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Nicotine increases cancer stem cell population in MCF-7 cells

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ABSTRACT

Epidemiological studies have suggested that cigarette smoking is related to increased breast cancer risk. Nicotine is most likely related to the risk in cigarette smoking. However, the mechanisms by which nicotine promotes cancer development are not fully understood. It has recently been suggested that development of breast cancer are originated from cancer stem cells, which are a minor population of breast cancer. In the present study, we investigated the effects of nicotine on the population of cancer stem cells in MCF-7 human breast cancer cells, using flow cytometry with a cancer stem cell marker aldehyde dehydrogenase (ALDH). We found that nicotine increased ALDH-positive cell population in a dose-dependent manner. We further demonstrated that a PKC-Notch pathway is involved in the effect of nicotine. In addition, the effect of nicotine was blocked by treatment with the α7 subunit-selective antagonist of nicotinic acetylcholine receptors (nAChR) α-Bungarotoxin. These data suggest that nicotine increases the stem cell population via α7-nAChR and the PKC-Notch dependent pathway in MCF-7 cells. These findings reveal a relationship between nicotine and the cancer stem cells in human breast cancer.

1. Introduction

Epidemiological studies have suggested that smoking is related to increased breast cancer risk [1]. Nicotine is considered to be most likely related to the risk in cigarette smoking [2]. Nicotine exerts its cellular functions through nicotinic acetylcholine receptors (nAChRs), which are heterodimers of nine different types of α subunits (α2–α10) and three kinds of β subunits (β2–β4). Recent studies have shown that nAChRs are present in a variety of cells, such as cancer cells, brain, and vascular smooth muscle cells [2–4]. However, little is known about the relationship between nicotine and the increased risk of breast cancer.

There is increasing evidence that many types of cancer, including breast cancer, are initiated from and maintained by a small population of cancer stem cells [5,6]. This minor population produces the cancer bulk through continuous self-renewal and differentiation, which contributes to the cancer cellular heterogeneity. Aldehyde dehydrogenase (ALDH), which is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, has been recently used as a functional marker for identification of cells with enhanced tumorigenic/metastatic potential [7–9]. In addition, ALDH-positive cells from breast tissue can be used as a predictor of clinical outcome in breast cancer patients [10].

The self-renewal behavior of cancer stem cells has been reported to be mediated by several signaling pathways, such as Notch, Hedgehog, and Wnt [11–13]. Notch might be important for breast cancer stem cells. Transgenic mice overexpressing isoforms of Notch, a signaling pathway active in stem cells, are more prone to develop mammary tumors [14]. Furthermore, the expression level of Notch-1 and Jagged-1 has been shown to correlate with poor prognosis [15]. Aberrant Notch-1 activation was reported in breast carcinomas of various subtypes [16].

In the present study, we investigated the effects of nicotine on the population of cancer stem cells in MCF-7 human breast cancer cells, using flow cytometry with the cancer stem cell marker aldehyde dehydrogenase (ALDH). We report here that nicotine increases stem cell population in MCF-7 cells. In addition, our data suggest that the effect of nicotine is mediated via the α7 subunits of nAChR and the PKC-Notch dependent pathway in MCF-7 cells. These findings might explain the development of breast cancer in cigarette smokers.

2. Materials and methods

2.1. Materials

Nicotine was from Wako Pure Chemicals (Osaka, Japan). Mecamylamine hydrochloride (MCA) and PHA543613 (*N*-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]furo[2,3-c]pyridine-5-carboxamide) were from Sigma–Aldrich (St. Louis, MO, USA). α-Bungarotoxin was from Tocris Bioscience (Bristol, UK). GF109203X (3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-

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dione monohydrochloride) and DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) were from Enzo Life Sciences (Farmingdale, NY, USA). All other reagents were of analytical grade and obtained from commercial sources.

2.2. Cell culture

MCF-7 cells were provided from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM, #D6046, Sigma–Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries, Ashrat, Israel), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Invitrogen Corp., Carlsbad, CA, USA).

2.3. Aldefluor assay

The Aldefluor kit (Stem Cell Technologies, Durham, NC, USA) was used to detect stem cell population with high ALDH enzyme activity. Briefly, the cells were plated at 3×10^5 cells/100 mm dish. After serum deprivation for 3 days, the cells were suspended in Aldefluor assay buffer containing the ALDH substrate BAAA (1 μM) and incubated for 30 min at 37 °C. As a negative control, cells were treated with diethylaminobenzaldehyde (15 μM), which is a specific ALDH inhibitor. FACS Aria II cell sorter (BD Biosciences, San Diego, CA, USA) was used to measure the ALDH-positive cell population.

2.4. Mammosphere-forming assay

Mammosphere-forming assay was performed as previously described with slight modification [17]. Briefly, MCF-7 cells were plated on ultra-low attachment 6-well plates (Corning, Acton, MA, USA) at a density of 10,000 cells/ml in serum-free DMEM supplemented with N_2 supplement (Gibco) and 20 ng/ml basic Fibroblast Growth Factor (R&D Systems, Minneapolis, MN, USA). The number of spheres was microscopically analyzed.

2.5. Real-time RT-PCR

Total RNA was isolated from MCF-7 cells using ISOGEN (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. Quantitative real-time reverse transcription (RT)-PCR with a QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Valencia, CA, USA) was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The relative changes in the amount of transcripts in each sample were determined by normalizing with the GAPDH mRNA levels. The sequences of the primers used for real-time PCR analysis are as follows: Hes-1 (forward, 5′-AGCGGGCGCAGATGAC-3′; reverse, 5′-CGTTCATGCACTCG CTGAA-3′), GAPDH (forward, 5′-GTCTCCTCTGACTTCAACAGCG-3′; reverse, 5′-ACCACCCTGTTGCTGTAGCCAA-3′).

2.6. Conventional RT-PCR

RT was performed in a 20-μl reaction volume comprising 1 μg total RNA, 1× reaction buffer, 0.5 mM dNTP mixture, 2.5 μM oligo(dT) primer, 40 U RNase OUT, and 200 U Superscript III reverse transcriptase (Invitrogen). PCR was performed using a thermal cycler (Gene Amp PCR System 2400-R, Perkin–Elmer, Norwalk, CT, USA) in a 20-μl reaction volume comprising 0.2 μl RT product, 1× PCR buffer, 0.5 μM each of the sense and antisense primers, and 0.1 U long and accurate Taq (Sigma–Aldrich). The following primers were used: α7-nAchR (forward, 5′-CGGAGTCAACGGATT GGTCGTAT-3′; reverse, 5′-CAGCGTACATCGATGTAGCA-3′); GAPDH (forward, 5′-CGGAGTCAACGGATTGGTCGTAT-3′; reverse, 5′-AGCCT TCTCCATGGTGGTGAAGAC-3′). The cycling conditions were as follows: 1 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C, followed by 7 min at 72 °C.

2.7. PKC activity

PKC activity was measured using a Pep Tag Non-Radioactive PKC Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, MCF-7 cells were starved for 4 h and then stimulated with nicotine for 30 min. After the cells were lysed in a PKC extraction buffer, the cell lysates were incubated with the specific peptide substrate and the reaction buffer for 30 min at 30 °C. The reaction was stopped by placing the tube in a 95 °C heating block for 5 min. The samples were then separated on a 1% agarose gel at 100 V for 15 min. The phosphorylated bands were extracted from the gel and the intensity of the bands was measured at 570 nm using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA).

2.8. Statistical analysis

The values are expressed as the arithmetic mean ± SD of three independent experiments. The data were statistically analyzed using two-tailed Student's *t*-test. Results were considered statistically significant if $P < 0.05$.

3. Results

3.1. Nicotine increases ALDH-positive cell population in MCF-7 cells

To investigate whether nicotine affects the size of stem cell population, we performed the Aldefluor assay, which is used for the identification of cancer stem cells, in human MCF-7 breast cancer cells. As shown in Fig. 1A, stimulation with 1 μM nicotine increased the ratio of ALDH-positive cell population. Nicotine increased both ALDH-positive cells (from 3.52×10^2 to 9.43×10^2 cells; approximately 2.7-fold) and ALDH-negative cells (from 3.16×10^5 to 3.47×10^5 cells; approximately 1.1-fold), suggesting that the effect of nicotine is due to the proliferation of the ALDH-positive cells. As shown in Fig. 1B, the effect of nicotine was observed in a dose-dependent manner and maximal effects were achieved at a concentration of 1 μM. To confirm whether nicotine increases the stem cell population, we measured the mammosphere-forming ability, which is used for self-renewal capability [17]. We found that the nicotine-treated MCF-7 cells formed approximately 2.0-fold more mammospheres than untreated control cells (Fig. 1C), suggesting that nicotine induces the stem cell proliferation. To examine whether the effect of nicotine is mediated through its nAChR, we tested the effects of a nAChR antagonist on the ALDH-positive cell population. As shown in Fig. 1D, a nAChR antagonist MCA inhibited the nicotine-induced increase in ALDH-positive cell population. MCA alone did not affect the ALDH-positive cell population. Taken together, these data suggest that nicotine increases ALDH-positive cell population via its nAChR in MCF-7 cells.

3.2. Nicotine induces a Notch-Hes1 pathway in MCF-7 cells

To investigate whether the effects of nicotine is involved in a stem cell-dependent pathway, we examined the Notch pathway, which is a feature of cancer stem cells. We found that nicotine induced an expression of Notch target gene Hes1 in MCF-7 cells (Fig. 2A). We next tested the effect of DAPT, which inhibits cleavage of activated Notch receptors by γ-secretase, and thereby prevents Notch signaling. DAPT reduced the nicotine-induced Hes1 expression (Fig. 2A) and the nicotine-induced increase in ALDH-positive cell population (Fig. 2B). These data suggest that nicotine in-

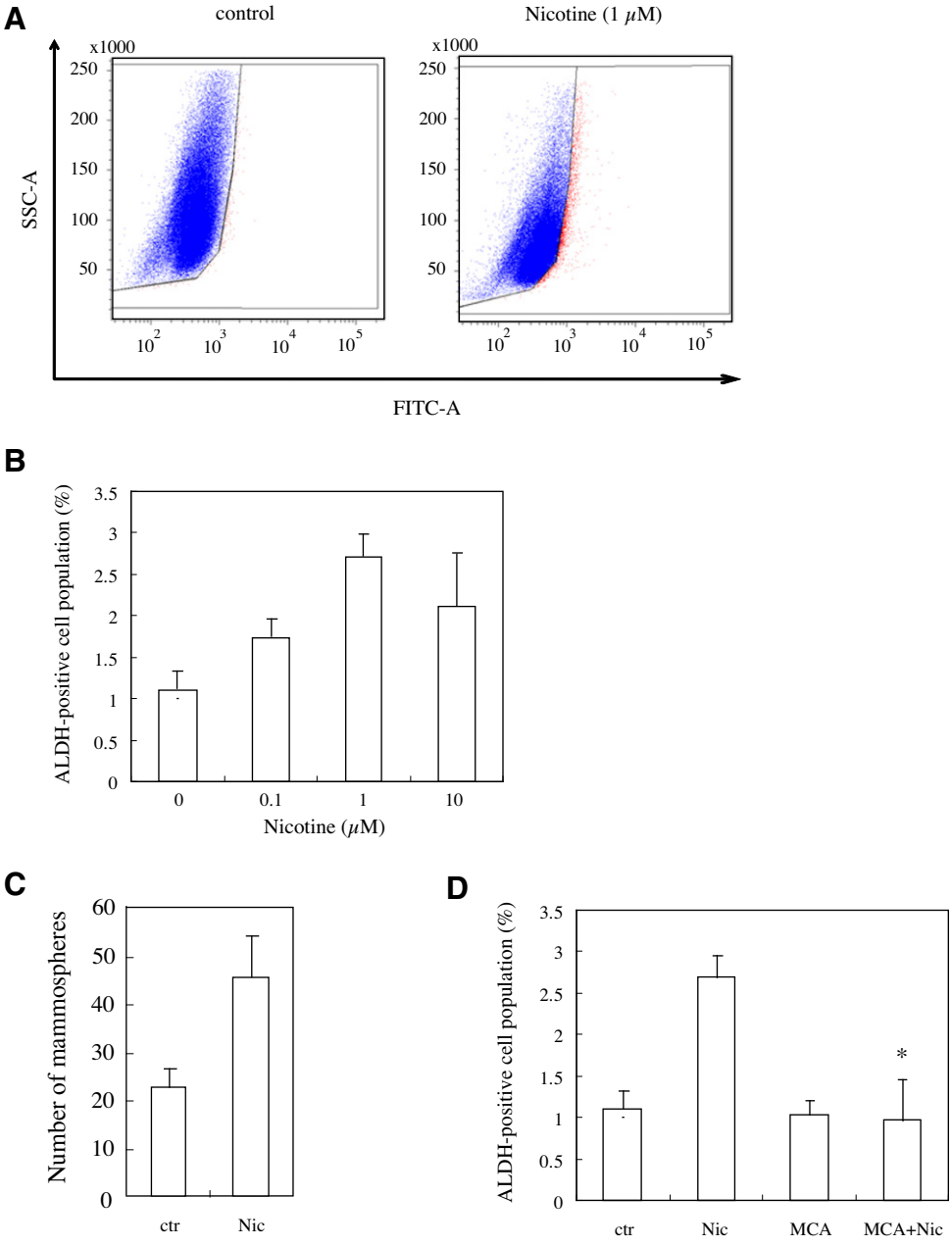

Fig. 1. Nicotine increases the stem cell population in MCF-7 cells. (A) MCF-7 cells were incubated with 1 μM nicotine for 72 h and the ALDH-positive cell population was measured by the Aldefluor assay kit and flow cytometry. (B) Effects of various concentrations of nicotine on ALDH-positive cell population. (C) After MCF-7 cells were stimulated with 1 μM nicotine, mammosphere-forming assay was performed in ultra-low attachment plates. (D) After MCF-7 cells were pretreated with the nicotine antagonist Mecamylamine (MCA, 10 μM), the cells were stimulated with 1 μM nicotine and then ALDH-positive cell population was measured. Values represent the mean ± SD from three independent experiments. $^{*}P < 0.05$ as compared with the respective control.

creases the ALDH-positive cell population via a Notch-Hes1 pathway in MCF-7 cells.

3.3. Effect of PKC on stem cell population in MCF-7 cells

Since protein kinase C (PKC) has been shown to be activated by nicotine and correlated with aggressiveness in breast tumor [18,19], we studied whether PKC is involved in the ALDH-positive cell population in MCF-7 cells. The potent PKC inhibitor GF109203X inhibited the nicotine-induced Hes1 expression (Fig. 3A) and nicotine-induced ALDH-positive cell population (Fig. 3B), suggesting that Notch act at a downstream of PKC. In addition, nicotine induced PKC activation via nAChR in MCF-7 cells. GF109203X inhibited the nicotine-induced PKC activation in MCF-7 cells (Fig. 3C). These data suggest that nicotine-induced PKC activation mediates the Notch pathway to drive the ALDH-positive cell population in MCF-7 cells.

3.4. Effect of α7 nAChR on stem cell population

We further investigated the subtype of nAChR. Since A549 human alveolar epithelial cells and other cancer cells are known to express α7-nAChR [4], we examined the involvement of α7-nAChR in stem cell population. As shown in Fig. 4A, α7-nAChR was detected in MCF-7 cells by RT-PCR. A549 cells were used as a positive control. As shown in Fig. 4B, the α7-selective nAChR agonist PHA543613 increased the ALDH-positive cell population in a dose-dependent manner. Furthermore, α-Bungarotoxin, an α7-

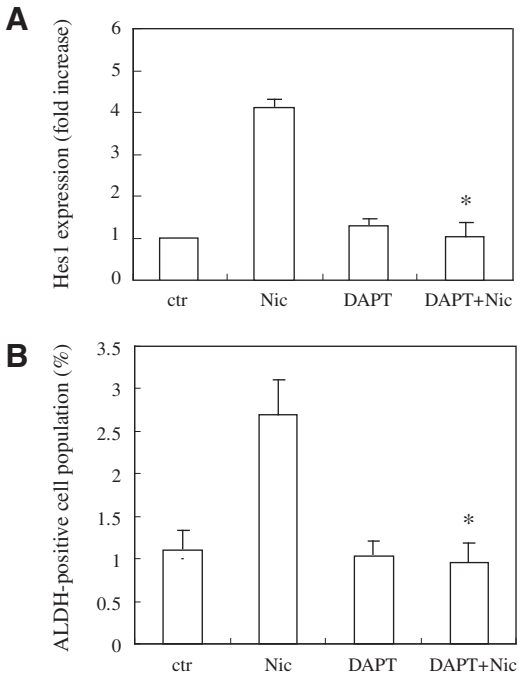

Fig. 2. Effect of a Notch-Hes pathway on the stem cell population in MCF-7 cells. (A) MCF-7 cells were pretreated with the α7-nAChR antagonist BTX (1 μM) or the Notch inhibitor DAPT (5 μM) and then stimulated with 1 μM nicotine. Hes1 expression was measured by real-time RT-PCR. (B) After MCF-7 cells were pretreated with DAPT, the cells were stimulated with 1 μM nicotine and then the ALDH-positive cell population was measured. Values represent the mean ± SD from three independent experiments. $^{*}P < 0.05$ as compared with the respective control.

selective nAChR antagonist, inhibited the nicotine-induced increase in ALDH-positive cell population in MCF-7 cells (Fig. 4C). α-Bungarotoxin also inhibited the nicotine-induced Hes1 expression and PKC activation (Fig. 4D and E). These data suggest that nicotine increases the ALDH-positive cell population via α7-nAChR in MCF-7 cells.

4. Discussion

In the present study, we demonstrated that nicotine increases the stem cell population in MCF-7 cells. We found the α7-nAChR as a mediator of the stem cell population. Furthermore, we showed that the Notch-Hes1 pathway act at the downstream of nAChR in the stem cell population.

There is increasing evidence regarding the existence of breast cancer stem cells and their central role in tumorigenesis. Clinical studies have suggested that smokers have enhanced metastasis of breast cancers to the lung [20,21]. These data raise the possibility that nicotine affect breast cancer stem cells. Our findings suggest that 1 μM nicotine increases ALDH-positive cell population via nAChR in MCF-7 cells. Since the plasma concentration of nicotine was reported to range between 10 nM and 10 μM in cigarette smokers [22], the concentrations of nicotine tested in our study are closely related to the blood concentrations of nicotine in cigarette smokers and might possibly induce growth or differentiation of breast cancer stem cells. Our observations support the idea that nicotine is related to the development of breast cancer in cigarette smokers.

We identified α7-nAChR as the major mediators of the stem cell population. Nicotine is known to activate many signaling cascade, such as Src, PI-3kinase, Akt signaling via nAChR [22]. Since the targeting of these pathways is not considered to be highly selective against cancer, α7-nAChR might be a good target for anti-cancer

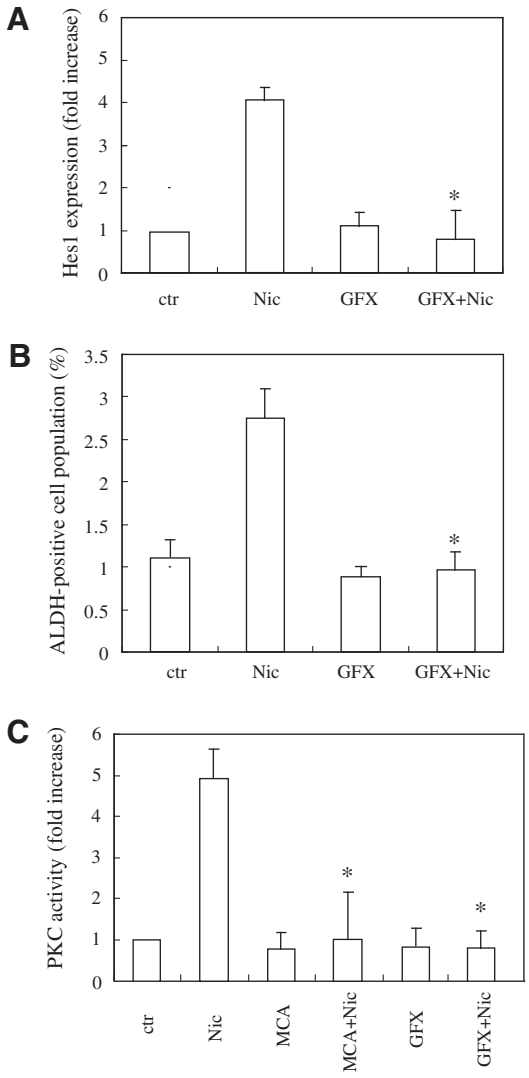

Fig. 3. Role of PKC on the stem cell population in MCF-7 cells. (A) After pretreatment with or without a PKC inhibitor GF109203X (GFX; 1 μM), MCF-7 cells were stimulated with 1 μM nicotine. Hes1 expression was measured by real-time RT-PCR. (B) After MCF-7 cells were pretreated with GFX, the cells were stimulated with 1 μM nicotine and then the ALDH-positive cell population was measured. (C) After MCF-7 cells were stimulated with 1 μM nicotine for 30 min, PKC activity was measured using a non-RI method. Values represent the mean ± SD from three independent experiments. $^{*}P < 0.05$ as compared with the respective control.

agents. We are currently conducting studies to examine the effect of α7-nAChR on in vivo tumorigenicity from breast cancer stem cells.

Our study demonstrated that nicotine increased the expression of the Notch target gene Hes1 and the stem cell population. Since Notch signaling has been shown to be required in mammosphere formation [12], our results using ALDH assay confirm a role for Notch signaling in breast cancer stem cells. However, the mechanism of how nicotine activates the Notch-dependent pathway is not well understood. ADAM protease has been implicated in the cleavage of Notch receptors [23]. Since PKC activity induces ADAM membrane translocation in glioblastoma [24], nicotine might activate ADAM by PKC and thereby induce Notch activation and the stem cell population in MCF-7 cells.

Cigarette smoking contains over 4000 different chemicals, many of which are toxic and/or carcinogenic in a variety of cells including breast epithelial cells [25,26]. Whereas we show nicotine

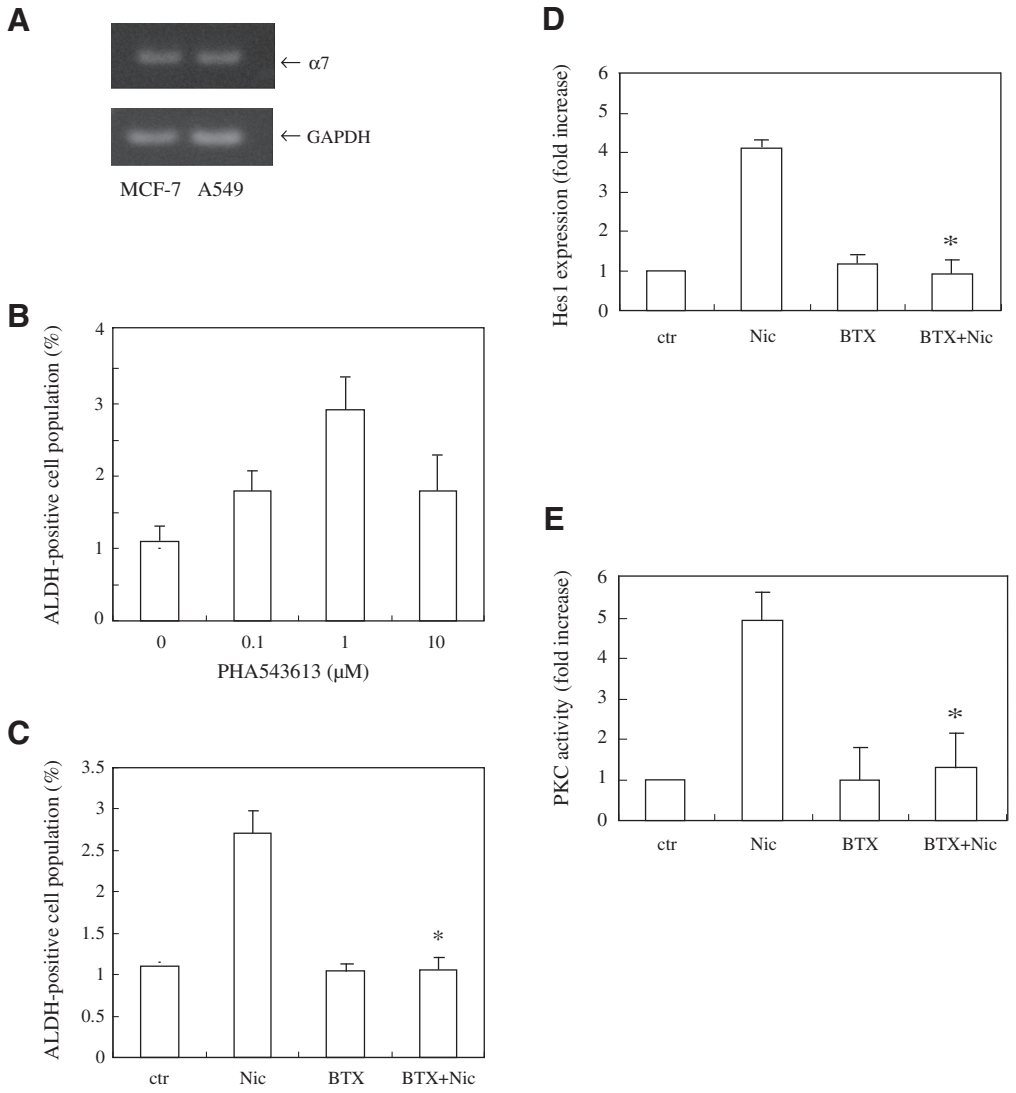

Fig. 4. Effect of α7-nAChR on the stem cell population in MCF-7 cells. (A) The α7-nAChR were detected by RT-PCR in MCF-7 cells. (B) MCF-7 cells were incubated with various concentrations of a α7-nAChR-selective agonist (PHA543613) and then the ALDH-positive cell population was measured. (C) After MCF-7 cells were pretreated with the α7-nAChR antagonist α-Bungarotoxin (BTX, 1 μM), the cells were stimulated with 1 μM nicotine and then the ALDH-positive cell population was measured.(D) MCF-7 cells were pretreated with the α7-nAChR antagonist BTX (1 μM) and then stimulated with 1 μM nicotine. Hes1 expression was measured by real-time RT-PCR. (E) MCF-7 cells were pretreated with the α7-nAChR antagonist BTX (1 μM) and then stimulated with 1 μM nicotine for 30 min. PKC activity was measured using a non-RI method. Values represent the mean ± SD from three independent experiments. $^{*}P < 0.05$ as compared with the respective control.

increases the stem cell population, there are numerous other molecules in cigarette smoking that can affect cancer stem cells. NNK, which is a nicotine-derived nitrosamino ketone, has been found to transform human normal breast cells [27]. Thus, NNK also might contribute to the observed stem cell population. Future studies would be required to examine whether the other cigarette components are involved in breast cancer stem cells.

In conclusion, we show here that nicotine increases the stem cell population via α7-nAChR in MCF-7 cells. These findings reveal a novel role for nicotine as a stimulant of the cancer stem cell population in human breast cancer. These findings might explain the development of breast cancer in cigarette smokers. Development of agents that can disrupt the α7-nAChR signaling pathway may provide a new therapeutic way for the treatment of breast cancer.

Conflicts of interest statement

None declared.

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