lymph nodes were isolated and slides were prepared as described previously (29).

Immunohistochemistry for xenograft tumor specimens. Paraffin blocks of specimens were cut at 4- μ m thickness. The sections were deparaffinized, and then autoclaved in 0.2% citrate buffer for 15 min for antigen retrieval. Sections were incubated with 3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity. Immunohistochemistry was performed using rabbit anti-human EGFR monoclonal IgG (Cell Signaling Technology, Inc.) and rabbit anti-human p-EGFR monoclonal IgG (Abcam), each at 1:100 dilution. The sections were incubated with the primary antibodies at 4°C for 16 h, and then treated with Envision System Labeled Polymer (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 60 min at a dilution of 1:100. The immunoreaction was visualized using a 3,3'-diaminobenzidine substrate-chromogen solution. Finally, the sections were immersed in an ethanol and xylene bath and mounted for examination. The extent of p-EGFR staining

was evaluated according to the percentage of cells with strongly stained nuclei in 3 visual fields under a light microscope (x200). All immunohistochemistry studies were evaluated by 2 experienced observers who were blind to the conditions of the experiments as reported previously (32).

Statistical analysis. Data were analyzed by using unpaired Student's t-test for analyses between two groups, and one-way analysis of variance with Bonferroni post hoc tests for the analysis of multiple group comparisons using SPSS

statistical software (version 22; IBM Corp., Armonk, NY, USA). Results were expressed as the mean \pm standard deviation. To control for multiple testing for data in the incidence of popliteal lymph node metastasis in mice, q-values were calculated with the false discovery rate method controlled by the Benjamini-Hochberg procedure. $P < 0.05$ and $q < 0.05$ were considered to indicate statistically significant differences.

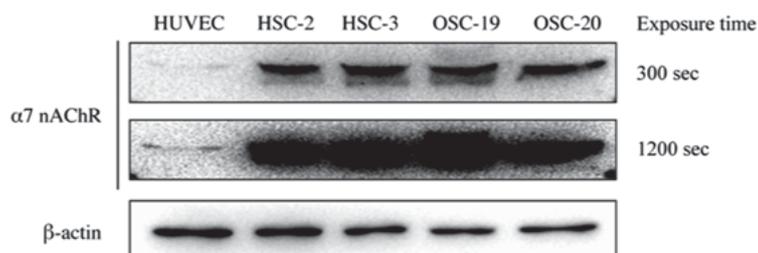


Figure 1. Effects of nicotine on the cell proliferation and expression of $\alpha 7$ nAChR of head and neck squamous cell carcinoma cells. Expression of $\alpha 7$ nAChR in HSC-2, HSC-3, OSC-19 and OSC-20 cells. HUVECs were used as a positive control of $\alpha 7$ nAChR. Data are presented as the mean \pm standard deviation of triplicates from a typical experiment. * $P < 0.05$. nAChR, nicotinic acetylcholine receptor; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell.

Results

Nicotine receptor was expressed in HNSCC cells. It is that Nicotine is known to exert its cellular functions through nicotinic acetylcholine receptors (nAChRs), whether nicotine receptors were expressed in HNSCC cells was firstly examined. Among nicotinic acetylcholine receptors, $\alpha 7$ nAChR has been reported as the primary receptor that mediates the proliferative effects of nicotine in various cancer cells (18). In the present study, it was demonstrated that all four HNSCC cell lines exhibited stronger $\alpha 7$ nAChR expression than that of the HUVEC used as the positive control for $\alpha 7$ nAChR (Fig. 1).

Nicotine upregulated cell activities and nAChR inhibitors suppressed the effects of nicotine in HNSCC cells. The effects of nicotine and nAChR inhibitor on HNSCC cells were evaluated. As HSC-3 and OSC-19 have been reported to metastasize to the lymph nodes (33,34), it was decided to use these cell lines in later experiments. HSC-3 and OSC-19 were treated with MCA (a non-selective nAChR inhibitor) and α -BTX (an $\alpha 7$ nAChR inhibitor) in the presence of nicotine. Nicotine increased cell viability 1.3-fold in both HSC-3 and OSC-19 cells. MCA and α -BTX inhibited nicotine-induced viability to the same level as in the control group (Fig. 2A). In regard to cell migration, nicotine increased cell migration 1.4- and 1.2-fold in HSC-3 and OSC-19 cells, respectively, and MCA and α -BTX suppressed these nicotine-induced effects (Fig. 2B). Finally, invasion of HSC-3 and OSC-19 cells was increased 1.4- and 1.3-fold by nicotine, respectively, and MCA and α -BTX counteracted these effects as well (Fig. 2C).

Nicotine counteracted the anti-tumor effects of cetuximab in HNSCC cells. Nicotine contributes to resistance to anti-cancer drugs of various cancer cells. In lung cancer cells, nicotine serves a role in the resistance against EGFR inhibitors. It was speculated that Cancer cells can utilize nicotine-nAChR signaling to acquire resistance to cetuximab, which is first molecular target drug for HNSCC. Cetuximab-induced skin reactions are suppressed by cigarette smoking in patients with advanced colorectal cancer. To test this hypothesis, HNSCC cells were treated with cetuximab and nicotine, then the cell activities were evaluated. In the presence of cetuximab alone, the proliferation of HSC3 and OSC-19 cells was significantly inhibited. When nicotine was added, however, the proliferation of both HSC-3 and OSC-19 cells increased to 1.4-fold of that in the group treated with cetuximab alone (Fig. 3A). Cetuximab also significantly inhibited the cell migration of HNSCC cells. However, when nicotine was added, the cell migrations of HSC-3 and OSC-19 cells were increased to 1.7- and 1.3-fold of those in the cells treated with cetuximab alone (Fig. 3B). In a similar manner, the levels of invasion of HSC-3 and OSC-19 cells treated with cetuximab and nicotine were 1.9- and 1.6-fold of those in the cells treated with cetuximab alone, respectively.

An nAChR inhibitor suppressed the tumor growth and lymph node metastasis of xenografted HNSCC in athymic mice. To further investigate whether nicotine was responsible for tumor growth and metastasis by HNSCC cells, an animal model of lymph node metastasis was utilized (29). To inhibit nAChRs, only MCA was used in animal experiments. MCA (35) is well-known as an antihypertensive drug under the name Inversine[®] (36), whereas α -BTX is famous as a snake venom (37); therefore, MCA was speculated to be more suitable for future clinical application.

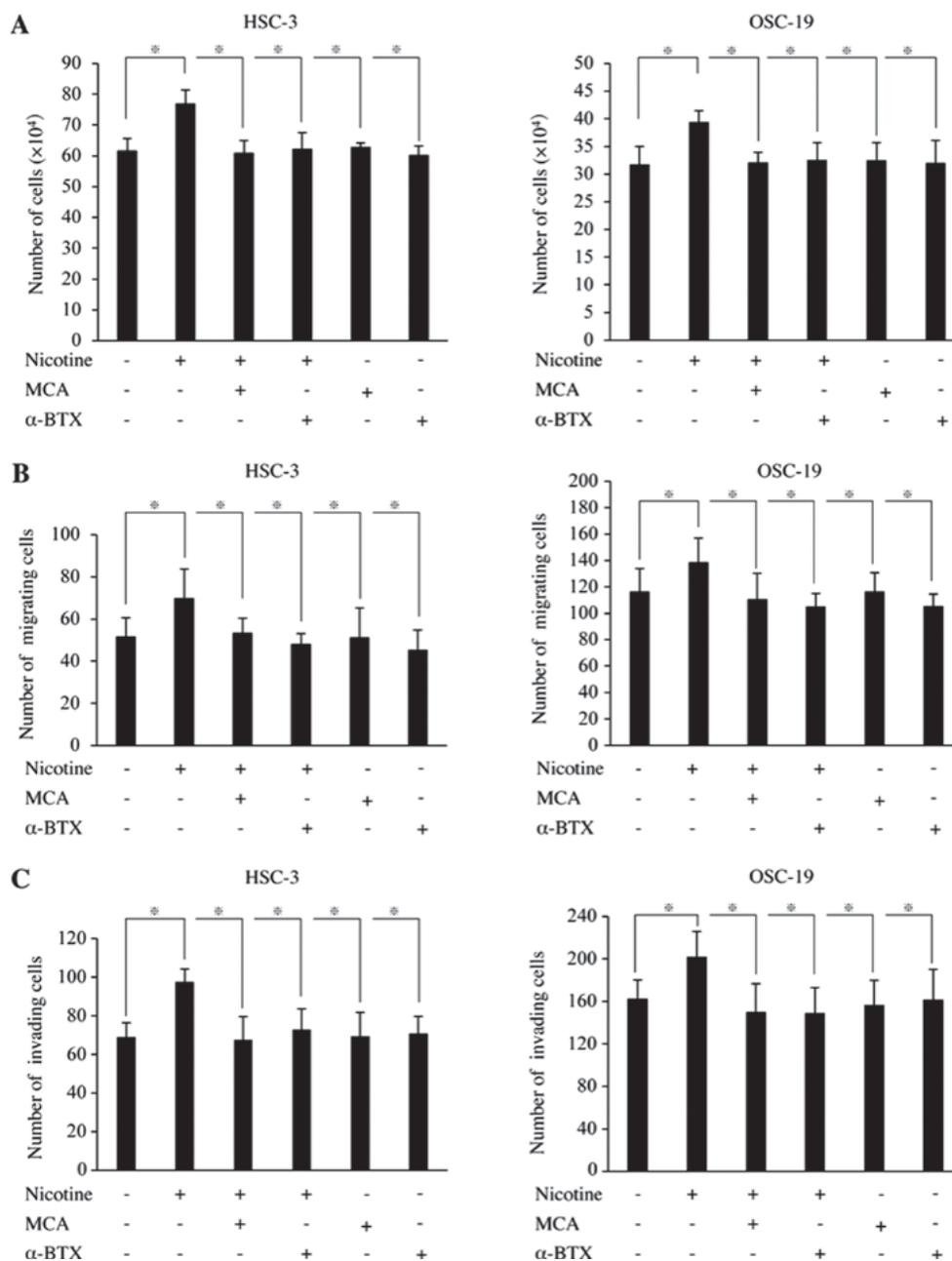


Figure 2. Effects of nicotine and nAChR inhibitors on the cell viability, migration and invasion of HNSCC cells. (A) Viability. Cells were seeded at a density of 1×10^5 cells in a 6-well plate. After becoming subconfluent, cells were cultured for 24 h in DMEM/F-12 without FBS. They were then cultured in the presence or absence of nicotine, MCA or α -BTX in DMEM/F-12 supplemented with 0.5% FBS. The cell number was measured at day 5. (B) Migration. Migration was evaluated using Boyden chambers. Cells were seeded at a density of 3×10^4 in medium with or without MCA, α -BTX or cetuximab on polycarbonate membranes. Nicotine was added to the lower chamber, and the system was incubated for 24 h. Following incubation, the number of cells on the lower side of the membrane was counted. (C) Invasion. Invasion was evaluated using Boyden chambers with Matrigel[®]. Incubation and cell counting were performed as in the migration assay. All experiments were repeated 3 times. Data are presented as the mean \pm standard deviation of triplicates from a typical experiment. * $P < 0.05$. nAChR, nicotinic acetylcholine receptor; HNSCC, head and neck squamous cell carcinoma; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture; FBS, fetal bovine serum; MCA, mecamylamine hydrochloride; α -BTX, α -bungarotoxin.

In the animal experiments, nicotine increased the growth rate of xenografted tumors compared with the rate in the control group, and MCA decreased tumor growth compared with the nicotine-treated group (Fig. 4A). The tumor volumes at the end of the experiment (day 42) for the control group, nicotine-treated group and MCA group were 615.2 ± 65.3 , 950.0 ± 188.9 and 686.5 ± 156.4 mm³, respectively. These results indicated an $\sim 54.5\%$ increase in the tumor growth rate for

the nicotine-treated group. The findings also suggested that MCA effectively inhibited the nicotine-induced xenograft tumor growth of HNSCC cells in athymic mice to the same level as in the control group (Fig. 4A). Furthermore, nicotine increased cetuximab-suppressed xenografted tumor growth from 1.2 ± 1.8 to 32.9 ± 37.7 mm³, suggesting that nicotine may contribute to local relapse following anti-EGFR therapy.

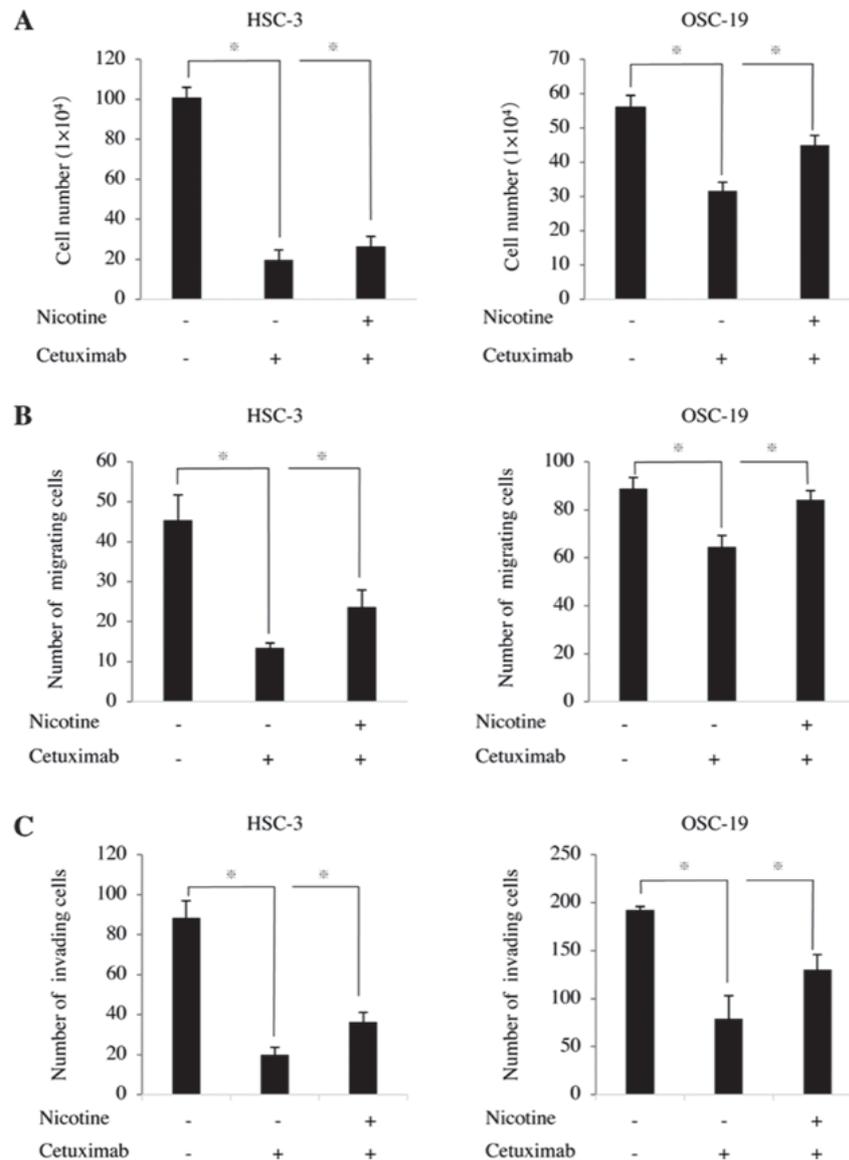


Figure 3. Effects of nicotine on cetuximab-suppressed viability, migration and invasion of head and neck squamous cell carcinoma cells. (A) Viability. Cells were seeded at a density of 1×10^5 cells in a 6-well plate. After becoming subconfluent, cells were cultured for 24 h in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture without fetal bovine serum. They were treated with cetuximab for 30 min, and then cultured in the presence or absence of nicotine. The cell number was measured at day 6. (B) Migration. Migration was evaluated using Boyden chambers. Cells were seeded at a density of 3×10^4 in medium with cetuximab on polycarbonate membranes. Nicotine was added to the lower chamber, and the system was incubated for 24 h. Following incubation, the number of cells on the lower side of the membrane was counted. (C) Invasion. Invasion was evaluated using Boyden chambers with Matrigel[®]. Incubation and cell counting were performed as in the migration assay. All experiments were repeated 3 times. Data are presented as the mean \pm standard deviation of triplicates from a typical experiment. * $P < 0.05$.

To determine whether nicotine influences p-EGFR localization *in vivo*, the percentage of p-EGFR-positive nuclei in the tumor specimens was evaluated. As presented in Fig. 4B, the percentage of p-EGFR-positive nuclei was increased in the nicotine-treated group ($57.7 \pm 8.4\%$) compared with the control group ($42.6 \pm 9.9\%$), which was consistent with the *in vitro* analysis. Conversely, MCA decreased the percentage of p-EGFR-positive nuclei to the level of the control group ($46.8 \pm 9.6\%$). Nicotine also increased the rate of popliteal lymph node metastasis from 10 to 60%. MCA decreased the rate of metastasis to 20%. Although there were no significant differences between the any two groups, there was a trend toward higher metastasis rate with nicotine compared with control. (control vs. nicotine, $q=0.057$; nicotine vs. nicotine+MCA, $q=0.159$; nicotine+MCA vs. control, $q=1.593$; Fig. 4C). There was no lymph node metastasis in either the cetuximab-treated

group or the cetuximab and nicotine-treated group. Taken together, the *in vivo* experiments revealed that nicotine increased the tumor growth and lymph node metastasis of HNSCC, whereas MCA suppressed them. It was also demonstrated that nicotine restored the cetuximab-inhibited tumor growth of HNSCC. The activation and nuclear localization of EGFR may contribute to these tumor-promoting effects of nicotine.

Discussion

Nicotine, one of the crucial components in the addictiveness of tobacco, also has important roles in the invasion and metastasis of various cancers, including lung cancer, breast cancer, glioma, bladder cancer, pheochromocytoma and colorectal cancer (15,18). In lung cancer cells nicotine exposure

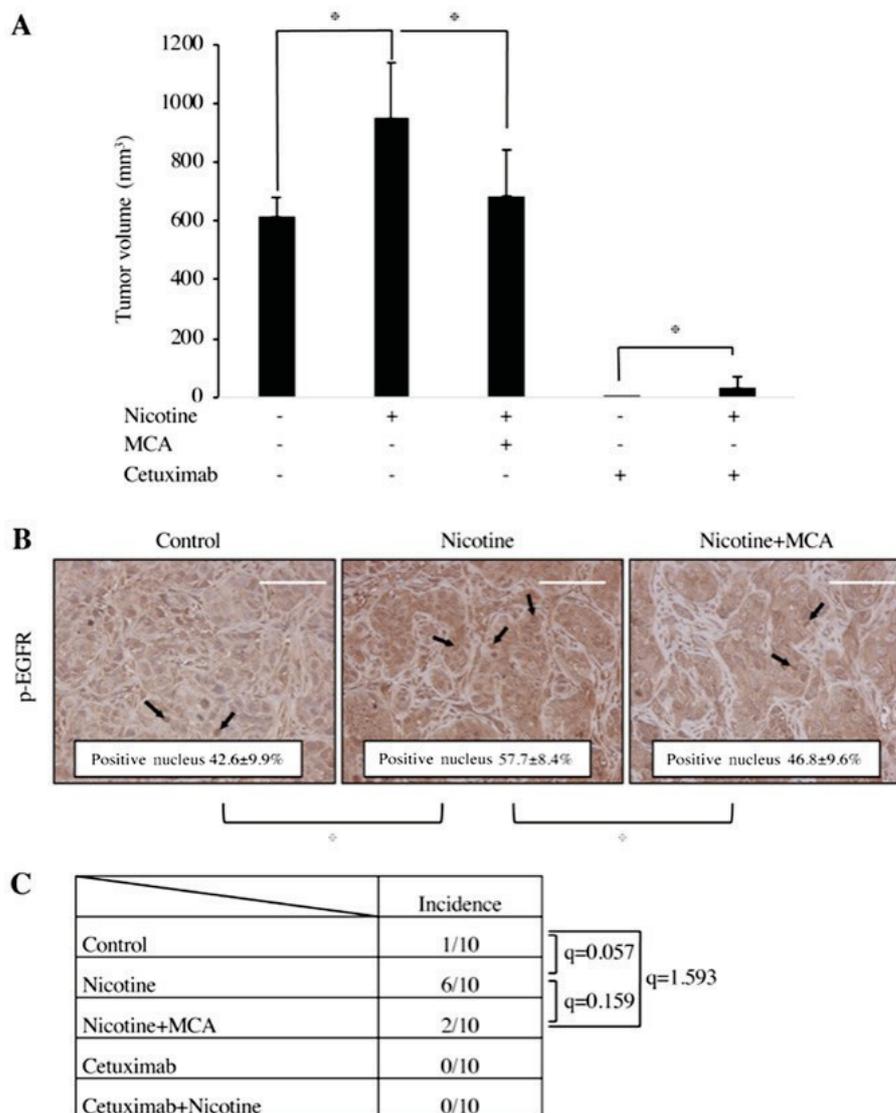


Figure 4. Effect of nicotine, MCA and cetuximab on the growth of OSC-19 xenografts and lymph node metastasis in athymic mice. (A) A total of 8×10^5 OSC-19 cells per mouse (10 mice/group) were inoculated into the hind footpad of athymic mice. Mice were peritoneally administered with PBS, nicotine (30 $\mu\text{g}/\text{mouse}$), MCA (20 $\mu\text{g}/\text{mouse}$), nicotine and MCA, cetuximab (1 mg/mouse), or cetuximab and nicotine every day. Mice were sacrificed at day 42. In bar chart, the data are presented as tumor volume in mm^3 (length \times width² / 2). Data are presented as the mean \pm standard deviation. * $P < 0.05$. (B) IHC staining of OSC-19 xenograft tumor specimens from the hind footpad. IHC staining of EGFR and p-EGFR in tumors from the OSC-19 treated with or without nicotine or MCA is presented. Nicotine induced nuclear localization of p-EGFR (arrows) whereas MCA suppressed nicotine-induced nuclear localization of p-EGFR. Scale bar, 100 μm . (C) The rate of popliteal lymph node metastasis is presented as the number of mice with metastases/number of mice injected. Lymph node metastasis was observed in the control group (10%), nicotine-treated group (60%) and nicotine and MCA-treated group (20%). MCA, mecamylamine hydrochloride; IHC, immunohistochemical; EGFR, epidermal growth factor receptor; p, phosphorylated.

induces epithelial-mesenchymal transition (38). In breast cancer cells, nicotine promotes cell motility via PKC and cdc42, leading to lung metastasis (20). However, its effect in HNSCC cells is unknown. To determine whether nicotine influences HNSCC *in vivo*, a mouse model of lymphatic metastasis was prepared (29). In this model, nicotine increased the tumor volume and incidence of metastasis in regional lymph nodes compared with the control groups. Consistent with the *in vivo* experiments, in *in vitro* studies, cell proliferation, migration and invasion were significantly elevated in the nicotine-treated groups than the control groups in all four HNSCC cell lines. In the animal model, nicotine failed to induce lymphangiogenesis in tumor tissues (data not shown). This suggests that nicotine-induced lymphatic metastasis was attributable to nicotine-driven invasive cell activities. Conversely, MCA and α -BTX, two nAChR

inhibitors, reversed the nicotine-driven invasive cell activities, suggesting that nicotine stimulation of cell activities occurs through nAChRs. The head and neck mucous epithelia express $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\beta 2$ and $\beta 4$ nAChRs (16,17). Among these nAChR subunits, $\alpha 7$ has been implicated as the most powerful nAChR subunit in terms of mediating the proliferative effects of nicotine in various cancer cells (18). Furthermore, nicotine activates $\alpha 7$ nAChR of keratinocytes in the head and neck region (17,39,40). These findings indicate that nicotine accelerates cell activity of HNSCC cells via $\alpha 7$ nAChR. This is consistent with the present *in vitro* finding that MCA and α -BTX inhibited nicotine-increased cell proliferation, migration and invasion to the level of the control groups. Cetuximab, a chimeric human mouse anti-EGFR monoclonal antibody, was the first molecular target drug used in head and neck cancer therapy (7,8). In our experiments,

nicotine restored the cetuximab-inhibited cell proliferation, migration and invasion of HNSCC cells. Nicotine is likely to attenuate the anti-tumor effect of cetuximab. However, we note here that cetuximab resistance might also arise through another mechanism, since nicotine failed to restore the cetuximab-inhibited cell activities to the level seen in the untreated control groups.

To date, no study has examined the direct effect of nicotine on cetuximab sensitivity, to the best of our knowledge. However, a few studies have demonstrated the effect of nicotine on anti-EGFR cancer therapy. In non-small cell lung cancer, nicotine induces resistance to erlotinib and gefitinib, which are EGFR tyrosine kinase inhibitors (23,41). In colorectal cancer, cigarette smoking during anticancer treatment with a cetuximab-based regimen reduces the therapeutic benefit (42). These findings support the present hypothesis that nicotine serves a role in cetuximab resistance.

In an *in vivo* experiment, cetuximab inhibited metastasis. However, nicotine failed to restore the cetuximab-inhibited metastasis. Therefore, the effect of nicotine in metastatic HNSCC treated with cetuximab remains unclear, and further investigation of this effect is required. In human papillomavirus-positive oropharyngeal cancer, increased distant metastases rates were noted in active smokers vs. never/former smokers (22 vs. 5%), and cetuximab-based bio-radiotherapy (BRT) vs. cisplatin-based chemoradiotherapy (CRT) (23 vs. 5%) (43). Although there was no difference in nodal disease extent between CRT and BRT groups in that previous study, nicotine and cetuximab may have synergistic negative effects in certain types of HNSCC.

In conclusion, nicotine-nAChR signaling pathways elevated the cell activities of HNSCC cells, causing lymph node metastasis and serving a role in cetuximab resistance. Blockade of nicotine-nAChR signaling may be of clinical benefit in cases of advanced HNSCC, especially HNSCC in the oral cavity, which is the primary site of exposure to nicotine, by inhibiting lymph node metastasis and releasing cetuximab resistance.

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