Overexpression and Activation of the α9-Nicotinic Receptor During Tumorigenesis in Human Breast Epithelial Cells

Chia-Hwa Lee, Ching-Shui Huang, Ching-Shyang Chen, Shih-Hsin Tu, Ying-Jan Wang, Yu-Jia Chang, Ka-Wai Tam, Po-Li Wei, Tzu-Chun Cheng, Jan-Show Chu, Li-Ching Chen, Chih-Hsiung Wu, Yuan-Soon Ho

Background Large epidemiological cohort studies in the United States have indicated that active and passive smoking are associated with increased breast cancer risk. However, there was no direct evidence of an effect of tobacco carcinogens on the cellular molecules involved in breast tumorigenesis.

Methods Reverse transcription–polymerase chain reaction was used to determine the expression of all of the nicotinic acetylcholine receptor (nAChR) subunits in 50 human breast cancer samples and to determine the expression of the α9-nAChR subunit in 276 surgical and laser capture microdissected breast tumor vs normal tissue pairs. Stable MDA-MB-231 breast cancer cell lines were established in which expression of the α9-nAChR subunit was inhibited using short interfering RNA. MCF-10A normal human breast epithelial cells were established in which the α9-nAChR subunit could be conditionally overexpressed by removal of doxycycline from the culture fluid. Cell proliferation and soft agar assays and tumor growth in nude mice were used as measures of cell transformation. All statistical tests were two-sided.

Results In 186 (67.3%) of the 276 paired samples, α9-nAChR mRNA was expressed at (mean 7.84-fold) higher levels in breast cancers than in surrounding normal tissue. Stable expression of α9-nAChR short interfering RNA in MDA-MB-231 cells attenuated nicotine-stimulated proliferation and growth in soft agar and reduced tumor volume when the cells were introduced as xenografts in SCID mice (n = 5 mice per group; mean tumor volume at 6 weeks treatment in mice injected with Si α9 cells = 995.6 mm³, in mice injected with parental cells = 2993.2 mm³, difference = 1997.6 mm³, 95% confidence interval [CI] = 112.7 to 2290.2 mm³, P = .009). Long-term treatment of MCF-10A normal breast epithelial cells with either nicotine or its active metabolite, 4-(methylamino)-1-(3-pyridyl)-1-butanone, triggered precancerous transformation as defined by soft agar assay. Inducible overexpression of α9-nAChR in MCF-10A cell xenografts in nude mice substantially increased tumor growth (n = 5 mice per group; DOX+, mean tumor volume without nicotine vs with nicotine = 266.2 vs 501.6 mm³, difference = 235.4 mm³, 95% CI = 112.7 to 358 mm³, P = .009; DOX−, mean tumor volume without nicotine vs with nicotine = 621.2 vs 898.6 mm³, difference = 277.4 mm³, 95% CI = 98.1 to 456.7 mm³, P = .016; mean tumor volume in the presence of nicotine, DOX+ vs DOX− = 501.6 vs 898.6 mm³, difference = 397 mm³, 95% CI = 241.3 to 552.6 mm³, P = .009).

Conclusion The α9-nAChR is important for nicotine-induced transformation of normal human breast epithelial cells.


Breast cancer is the second leading cause of cancer death among women in the United States. Approximately 215,000 women were diagnosed with invasive breast cancer, 50,000 women were diagnosed with ductal carcinoma in situ, and 40,000 women died of invasive breast cancer in 2005 (1). Worldwide, it is estimated that more than 1 million women are diagnosed with breast cancer every year, and more than 410,000 will die of the disease (2). Tobacco, one of the most widely examined environmental factors, contains human carcinogens and may contribute to a woman’s risk of developing breast cancer (3). Epidemiological cohort studies with large numbers of participants in the United States and Japan have indicated that breast cancer risk is associated with active and passive smoking (4,5).

Cigarette smoke is a complex mixture of more than 4000 chemical constituents. On average, approximately 1.0 mg (range 0.3–2.0 mg) of nicotine is absorbed systemically during the
smoking of a single cigarette (6). Studies using 14C-nicotine have shown that from 80% to 90% of inhaled nicotine is absorbed (7). Nicotine concentrations in plasma average about 15 ng/mL immediately after smoking and are extremely high in the saliva and gastric juice (>1300 and >800 ng/mL, respectively) (8).

Previous studies using a soft agar transforming assay and a mouse xenograft model demonstrated that noncancerous MCF-10A human breast epithelial cells can become neoplastically transformed by exposure to either a cigarette smoke condensate or the tobacco-specific carcinogen 4-(methylnitrosamino)-1-((3-pyridyl)-1-butanone (NNK) (9,10). Repeated exposure of MCF-10A cells to NNK resulted in anchorage-independent growth and reduced growth factor dependence (10). In vivo studies have demonstrated that nicotine promotes the growth of solid tumors, suggesting that nicotine might contribute to the progression of cell proliferation, invasion, and angiogenesis in tumors (11–13). These results imply that nicotinic alteration of normal breast epithelial cells may also contribute to breast tumorigenesis.

Human neuronal tissues have been reported to have the most abundant expression of the nicotinic acetylcholine receptor (nAChR) subunit. nAChRs occur as homotetramers comprising a combination of α subunits (α1–α6) and β subunits (β2–β4) or as homopentamers derived from subunits α7–α10 symmetrically arranged around a central ion pore (14,15). However, reverse transcription–polymerase chain reaction (RT-PCR), immunoblotting, and flow cytometry analyses have provided considerable evidence for the expression of nAChRs in nonneuronal cells outside the nervous system, including bronchial epithelium membranes and endothelial cells (16,17). The physiological ligand of nAChRs is acetylcholine; however, tobacco components like nicotine and NNK are also known to be high-affinity nAChR agonists (18,19). Several studies have reported roles for the nAChRs in carcinogenesis, which include angiogenesis (13), proliferation (20,21), and the inhibition of apoptosis (22,23). Cigarette smoking is known to be a prominent risk factor for lung (16), colon (24), and bladder cancers (25), all of which express α7 as a major nAChR, as well as breast cancers, which express α9-nAChR, suggesting that agents like nicotine and NNK may function in a receptor-dependent manner (11,16,20,26).

Unlike the nAChRs that are expressed in normal neuronal cells, most of the nAChRs present in cancer cell lines have not been functionally characterized (17). Characterization is important because nonneuronal nAChRs in human cancer cells could be potential molecular targets for clinical therapeutic purposes (27). Therapeutic strategies involving activation of neuronal α7-nAChRs have been considered for the treatment of Alzheimer disease and schizophrenia (28). In cancer cells, nAChRs could also play a role in the acquisition of chemotherapy drug resistance: Nicotine has been shown to protect cells against apoptosis, so nAChR antagonists could potentially be used in combination with established chemotherapeutic drugs to enhance therapeutic responses to chemotherapy (27,29).

In this study, we sought to determine whether inhibition of α9-nAChR subunit expression in human breast cancer cells can substantially inhibit tumor growth in vivo. To explore the potential carcinogenic effects of the α9-nAChR subunit, which is expressed in normal human breast epithelial cells, we established MCF-10A normal human breast cells with tetracycline-regulated (Tet-off) overexpression of α9-nAChR. We then performed in vivo studies that used the MCF-10A-Nic cells with conditional overexpression of the α9-nAChR or MDA-MB-231 human breast cancer cells with short interfering RNA (siRNA) to reduce α9-nAChR expression levels to investigate the role of α9-nAChR in nicotine-induced breast carcinogenesis.

**Subjects and Methods**

**Cell Culture and Patient Samples**

All human breast tumor samples (n = 276) were obtained as specimens from anonymous donors from Taipei Medical University Hospital and Cathay General Hospital, Taipei, Taiwan, according to a protocol approved by the Institutional Review Board (P950012). On histological inspection, all patient samples consisted of more than 80% tumor tissue. All samples (each paired tumor vs normal tissue) were collected and categorized according to the clinical information such as stage status. Human mammary gland epithelial...
adenocarcinomas (MCF-7, MDA-MB-231, AU-565, MDA-MB-453, and BT-483) and human normal mammary gland epithelial fibro-cystic cell lines (MCF-10A and HBL-100) were purchased from the American Tissue Cell Culture collection (Manassas, VA). MCF-10A cells were maintained in complete MCF-10A culture medium, that is, a 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham F12 supplemented with 100 µg/mL cholera enterotoxin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisone, and 20 ng/mL epidermal growth factor (Life Technologies, Rockville, MD). MCF-7, MDA-MB-231, HBL-100, and MDA-MB-453 cells were maintained in DMEM, whereas AU-565 and BT-483 cells were maintained in RPMI-1640.

**Cell Proliferation and Viability Assays**

Cell growth, proliferation, and viability were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (30). Stock solutions of 10 mM nicotine and NNK (Chemsyn, Lenexa, KS) were prepared in dimethyl sulfoxide. This assay was repeated four times with duplicate samples.

**RNA Isolation and Real-Time Quantitative PCR**

Total RNA was isolated from both human cell lines and breast tumor tissue samples acquired directly from patients (n = 276) using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. A portion of the samples from the original group (n = 50 of 276) were randomly selected for the determination of nAChR expression profiles by RT-PCR. After random selection, the clinical information associated with these samples was carefully checked to ensure that there were no substantial differences between the selected and the original groups (Supplementary Table 1, available online). The nAChR subunit–specific primers were synthesized as previously described (16) (Supplementary Table 2, available online). A LightCycler thermocycler (Roche Molecular Biochemicals, Mannheim, Germany) was used for the real-time quantitative PCR. The α9-nAChR mRNA fluorescence intensity was measured and normalized to β-glucuronidase expression using the built-in software (Roche LightCycler Version 4).

**RNA Interference**

Both α5- and α9-nAChR expression were each ablated in MDA-MB-231 breast cancer cell with at least two independent siRNAs. Scrambled sequences of each siRNA were used as controls (Supplementary Table 2, available online). After BLAST analysis to verify the absence of significant sequence homologies with other human genes, the selected sequences were inserted into pBII and HindIII-cut pSUPER vectors to generate the pSUPER-Si α5-nAChR, pSUPER-Si α9-nAChR, and pSUPER-scramble vectors. The identities of all constructs were confirmed by DNA sequence analysis. The transfection protocol has been described previously (31). Briefly, 1.5 × 10⁶ cells were washed twice with phosphate-buffered saline and mixed with 0.5 µg of plasmid. One pulse was applied for a duration of 20 milliseconds under a fixed voltage of 1.2 kV on a pipette-type microporator MP-100 (Digital Bio, Seoul, Korea).

**Generation of Stable nAChR siRNA–Expressing Cell Lines**

At least three clones of the MDA-MB-231 cell lines were generated that stably expressed siRNAs to α5-nAChR or α9-nAChR or scrambled control siRNA. All experiments were performed using multiple subclones of each cell line, with reproducible results. The pSUPER-Si α5-nAChR, pSUPER-Si α9-nAChR, and pSUPER-scramble vectors were transfected, and stable integrants were selected 72 hours later with G418 (4 mg/mL). After 30 days in selective medium, two G418-resistant clones, referred to as Si α5-nAChR (Si α5) and Si α9-nAChR (Si α9), were isolated; these clones demonstrated more than 80% reduction in mRNA and protein levels when compared with the control clones (scramble control, Sc).

**In Vivo Treatment of Mice With α9-nAChR siRNA–Expressing Breast Cancer Cell Xenografts**

MDA-MB-231 cell lines with stable integration of pSUPER-Si α9 or pSUPER-Si α9 scramble sequences were established by G418 selection. The cells (5 × 10⁶) were implanted subcutaneously into each 6-week-old NOD.CB17-PRKDC(Scid)/J (NOD-SCID) mice (n = 5) (purchased from National Science Council Animal Center, Taipei, Taiwan). After tumor transplantation, nicotine (10 mg/mL) was administered via the drinking water for 6 weeks until the mice were killed by anesthesia with ether. During the experiment, the tumor size was measured using calipers and the tumor volume was estimated by using the formula: tumor volume (mm³) = 1/2 × L × W², where L is the length and W is the width of the tumor (32). At the end of experiment, subcutaneous tumor masses were dissected from the mice and weighing them. All mouse protocols were performed according to an Association for Assessment and Accreditation of Laboratory Animal Care–approved protocol.

**Generation of Nicotine- and NNK-Transformed MCF-10A Cells**

We treated MCF-10A cells with a low dose of nicotine (10 µM) and NNK (1 µM) to mimic long-term exposure of cells to these carcinogens (9,33). Cells were subcultured every 4 days, and cells were treated with nicotine and NNK for 48 hours after every passage. After 2 months, the nicotine- and NNK-transformed cells (MCF-10A-Nic and MCF-10A-NNK) were transfected with adenoviruses carrying conditionally regulated (Tet-off) α9-nAChR transgenes.

**Construction of α9-nAChR Adenovirus Tet-Off Expression Vectors**

A PCR fragment encompassing the coding region of the α9-nAChR gene was generated using a forward primer, 5′-GGTGAATCCATGAACTGCTGTTCCATTTTGCCTG-3′, and a reverse primer, 5′-GATGGATCTCTAATCGCTGATATCCTGATC-3′. The fragment was ligated into the pTRE-Adeno vector (Supplementary Table 1, available online). After digestion with EcoRI and BamHI, the fragment was ligated into the pTRE-Adeno vector. The integrity of the constructed vector was verified by restriction digestion and DNA sequence analysis. The Tet-responsive expression cassette was excised from the recombinant pTRE-Adeno plasmids using the I-CeuI and I-SceI enzymes and ligated into the predigested Adeno-X viral DNA. Recombinant Adeno-X viral DNA was propagated in Escherichia coli, linearized by digestion with PacI, and transfected into low-passage HEK 293 cells. HEK 293 cells were infected with the recombinant virus, and the growth...
medium was collected when 80% of the cells had detached from the culture plate to produce high-titer adenovirus stocks.

**Generation of Adeno-X Tet-off α9-nAChR–Overexpressing Cells**

The nicotine- and NNK-transformed (MCF-10A-Nic and MCF-10A-NNK) cells were plated in 60-mm dishes at a density of 10^6 cells per dish. Two days later, the cells were coinfected with the Tet-responsive recombinant virus and the tetracycline-controlled transactivator virus (BD Adeno-X Tet-off system; Clontech, Palo Alto, CA) at a multiplicity of infection of approximately five plaque-forming units of each virus strain per cell. After incubation for 12 hours at 37°C in a CO_2_ incubator, the virus-containing medium was removed and fresh growth medium containing 10% serum was added in both the presence and absence of 1 µg/mL of the tetracycline analog doxycycline (DOX) (DOX+ or DOX−). This resulted in the establishment of MCF-10A-Nic (DOX) and MCF-10A-NNK (DOX) cells in which α9-nAChR gene expression was induced by removal of DOX.

**Isolation of Transformed Adeno-X Tet-off α9-nAChR–Overexpressing Cells**

The transformed MCF-10A-Nic (DOX) and MCF-10A-NNK (DOX) cells were plated onto soft agar (see below). After 21 days, colonies were isolated from soft agar and incubated with 0.5% trypsin for 10 minutes as in previous studies (9,33). Cells were dispersed in complete MCF-10A culture medium, maintained at 37°C, and cultured as cell lines.

**Soft Agar Growth Assay**

Anchorage-independent growth of α9-nAChR overexpressing [MCF-10A-Nic (DOX) and MCF-10A-NNK (DOX)] and siRNA-expressing MDA MB-231 Si α5, Si α9, and Sc cells were examined in soft agar assays. The base layer consisted of 0.9% low-melting point SeaPlaque agarose (Sigma, St Louis, MO) in complete MCF-10A culture medium. Soft agar composed of 0.4% SeaPlaque agarose in complete DMEM, and F12 culture medium was mixed with 1 × 10^4 cells and plated on top of the base layer in 60-mm-diameter culture dishes. Cells were treated with NNK (1 µM) or nicotine (10 µM) before plating in soft agar. Soft agar cultures were maintained at 37°C for an additional 21 days and observed for the appearance of colonies with a Leica DMI 4000B Microscope Imaging System (Leica Microsystems, Wetzlar, Germany). The assay was repeated four times with duplicate samples.

**Nicotine- and NNK-Transformed MCF-10A Cells and Mice With α9-nAChR–Overexpressing Nicotine-Transformed Xenografts**

BALB/c-nu/nu mice (female, 4 weeks old, n = 5 per group) purchased from National Science Council Animal Center (Taipei, Taiwan) were injected subcutaneously with MCF-10A-Nic (DOX) and vector control (5 × 10^6) cells. After transplantation, mice bearing tumors were treated with DOX (0.5 mg/mL) via the drinking water for 14 days. After that, all mice bearing tumors (200 mm^3) were divided into either α9-nAChR mRNA expressing (DOX−) or noninduced (DOX+) groups, the latter of which could express the α9-nAChR mRNA at basal level. The mice were simultaneously treated with or without nicotine (10 mg/mL) in their drinking water for an additional 6 weeks. The xenografts were weighed and either snap-frozen in dry ice and stored at −80°C for RNA and protein analysis or formalin-fixed and paraffin-embedded for immunohistochemical observation.

**Protein Extraction, Western Blotting, and Antibodies**

For determination of α9-nAChR protein expression, the Si α5, Si α9, Sc, and MCF-10A-Nic (DOX+) cells were washed once with ice-cold phosphate-buffered saline and lysed on ice in cell lysis buffer (50 mM Tris–HCl, pH 8.0; 120 mM NaCl; 0.5% Nonidet P-40; 100 mM sodium fluoride; and 200 µM sodium orthovanadate) containing protease inhibitors, as previously described (21). Xenograft tumor tissues were thawed in 750 µL of lysis buffer containing protease inhibitors to examine protein expression. The samples were homogenized three times at setting 3 (18 000 rpm) on ice using a PRO 200 homogenizer (PRO Scientific, Inc, Monroe, CT). 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-α9-nAChR antibody 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-α9-nAChR primary antibodies were incubated at 1:2000 and 1:8000 dilution, respectively, for 2 hours, and the secondary antibodies were incubated at 1:4000 dilution for 1 hour. The assay was repeated twice with duplicate samples.

**[3H]-Nicotine Equilibrium Binding**

1-(−)[N-methyl-[3H]-nicotine (71–75 Ci/mmol) was purchased from Dupont/NEN Research Products (Boston, MA), and free base nicotine (99% pure) was purchased from the Eastman Kodak Co (Rochester, NY). To study the uptake of [3H]-nicotine in MDA-MB-231 cell monolayers (2 × 10^5 cells per well), cells were rinsed three times with a buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2, 0.8 mM MgSO_4, 5 mM glucose, and 25 mM HEPES (pH 7.4). Saturation binding studies were conducted for 2 hours at 37°C in six-well plates and used at least eight different concentrations of [3H]-nicotine ranging from 1 to 15 nM. Time-dependent association kinetic studies in MDA-MB-231, Si α9, Sc cells were conducted using a single concentration of [3H]-nicotine (7 nM) for treatments of 5, 10, 15, 30, 60, and 90 minutes. For determination of nonspecific binding, 10 µM unlabeled nicotine was pre-added to medium, cells were then washed three times with ice-cold buffer and then exposed to [3H]-nicotine. [3H]-nicotine uptake was stopped by aspiration of the uptake medium and washing the wells three times with ice-cold buffer. The cells were lysed in 1 mL of 0.5% Triton X-100, and aliquots of the cell lysates were transferred to scintillation vials to determine the incorporated radioactivity by scintillation counting. The assay was repeated four times with duplicate samples.

**Laser Capture Microdissection**

Frozen sections from the breast tumor samples were prepared for laser capture microdissection experiments. In this study, tumor
tissues diagnosed as different stages (stage 1–4, n = 11) were collected. The sections stained with HistGene (Arcturus Engineering, Mountain View, CA) were subjected to laser capture microdissection by using a PixCell IIe system (Arcturus Engineering) (34). The parameters used for laser capture microdissection included a laser diameter of 8 µm and laser power of 48–65 mW. For each specimen, 15,000 laser pulse discharges were used to capture ~10000 morphologically normal epithelial cells or malignant carcinoma cells. Each population was visualized under a microscope to make sure that the captured cells were homogeneous. The caps with the captured cells were then fitted onto 0.5 mL Eppendorf tubes containing 42 µL of lysis buffer, and RNA was isolated by following a standard protocol (PicoPure RNA Isolation Kit; Arcturus Bioscience, Mountain View, CA). The purified RNA was then measured by reverse transcription and real-time quantitative PCR analysis.

Immunohistochemistry and Confocal Microscopy
To investigate whether the α9-nAChR could be detected in human breast cancer cell lines, confocal microscopy assay were performed by seeding human breast cancer (MCF-7) cells onto poly-l-lysine–coated slides. The slides were incubated with fluorescein isothiocyanate-labeled anti-α9-nAChR antibodies and rhodamine-labeled anti-caveolin-1 antibodies for 1 hour at room temperature, washed twice with phosphate-buffered saline, and incubated with secondary antibodies for an additional 30 minutes in a moist chamber at room temperature. The slides were then examined with a Leica TCS SP5 Confocal Spectral Microscope Imaging System (Leica Microsystems, Wetzlar, Germany).

The α9-nAChR protein localization in breast tumor tissues was further detected by immunohistochemistry. Paraffin-embedded breast tumor tissues that had been excised either from patients or from xenografted tumors were cut into 8-µM slides. Sections were microwaved in Tris buffer (pH 6) for 10 minutes. Following incubation with the primary antibodies, sections were washed in Tris buffer (pH 6) for 10 minutes. Following this step, sections were blocked in 5% horse serum (Chemicon, Temecula, CA) for 30 minutes and subsequently incubated with 1:400 diluted α9-nAChR antibody for 2 hours at room temperature. Following incubation with the primary antibodies, staining was developed according to the streptavidin–biotin–peroxidase method using a LSAB 2 kit purchased from DAKO (Carpinteria, CA). Briefly, sections were washed in phosphate-buffered saline and incubated with biotinylated anti-rabbit secondary antibody. They were then washed again in the same buffer and incubated in streptavidin–biotin–peroxidase complex. Staining was completed after incubation with substrate–chromogen solution. The length of incubation in solution with 3,3′-diaminobenzidine was determined by low-power microscopic inspection. Slides were then washed, dehydrated, and coverslipped using DPX (Sigma-Aldrich, St Louis, MO). Both adjacent sections and same slides were counterstained with hematoxylin for general histological orientation.

Statistical Methods
All data are expressed as means with 95% confidence intervals (CIs) of at least three determinations, unless stated otherwise. A paired t test was used to compare α9-nAChR mRNA expression in paired normal vs tumor tissues from breast cancer patients. A Mann–Whitney test was used to evaluate the effects of α9-nAChR mRNA expression on cell lines and on growth of tumor xenografts with increased (Tet-off) or diminished (siRNA) α9-nAChR expression in mice. The fold ratios of α9-nAChR mRNA expression detected in tumor vs normal samples (from surgical or laser capture microdissected samples with different clinical staging criteria), were compared using the Scheffe test. Statistical differences in tumor cell proliferation, in vitro Tet-regulated α9-nAChR gene induction, [3H]-nicotine receptor–binding activity, and soft agar assays were analyzed by the Kruskal–Wallis (nonparametric) test, and each pairwise comparison was made with the Mann–Whitney test. All statistical comparisons were performed using the SigmaPlot graphing software (San Jose, CA) and Statistical Package for the Social Sciences v.11.0.0 (SPSS, Chicago, IL). All statistical tests were two-sided. A P value of .05 or less was considered to indicate statistical significance.

Results

nAChR Expression in Human Breast Tumor Tissues and Breast Cell Lines
Previous studies have demonstrated that nicotine and its metabolites (eg, NNK) bind to nAChR subunits, which may mediate the carcinogenic effects of these tobacco components (16,21). Therefore, we characterized the expression of nAChR subunits in normal (nonmalignant) human breast cell lines (MCF-10A and HBL-100) and human breast cancer cell lines (MDA-MB-231, MDA-MB-453, AU-565, BT-483, and MCF-7). To evaluate the expression of nAChR subunits among Taiwanese breast cancer patients, human breast tumors (n = 50) and the surrounding normal tissues were dissected and subjected to RT-PCR separately. All breast cell lines were found to express similar (α5, α9, and α10) nAChR subunits (Figure 1, A). The same three nAChR subunits (α5, α9, and α10) predominated in normal and malignant breast tissues (Figure 1, B). We found increased α9-nAChR mRNA levels in nearly all tumor tissues compared with normal tissues (Supplementary Figure 1, A, available online). By contrast, mRNA levels for the α5- and α10-nAChR subunits were not substantially different between tumor and normal paired samples (data not shown).

Role of α9-nAChR in Growth of Human Breast Cancer Cells
To explore the possibility that the α9-nAChR subunit might play a role in smoking-induced human breast tissue tumorigenesis, we established a stable MDA-MB-231 cell line in which the expression of α9-nAChR was reduced by RNA interference. An MDA-MB-231 cell line with reduced expression of the α5-nAChR subunit was also generated as a control (Figure 1, C). A cell line in which expression of both the α5- and α9-nAChR subunits were silenced could not be established because of the essential role of these subunits in cell survival. Rates of cell proliferation in parental MDA-MB-231 cells (231) and in such cells stably transfected with scrambled vector (Sc) or α5-nAChR (Si α5) siRNAs were statistically significantly increased after treatment with 1 µM NNK or 10 µM nicotine (for Sc cells on day 11, mean optical density |OD|140 nm
Figure 1. Role of nicotine-α9-nicotinic acetylcholine receptor (nAChR) binding in human breast cancer cell proliferation. A) Detection of nAChR subunits by reverse transcription–polymerase chain reaction (RT-PCR) in normal and cancerous human breast cell lines. MCF-7, MDA-MB-231, AU-565, MDA-MB-453, and BT-483 are transformed human breast cancer cells; MCF-10A and HBL-100 are considered normal human breast cells. The expression profiles of nAChR subunits in human SAEC (small airway epithelial), NHBE (normal human bronchial epithelial), and H157 (lung cancer) cells were also examined as described previously (16). B) Relative mRNA expression of different nAChR subunits in normal and tumor human breast tissues isolated from 50 breast cancer patients. The cDNA was used for RT-PCR analysis, and the experiment was repeated twice. Percentage of occurrence is shown. C) Expression of the α5- and α9-nAChR subunits in stable MDA-MB-231 cell lines that express α9-nAChR (Si α9), α5-nAChR (Si α5), or scrambled (Sc) short interfering RNAs (siRNAs). Cell lines in which expression of the α5- or α9-nAChRs was specifically reduced were generated by transfection and G418 (4 mg/mL) selection. Levels of α5 and α9 mRNAs were determined by RT-PCR, and levels of the α5 and α9 proteins were determined by western blotting (WB). In each case, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression served as a control. D) Cell proliferation in Si α5, Sc, and parental MDA-MB-231 cells treated with dimethyl sulfoxide (DMSO) vs nicotine, and in cells treated with DMSO vs the nicotine metabolite, 4-(methylthioamino)-1-(3-pyridyl)-1-butanone (NNK). Parental MDA-MB-231 cells (231) and 231 cells stably transformed the Si α5, Sc, or Si α9 siRNAs were treated with either nicotine (10 μM), NNK (1 μM), or vehicle alone in the absence of continued G418 selection afterward. Cells from each group were treated with G418 to confirm successful expression of plasmids. Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, in which a greater number of cells were reflected by an increased OD_{540} at the indicated time points. The experiment was repeated four times with duplicate samples. Data points represent the mean; error bars indicate 95% confidence intervals. The data were analyzed by nonparametric two-sided tests (Kruskal–Wallis and Mann–Whitney tests). On day 11 in MDA-MB-231, Sc, or Si α5 cells, the mean OD_{540} of DMSO-treated cells was statistically significantly different than that for nicotine- and NNK-treated cells (P = .009 for all comparisons). E) Confocal microscopy of α9-siRNA expression in human breast cancer (MCF-7) cells. Immunofluorescence with a fluorescein isothiocyanate–conjugated secondary antibody was used to detect the α9-nAChR, whereas a rhodamine-conjugated fluorescent antibody was used for caveolin-1 labeling. Localization of α9-nAChR (left, green), the membrane protein caveolin-1 (middle, red), and the merged image (right, yellow) are shown. Scale bar = 25 μm. F) Dose-dependent binding of [3H]-nicotine to the endogenous α9-nAChR receptor in human breast cancer MDA-MB-231 cells (left panel) and time-dependent [3H]-nicotine binding in parental MDA-MB-231 cells (231) or in 231 cells stably transformed with α9-nAChR siRNA (Si α9) or scrambled control (Sc) siRNA (right panel). The experiment was repeated four times with duplicate samples. Data points represent the mean; error bars indicate the 95% confidence intervals. The data were analyzed by two-sided nonparametric tests (Kruskal–Wallis and Mann–Whitney test): Si α9-expressing cells bound statistically significantly less [3H]-nicotine than parental (231) or control (Sc) cells (for both comparisons, P = .009).

with dimethyl sulfoxide = 0.77, with nicotine = 1.35 and with NNK = 1.77; difference, nicotine vs control = 0.58, 95% CI = 0.48 to 0.68, P = .009; difference, NNK vs control = 1.00, 95% CI = 0.9 to 1.1, P = .009; for Si α5 cells on day 11, mean OD_{540} with dimethyl sulfoxide = 0.77, with nicotine = 1.35, and with NNK = 1.78; difference, nicotine vs control = 0.58, 95% CI = 0.48 to 0.68, P = .009; difference, NNK vs control = 0.99, 95% CI = 0.89 to 1.09, P = .009) (Figure 1, D). The rates of cell proliferation in Si α9 cells were statistically significantly decreased after treatment with 10 μM nicotine, 1 μM NNK, and vehicle control when compared with Sc cells (for dimethyl sulfoxide treatment on day 11, mean OD_{540} of Sc cells = 0.77 and Si α9 cells = 0.63; difference = 1.34, 95% CI = 0.27 to 2.41, P = .009; for nicotine treatment on day 11, mean OD_{540} of Sc cells = 1.35 and Si α9 cells = 0.63; difference = 0.72, 95% CI = 0.65 to 0.79, P = .009; for NNK treatment on day 11, mean OD_{540} of Sc cells = 1.77 and Si α9 cells = 0.62; difference = 1.15, 95% CI = 1.11 to 1.19, P = .009).

The membrane-associated α9-nAChR protein was detected in human MCF-7 breast cancer cells by immunofluorescence staining followed by confocal microscopy (Figure 1, E, arrowhead). These results suggest that breast cancer cell proliferation induced by tobacco-specific carcinogenic components (such as NNK or nicotine) could be mediated through the endogenous α9-nAChR receptor. To test this hypothesis, MDA-MB-231 cells were treated with [3H]-nicotine to determine its ligand receptor–binding activity. Our results demonstrated that the dissociation constant (K_d) of [3H]-nicotine binding is 3 nM (Figure 1, F, left panel) and that its maximum binding activity is attained at 60 minutes in MDA-MB-231 cells (Figure 1, F, right panel). The mean [3H]-nicotine-binding activity was statistically significantly inhibited in (Si α9) MDA-MB-231...
cells that had been transfected with α9-nAChR siRNA compared with parental cells or with MDA-MB231 cells that had been transfected with scrambled (Sc) control siRNA ([3H]-nicotine bound by Si α9 cells = 201.7 DPM, by parental cells = 489.7 DPM, by Sc cells = 450.6 DPM, difference, Si α9 vs parental = 288 DPM, 95% CI = 275 to 301 DPM, \( P = .009 \); difference, Si α9 vs Sc = 248.3 DPM, 95% CI = 231 to 265.6 DPM, \( P = .009 \)).

Expression of α9-nAChR mRNA in Human Breast Tumor Tissues

As described above, the α9-nAChR subunit is important for nicotine-induced breast cancer cell proliferation. We next examined the mRNA levels of the α9-nAChR subunit in 276 tumor vs normal paired tissue samples by real-time PCR analysis (Figure 2, A and B). The PCR amplification curves were “left-shifted” in the tumor tissues (Figure 2, A, red lines) relative to the profiles of normal tissues (green lines), indicating that the tumor samples overall contained greater quantities of α9-nAChR mRNA. The real-time PCR results were calculated, and the tumors were divided into two groups according to their α9-nAChR mRNA expression patterns. Here, 186 (67.3%) of the 276 normal vs tumor tissue pairs fell into the group in which expression of the α9-nAChR was higher in tumor than in normal tissue (T > N) and 90 paired samples had somewhat higher expression in normal than tumor tissue (N > T) (Figure 2, C). In the group with higher tumor than normal expression (T > N) overall, the α9-nAChR expression

**Figure 2.** α9-nicotinic acetylcholine receptor (α9-nAChR) expression levels in normal and malignant human breast tissues. A) The α9-nAChR mRNA expression profiles of paired human breast tumor (red lines) and normal (green lines) tissues (n = 276) were detected by real-time polymerase chain reaction (PCR). B) α9-nAChR mRNA expression levels in 186 patient samples in which expression was higher in tumor than normal (T > N) vs 90 samples in which expression was higher in normal than tumor tissue (N > T). Copy numbers (x10^6 per µg mRNA) were calculated from mean real-time PCR data; error bars indicate the 95% confidence intervals. Normal vs tumor tissue in group 1 (T > N), \( P = .002 \); normal vs tumor tissue in group 2 (N > T), \( P = .16 \). Data were analyzed with paired \( t \) test, \( P \) values presented are two-sided. C) Paired tumor and normal tissue samples categorized according to the kind and degree of α9-nAChR mRNA expression differences. The levels of α9-nAChR mRNA calculated in Figure 2, B, were subdivided into four groups depending on the extent of the difference in expression between tumor and normal tissue (less than twofold, two- to fivefold, five- to 10-fold, and >10-fold). The percentage of occurrences and the total number of tumor-normal pairs are presented for each category. D) Relative expression of α9-nAChR mRNA in tumor and normal tissue pairs grouped according to the clinical breast cancer stage. The tumor-normal tissue pairs for which relative levels of α9-nAChR mRNA were established in Figure 2, B, were divided into five subgroups according to the clinical staging criteria as recommended by the American Journal of Critical Care. Data shown are the mean of the fold ratios of expression in paired tumor and normal tissues. Error bars indicate 95% confidence intervals. The numbers of paired samples at each stage are indicated above the bars. Data were analyzed with an overall nonparametric test (Kruskal–Wallis test), and multiple comparisons were assessed by the Mann–Whitney test. The comparison was carried out as follows: stage 0 vs stage 1, \( P = .66 \); stage 0 vs stage 2, \( P = .047 \); stage 0 vs stage 3, \( P < .001 \); stage 0 vs stage 4, \( P < .001 \). All \( P \) values are two-sided. E) Immunolocalization of the α9-nAChR protein in human invasive ductal and lobular carcinoma breast tumors. The tumor tissues were cut into 8-µm serial sections and stained with antibodies specific to human α9-nAChR. N = normal; T = tumor; I.H.C. = immunohistochemistry stain; H.E. = hematoxylin and eosin stain. The normal breast cells are indicated by green arrows in a green frame, whereas the malignant breast cells are indicated by yellow arrows in a red frame. Scale bar = 200 µm.
in tumor cells was 7.84-fold greater than that of normal cells (copy number for normal cells = 73,638 vs tumor cells = 497,655, difference = 424,017, 95% CI = 285,647 to 709,664, P < .002). Also, more than fivefold increased α9-nAChR mRNA expression was detected in 57 (30.6%) of the 186 tumor tissues (Figure 2, C, bars 3 and 4). However, in the group with higher normal than tumor expression (N > T), nearly all of the normal tissues had less than fivefold greater α9-nAChR expression than the paired tumor tissues (Figure 2, C, bars 5 and 6).

Expression of α9-nAChR in Advanced-Stage Breast Tumor Tissues

We next categorized each tumor vs normal tissue pair according to the clinical stage of the tumor (Figure 2, D). Advanced-stage tumors were associated with substantially higher levels of α9-nAChR mRNA expression. Data are presented as comparisons of the means of the fold ratios between paired tumor vs normal tissues and compared at each stage with the fold change of α9-nAChR mRNA expression levels in stage 0 (ductal carcinoma in situ) tumor vs normal paired tissues, as follows: stage 1 = 1.0-fold, stage 1 = 1.14-fold, stage 2 = 3.51-fold, stage 3 = 6.66-fold, stage 4 = 18.88-fold; difference, stage 0 vs 1 = 0.14-fold, 95% CI = 0.09- to 0.19-fold, P = .66; difference, stage 0 vs 2 = 2.51-fold, 95% CI = 1.39- to 3.63-fold, P = .047; difference, stage 0 vs 3 = 5.66-fold, 95% CI = 3.67- to 7.65-fold, P < .001; difference, stage 0 vs 4 = 17.88-fold, 95% CI = 9.22- to 26.54-fold, P < .001). To confirm these observations, laser capture–microdissected tumor and normal tissues were harvested separately from 11 tumor samples. The α9-nAChR mRNA expression levels in the laser capture–microdissected cells were determined by real-time PCR analysis. The α9-nAChR mRNA expression level increased in a differentiation stage-dependent manner (stage 1 = 2.55-fold, stage 2 = 11.6-fold, stage 3 = 35.66-fold; difference, stage 1 vs 2 = 9.08-fold, 95% CI = 1.85- to 16.3-fold, P = .05; difference, stage 1 vs 3 = 33.1-fold, 95% CI = 8.24- to 57.97-fold, P = .05) (Figure 2, D, and Supplementary Figure 2, C, available online). Next, α9-nAChR protein localization was determined by immunohistochemical staining of frozen tumor sections, which revealed an increase in α9-nAChR protein expression in advanced-stage tumor tissues diagnosed as invasive ductal and lobular carcinomas (Figure 2, E, brown stain in red square frame indicated by the yellow arrows). By contrast, normal tissues did not express substantial levels of α9-nAChR (Figure 2, E, green square frame indicated by the green arrows). In this study, no substantial changes in α9-nAChR mRNA and protein expression levels were detected in premalignant ductal carcinoma in situ lesions (diagnosed as stage 0) tumor vs normal paired samples (n = 10) (Figure 2, D, bars 1 and 2, and Supplementary Figure 1, B, available online).

Influence of α9-nAChR Expression on Growth of MDA-MB-231 Cells in Transformation Assays

In soft agar assays, the number of transformed colonies was statistically significantly reduced in MDA-MB-231 cells that carried α9-nAChR siRNA (Si α9) (Figure 3, A, bars 1, 4, and 7; No. of colonies: Si α9 cells = 140, Sc cells = 222, parental MDA-MB-231 cells = 272; Si α9 vs parental = 132 colonies, 95% CI = 93 to 170 colonies, P = .009; Si α9 vs Sc = 82 colonies, 95% CI = 33 to 130 colonies, P = .009). After treatment with nicotine and NNK, we observed a statistically significant increase in the number of transformed colonies arising from parental MDA-MB-231 cells and from those carrying the scrambled control siRNA (Sc) compared with those carrying α9-nAChR siRNA (Si α9) (Figure 3, A). After nicotine treatment, colony numbers increased particularly in α9-nAChR–expressing cells (bars 2, 5 and 8; No. of colonies: Si α9 cells = 157, Sc cells = 157, parental cells = 341; Si α9 vs parental = 184 colonies, 95% CI = 137 to 230 colonies, P = .009; Si α9 vs Sc = 200 colonies, 95% CI = 164 to 235 colonies, P = .009). After NNK treatment, colony numbers increased most in α9-nAChR–expressing cells (bars 3, 6 and 9; No. of colonies: Si α9 cells = 164, Sc cells = 398, parental cells = 406; Si α9 vs parental = 242 colonies, 95% CI = 218 to 266 colonies, P = .009; Si α9 vs Sc = 234 colonies, 95% CI = 202 to 266 colonies, P = .009). We next examined the effects of α9-nAChR siRNA on cell growth in vivo by treating SCID mice bearing MDA-MB-231, Sc, or Si α9 tumor xenografts with nicotine (10 mg/mL) in their drinking water. After 6 weeks, the tumor volumes and tumor weights in nicotine-treated MDA-MB-231 Si α9 tumor bearing mice were statistically significantly smaller than those in the nicotine-treated parental MDA-MB-231—tumor bearing mice (n = 5 mice per group; tumor volume at 6 weeks treatment, mice with Si α9 tumors vs mice with parental cell tumors, 995.6 vs 2939.2 mm3, difference = 1977.6 mm3, 95% CI = 1705 to 2290.2 mm3, P = .009; tumor weight at 6 weeks, mice with Si α9 tumors vs mice with parental cell tumors, 1.23 vs 4.38 g, difference = 3.14 g, 95% CI = 2.31 to 3.97 g, P = .009) (Figure 3, C). The tumor tissues were dissected from mice 6 weeks after tumor cell transplantation, and RT-PCR and western blot analysis revealed substantial inhibition of α9-nAChR mRNA and protein levels in tumors with α9-nAChR siRNA (Figure 3, E). The mRNA expression level of α5-nAChR was unaltered in the same tumors (Figure 3, E).

Effect of Overexpression of α9-nAChR on Transformation of Normal Human Breast Epithelial Cells and Tumor Growth in MCF-10A-Xenografted Mice

To investigate whether α9-nAChR is involved in smoking-induced transformation in normal human breast epithelial (MCF-10A) cells, we established MCF-10A (DOX) cells in which α9-nAChR gene expression was induced by removal of DOX. Real-time PCR analysis revealed that α9-nAChR mRNA expression in MCF-10A (DOX−) cells was maximally (>200-fold) induced 9–12 hours after removal of DOX (Supplementary Figure 3, A, upper right panel, and 3, B, available online, P = .009). After 24 hours of removal of DOX, the levels of α9-nAChR protein were still substantially increased in MCF-10A (DOX−) cells compared with control MCF-10A (DOX+) cells (Supplementary Figure 3, C, available online, lanes 1–4). The α9-nAChR–overexpressing MCF-10A (DOX−) cells exhibited increased cell proliferation compared with control MCF-10A (DOX+) cells (Figure 4, A, empty vs solid triangle, day 7, mean OD490 for DOX+ cells = 1.53, for DOX− cells = 2.65, difference = 1.12, 95% CI = 1.01 to 1.23, P < .009). However, nicotine- or NNK treatment–induced cell proliferation was observed only in the MCF-10A (DOX+) cell line that expressed normal levels of the α9-nAChR.
Figure 3. Tumorigenicity of MDA-MB-231 cells that express α9-nicotinic acetylcholine receptor short interfering RNAs (α9-nAChR siRNAs) as measured in soft agar assays and tumor growth in mice. A) Effect of α9-nAChR activation on anchorage-independent growth of MDA-MB-231 cells. Parental MDA-MB-231 cells (231) and cells that expressed α9-nAChR siRNA (Si α9) or a scrambled control siRNA (Sc) were treated with the nicotine metabolite, 4-(methylthiobenzyl)-1-(3-pyridyl)-1-butanone (NNK; 1 µM) or nicotine (Nic; 10 µM) before plating in soft agar. The number of colonies in soft agar 21 days after plating 10,000 cells per 3 mm diameter dish was counted under the microscope after crystal violet staining. The experiment was repeated four times with duplicate samples. Data represent the means of nine samples in each group; error bars indicate the 95% confidence intervals. Statistically significant differences are shown for wild-type MDA-MB-231 (231) cells exposed to dimethyl sulfoxide (DMSO) vs nicotine (P = .027) or DMSO vs NNK (P = .009) and for scrambled vector control (Sc) cells exposed to DMSO vs nicotine (P = .03) or DMSO vs NNK (P = .009). Colony number was also statistically significantly higher for MDA-MB-231 (231) cells and scrambled vector control (Sc) cells compared with α9-nAChR siRNA-carrying cells (Si α9) whether treated with DMSO, nicotine, or NNK (P = .009). Data were analyzed using nonparametric tests (Kruskal–Wallis and Mann–Whitney test); all P values are two-sided. B) Effect of α9-nAChR activation on tumorigenesis by MDA-MB-231 cells in nude mice. Wild-type 231 cells, Si α9 cells, or scrambled siRNA control cells (Sc) (1 × 10⁶) were injected subcutaneously into the back of each NOD.CB17-PRKDC(Scid)/J (NOD-SCID) mouse (n = 5). Two transformed cell lines were generated from soft agar colonies exposed to long-term treatment with nicotine or NNK: MCF-10A-Nic (DOX+) and MCF-10A-NNK (DOX) (Figure 4, B, bars 5 and 6; the cell morphologies are presented in Supplementary Figure 4, A, available online) (9). The NNK- or nicotine-treated MCF-10A (DOX+) or DOX(−) cells were then cultured in soft agar for an additional 21 days, and colony formation was evaluated microscopically (Figure 4, B). More colonies were formed by NNK-treated MCF-10A (DOX−) cells compared with NNK-treated MCF-10A (DOX+) cells (Figure 4, B, bars 6 vs 3). Interestingly, six colonies formed in the nicotine-treated MCF-10A (DOX−) cells (Figure 4, B, bar 5), an observation that has never been reported previously (9). Our results indicate that long-term exposure to lower concentrations of nicotine can induce transformation of normal breast epithelial cells and that α9-nAChR may play an important role in this process.

Previous studies in an animal model revealed that normal human breast epithelial (MCF-10A) cells can also be transformed by NNK in vivo (9). To mimic the long-term carcinogenic effects of nicotine to receptor binding on normal human breast epithelial cell transformation, MCF-10A (DOX+) or DOX(−) cells were treated long term (60 days) with NNK (1 µM) or with nicotine (10 µM) according to the previously described methods (Supplementary Figure 4, A, available online) (9). The NNK- or nicotine-treated MCF-10A (DOX+) or DOX(−) cells were then cultured in soft agar for an additional 21 days, and colony formation was evaluated microscopically (Figure 4, B). More colonies were formed by NNK-treated MCF-10A (DOX−) cells compared with NNK-treated MCF-10A (DOX+) cells (Figure 4, B, bars 6 vs 3). Interestingly, six colonies formed in the nicotine-treated MCF-10A (DOX−) cells (Figure 4, B, bar 5), an observation that has never been reported previously (9). Our results indicate that long-term exposure to lower concentrations of nicotine can induce transformation of normal breast epithelial cells and that α9-nAChR may play an important role in this process.

Two transformed cell lines were generated from soft agar colonies exposed to long-term treatment with nicotine or NNK: MCF-10A-Nic (DOX) and MCF-10A-NNK (DOX) (Figure 4, B, bars 5 and 6; the cell morphologies are presented in Supplementary Figure 4, A, available online). We then sought to determine whether induction of α9-nAChR in the presence or absence of nicotine stimulation in vivo would effectively promote tumor growth (Supplementary Figure 5, available online). BALB/c-nu/nu mice (female, 4 weeks old) were injected subcutaneously with transformed MCF-10A-Nic (DOX) cells (5 × 10⁶). After that, all mice bearing tumors (200 mm³) were divided into either α9-nAChR mRNA expressing (DOX−) or nonexpressing (DOX+) groups in the presence or absence of nicotine (10 mg/mL) in their
drinking water (n = 5, per group). A weekly measured tumor volume of MCF-10A-Nic (DOX)-xenografts in nude mice was statistically significantly increased after 7 weeks of nicotine treatment (+DOX, tumor volume without nicotine = 266.2 mm³ vs with nicotine = 501.6 mm³, difference = 235.4 mm³, 95% CI = 112.7 to 358 mm³, P = .009) (Figure 4, C, empty circle vs empty triangle symbol). Tumor growth induction in the nicotine-treated mice was potentiated by withdrawal of DOX (−DOX, tumor volume without nicotine = 621.2 mm³ vs with nicotine = 898.6 mm³, difference = 277.4 mm³, 95% CI = 98.1 to 456.7 mm³, P = .016; with nicotine, DOX+ vs DOX− = 501.6 vs 898.6 mm³, difference = 397 mm³, 95% CI = 241.3 to 552.6 mm³, P = .009) (Figure 4, C, empty triangle vs solid triangle symbol, and Supplementary Figure 5, available online). No tumors were observed in the vector control mice in either the presence or absence of DOX treatment. These results recapitulate the tissue culture data and show that α9-nAChR overexpression in normal human breast epithelial cells sensitizes them such that they are transformed in response to nicotine exposure (Figure 4, B, lanes 5 and 6). In vivo expression of α9-nAChR mRNA and protein was substantially induced in MCF-10A-Nic (DOX)-xenografted breast tumor tissues. Strong immunoreactivity for α9-nAChR was detected in the DOX− but not the DOX+ mouse tumor tissues (arrowhead). Scale bar = 100 μm. C, vehicle control; Tet-off, removal of tetracycline (DOX).

A previous article indicated that the tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation, which causes hypermethylation of the promoters of tumor suppressor genes in Taiwanese lung cancer patients (35). However, it is difficult to provide the direct evidence of smoking-related α9-nAChR-mediated...
carcinogenic effects in human breast cancer. We performed an epidemiological cohort study to assess the clinical significance of α9-nAChR expression in different stages of breast tumors and to correlate it to smoking history among Taiwanese women. In this study, 174 breast tumor patients were recruited for evaluation of tobacco smoking history, clinical staging criteria, and α9-nAChR mRNA expression analysis of tumor vs normal paired samples (Supplementary Table 3, available online). The results indicate that seven (38%) of the 18 breast tumor tissues were diagnosed as later stages (3–4) in the smoker group. By contrast, a similar occurrence ratio was detected but shifted to early-stage (0–1) tumors in the passive (19 of 52, 36.5%) and nonsmoker groups (40 of 104, 38%). Furthermore, we found increased expression of α9-nAChR mRNA in tumor tissues from current smokers compared with those from nonsmokers (6.62- vs 1.51-fold, difference = 5.11-fold, 95% CI = 2.67- to 7.55-fold, P = .003). By contrast, a lower fold ratio of α9-nAChR mRNA expression was detected in tumor tissues from passive smokers compared with nonsmokers (2.81- vs 1.51-fold, difference = 1.3-fold, 95% CI = 0.79- to 1.81-fold, P = .256). In this study, direct evidence for the nicotinic binding activity of α9-nAChR in human breast cancer was also provided by a [3H]-nicotine-binding assay (Figure 1, F). These observations have led to the conclusion that nicotine binding to nAChR may play a direct role in the promotion and progression of human breast cancers.

Discussion

In this study, our results demonstrate that reduction of α9-nAChR subunit expression by RNA interference in human breast cancer cells substantially inhibits tumor growth in vitro and in vivo. By contrast, we conclude that the α9-nAChR subunit is potentially carcinogenic in normal human breast epithelial cells in vitro and in vivo as shown by the properties of α9-nAChR-overexpressing MCF-10A cells. Observation of clinical specimens has indicated that α9-nAChR expression is generally higher in tumor cells relative to normal levels. Levels of α9-nAChR expression in human breast tumor cells are generally increased in more advanced-stage breast cancers.

A recent study demonstrated that binding of exogenous nicotine and acetylcholine to nAChRs and mAChRs, respectively, stimulates the growth of both small cell lung carcinomas (SCLCs) and non–small cell lung carcinomas (NSCLCs) (36). A similar study also showed that the autocrine interaction of acetylcholine with the nAChR stimulates SCLC cell proliferation (37). To identify genetic factors involved in smoking-mediated cancer risk, a genome-wide association study of 317 139 single-nucleotide polymorphisms was recently performed using DNA from 1989 lung cancer patients and 2625 control subjects from six central European countries (38). A locus in the 15q25 chromosome region was strongly associated with lung cancer (P = 9 × 10−10) (39). Interestingly, this region contains several genes, including three nAChR subunits (CHRN45, CHRN43, and CHRN48, encoding the α5, α3, and β4 subunits, respectively) that are expressed in neurons and other tissues (particularly alveolar epithelial cells, pulmonary neuroendocrine cells, and lung cancer cell lines) (38,40,41). These nAChR subunits also bind to N-nitrosonornicotine as well as potential lung carcinogens (18,42).

We present substantial evidence of the expression of the three major nAChR subunits (α5, α9, and α10) in human breast cancer tissues. However, we found that the mRNA expression levels of α10-nAChR were not substantially different between tumor and normal tissues. This could be explained by the fact that α10-nAChR is structurally similar to α9-nAChR, with 58% amino acid sequence identity. Based on the sequence similarity, the α9 subunit belongs to a family of ligand-gated channels that includes subunits for the receptors of the neurotransmitters acetylcholine, gamma-aminobutyric acid, glycine, and serotonin (43). Most of the neuronal-nAChR subunits (α1–α10 and β2–β4 nAChRs) and the muscle subunit (M1–M4 nAChRs) share between 48% and 70% sequence identity. However, the sequence identity between α9 and all other known nAChR subunits is less than 39% (43). As described above, α9-nAChR subunits can assemble into a homopentameric receptor-channel complex (14,15) or form a heteropentameric receptor with the α5-nAChR subunit (40), similar to the behavior of α7 and α8 neuronal-nAChR subunits. This observation implies that the structure of the α9-nAChR gene differs from those of known nAChR subunit genes (44–46), suggesting that α9-nAChR represents a divergent branch within the nAChR gene family.

The α9- and α10-nAChR subunits are expressed primarily within the cochlear and vestibular hair cells of the inner ear and have been implicated in auditory processing (43,47). Previous studies have demonstrated that the α10-nAChR subunit fails to produce functional receptors on its own (48,49). However, coinjection of α9- and α10-nAChR into Xenopus laevis oocytes results in the appearance of an unusual nAChR subtype that displays unusually fast and extensive agonist-mediated desensitization, a distinct current-voltage relationship, and a biphasic response to changes in extracellular Ca2+ ions (50). The observed effects could be because of the activation of the α9- and α10-nAChR subunits because they are most likely to be found in the functional receptor in vivo (47,48). Coexpression of the α9- and α10-nAChR subunits in normal cells has been described in very restricted cell types, such as bronchial epithelia (16), pituitary pars tuberalis (48), olfactory epithelia (48), cochlea (47,48), keratinocytes (51,52), and, in this study, breast tissue. A previous study indicated that the α9- and α10-nAChRs are expressed by the lung parenchyma and alveolar macrophages of isogenic lung transplants. Their expression increases during rejection in pulmonary allografts (53). Furthermore, α9-nAChR is one of a number of self-antigens targeted by autoantibodies produced in patients with pemphigus (54). These studies suggest that the α9- and α10-nAChR may represent a potential therapeutic target for ear disorders (49), pemphigus (54), and neuropathic pain (55); it may also inhibit the proinflammatory functions of alveolar macrophages and afford protection to pulmonary transplants (53). Our results, as well as results from other groups, suggest that the α10-nAChR subunit probably associates with the α9-nAChR subunit in vivo to form a novel subtype of nicotinic receptor involved in different physiological systems, further illustrating the potential involvement of these receptors in tumor carcinogenesis (48).

Previous studies have demonstrated that the α9- and α10-nAChRs are present in human lung airway epithelia (SAEC, NHBE) (16) and lung carcinoma cells (H157, SCLC, and NSCLC).
(16), which play a part in the autocrine-proliferative network that facilitates the growth of neoplastic cells (37). Another study demonstrated that activation of α9-nAChR signaling elevated the phosphorylation status of adhesion molecules, which directly regulate cell–matrix and cell–cell adhesion in normal human keratinocytes (51). These results suggest the existence of a novel biological mechanism of α9-nAChR signaling in normal epithelial cell motility that has clinical implications for cancer metastasis. Our data also demonstrate that overexpression of the α9-nAChR subunit in transformed normal human breast epithelial MCF-10A-Nic (DOX−) cells increased anchorage-independent colony formation in soft agar assays in response to nicotine treatment. These results are consistent with those described above (36,37), implying that overexpression of α9-nAChR signaling triggers an autocrine loop in which nicotine more readily interacts with and activates the α9-nAChR. This loop stimulates premalignant transformation in normal human breast epithelial cells and increases tumor growth in MCF-10A-xenografted mice.

There are some limitations to our study. First, the breast tumor tissues in this study were limited in number and presumably all from Asian patients. Our data highlight the urgent need for demographic studies of α9-nAChR expression in breast tumor patients from diverse genetic backgrounds. Breast cancer in Taiwan is particularly characterized by its low incidence rate and its early age of tumor onset (56). Additional multiracial cohort studies should be performed to investigate whether this disparity in breast cancer occurrence is because of differential racial risks related to α9-nAChR expression and/or differences in socioeconomic status (education, occupation, or cigarette smoking). Also, to examine the prognostic value of α9-nAChR mRNA expression in breast cancer patients with different stages, we measured α9-nAChR mRNA by real-time PCR either in surgical and laser capture–microdissected tumor samples. Our results suggest that higher α9-nAChR mRNA expression is associated with later-stage disease among breast cancer patients. However, our study included only a small number of stage 4 samples (n = 4) and should therefore be considered as exploratory. Further investigations that include more late-stage (stage 4) patient tumors are urgently needed.

Nicotine, from smoking, and its metabolite NNK are considered to be a carcinogens that react with DNA, and most reports have suggested the chemical properties of the resulting DNA adducts to cause the many genetic changes known to exist in human cancers (35,57,58). In this study, to explore whether the nicotinic receptor–mediated biological mechanisms may play a decisive role in tumor formation, the α9-nAChR expression in human breast cancer (MDA-MB-231) and normal (MCF-10A) cells were forcibly modified sham feeding in duodenal ulcer patients. An analysis of nicotine, acid secretion, gastrin, catecholamines, epidermal growth factor, prosta-glandin E2, and bile acids. Scand J Gastroenterol. 1993;28(6):487–494.


Supplementary Data

Supplementary data can be found at http://www.jnci.oxfordjournals.org/.

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**Affiliations of authors:** Graduate Institute of Medical Sciences, College of Medicine (C-HL, T-CC, L-CC, Y-SH), Department of Medical Technology (C-HL, T-CC, Y-SH), Graduate Institute of Clinical Medicine, College of Medicine (Y-JC), Center of Excellence for Cancer Research (Y-JC, P-LW, C-HW, Y-SH), and Department of Pathology, School of Medicine (J-SC), Taipei Medical University, Taipei, Taiwan; Department of Surgery, Cathay General Hospital, Taipei, Taiwan (C-SH, S-HT); Department of Surgery, School of Medicine (C-SH, C-SC, S-HT, K-WT, P-LW) and Center of Quality Management and Breast Health Center (C-SC), Taipei Medical University Hospital, Taipei, Taiwan; Department of Environmental and Occupational Health, National Cheng Kung University Medical College, Tainan, Taiwan (Y-JW); Department of Surgery, School of Medicine, Taipei Medical University-Shuang Ho Hospital, Taipei, Taiwan (C-HW).