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尼古丁暴露與台灣婦女乳癌致癌之分子機制研究

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一、中文摘要

本實驗室利用 PGL3 plasmid 將 $\alpha 9$ -nAChR promoter 送入乳癌細胞株，同時給予不同濃度的尼古丁刺激，結果證實在 10 M 尼古丁刺激下 9-nAChR promoter 之 luciferase 活性增加達十倍之多(圖 2)。Time-dependent 實驗證實 1 M 尼古丁可以在 2-6 小時間讓 9-nAChR promoter 之 luciferase 活性增加達 2.5 倍左右(圖 2)。有趣的是乳癌細胞株 MCF-7(ER+)，MDAMB231 (ER-)在接觸到 10 nM E2 刺激後只有 MCF-7(ER+)細胞株的 $\alpha 9$ -nAChR promoter 之 luciferase 活性有被活化表現。我們的結果證實香菸中尼古丁可在兩種乳癌細胞上活化 9-nAChR promoter 之表現，然而女性荷爾蒙 E2 則只能活化 MCF-7 (ER+)細胞 $\alpha 9$ -nAChR 表現。結果證實尼古丁(10 M)可以讓 MDAMB231 (ER-) MCF-7(ER+)及在 12 小時內活化 mRNA 表現。E2 (10 nM)在 MCF-7(ER+) 12 小時內活化 mRNA 表現，反之對 MDAMB231 並無任何作用。兩者是否有交互調控之作用，需要進一步證實。

Abstract

Nicotine[3-(1-methyl-2-pyrrolidiny)-pyridine], a major alkaloid in tobacco, has been implicated as playing a role in carcinogenesis. Our previous study showed that cigarette smoking promoted inflammation-associated lung adeno-carcinoma formation in vivo and in

vitro, and MAPK plays an important role in this process 1. In the present study, we aimed to investigate whether nicotine and its specific nicotinic acetylcholine receptor (nAChRs) could stimulate breast cancer cell proliferation and tumour growth and the possible mechanisms involved. We first demonstrated that the 5 and 9 nAChRs were detected in both the MCF-7 and MDA-MB-231 breast cancer cell lines. We further study the expression of the nAChR mRNA levels in breast tumor tissues collected from a hundred and fifty cases of breast cancer patients in Taiwan. The data was found that the $\alpha 9$ subunit of the nAChR expression was more significant higher in tumor tissue than in normal tissue. Western blotting analysis demonstrated that the Akt-regulated proteins were the major signal pathway that involved in breast cancer cell proliferation stimulated by nicotine treatment. Constructively express

9-nAChR knock down breast cancer cell line caused a similar result as treatment with LY294002, a Akt/protein kinase B (PKB) inhibitor, it was not only blocked PKB downstream signal but also reduced the tumor formation and growth curve in vitro, soft agar assay and growth curve assay. In vivo, nude mice tumor formation assay also proved the knocked-down nAChR 9 breast cancer cell line had a significant tumor growth inhibition, whether or not nicotine treatment. Another important carcinogenic factor was found to be Estrogen (E2) which directly bound 9-nAChR promoter binding

site at -44 bp and produced more mRNA expression in breast cancer cell line. Thus, this study provides evidence for a novel signal route coupling the stimulation of $\alpha 9$ nAChR to the activation of PKB dependent manner, which implied that the $\alpha 9$ -nAChR may play some important roles involved in nicotine-mediated breast tumor carcinogenesis.

Key words: nicotinic receptor, smoking breast cancer

二、緣由與目的

Breast cancer is the most frequently diagnosed in the United States, accounting for almost 21% of all diagnosed cancers in women. The link between smoking and breast cancer has been elusive during past two decades [2, 3]. Even some studies have suggested that smoking has a little or no relationship with female breast cancer incidence, [4] more previous studies have been reported to have positive association by cohort studied or case-control studies [5, 6]. Recently, breast cancer risk has been associated with active and passive smoking in two large number cohort studies in USA [7] and Japan [8]. The two studies both showed tobacco smoking significantly increases the risk of female breast cancer. There are many considerable factors also involved in the event of smoking caused breast cancer, such as active or passive smoking, the age of started smoking, the number of cigarettes smoked, smoking duration, certain stages of pregnancy and ethnic group [2, 6, 9, 10, 11]. With the enormous epidemiological studies, Mei and Narayan gave the cell biology evidence to prove the transformation possibility of human breast epithelial cells by either cigarette smoke condensate or tobacco-specific carcinogen [12, 13]. However, detailed mechanism of breast epithelial cell transformation and development by smoking still need remained addressed.

Tobacco is one of the most widely examined environmental exposures for disease risk. Despite considerable research, however, the relationship of tobacco exposures to breast cancer incidence remains controversial.

Tobacco smoke contains at least 2550 known compounds, more than 60 of which have been found to be carcinogenic in humans and the metabolites of cigarette smoke have been found in the breast fluid of smokers [14, 15, 16]. Nicotine, although addictive and likely responsible for the substance dependence resulting from tobacco use [17], is considered to be one of the less dangerous components of tobacco smoke [18]. It has been suggested that the amount of Nicotine to which one is exposed as a result of tobacco smoking may not pose a serious health risk. Low tar cigarettes have been considered an acceptable solution to satisfy the smoker's craving for Nicotine. Devices for aerosol delivery of Nicotine without tar-related carcinogens are actively developed and tested as safe alternatives to tobacco smoking. Nicotine is highly soluble in water, and its concentration in the saliva of tobacco smokers can be very high, since average steady-state serum concentration of nicotine have been reported at 200 nM and acute increases to 10-100 μ M in serum or to 1mM at mucosal surface immediately after smoking have been reported [19, 20, 21]. Comparable concentrations are likely present on the bronchial and lung surface. In human, neuronal acetylcholine receptors (nAChRs) are expressed in nervous system, muscle, lymphoid tissue, skin, neurosecretory and vascular endothelial cells [22, 23, 24]. The neuronal nAChRs comprise a rather heterogeneous group of different subtypes that are built from various combinations of α ($\alpha 2$ - $\alpha 10$) and β ($\beta 2$ - $\beta 4$) subunits. Each nAChR has specific pharmacological and physiological responses to ACH, nicotine or other nicotinellike agonists or antagonists such as nicotine-derived nitrosamines N'-Nitrosonornicotine (NNN) 4(methylnitrosamino-1-(3-pyridyl)-1-butanol (NNK), which are potent lung carcinogens [23, 25, 26]. Nicotine has been known to promote cell proliferation and neural angiogenesis through nAChRs by activate AKT (PKB) and Raf/MAPK kinase/ERK (RAF/MEK/ERK) signaling pathway [27, 28, 29, 30, 31]. While there is no evidence that nicotine contributes to the

induction of tumors, it has been demonstrated that nicotine promotes the growth of solid tumors in vivo, suggesting that nicotine might be contributing to the progression of tumors already initiated 32 33.

In this study, we found that either human breast cancer cell line and breast tissue both express nAChRs which sensitive to nicotine, similar to those expressed by ganglionic neurons, that compose the $\alpha 3$ subunit 34. By using RT-PCR and realtime PCR with laser capture microdissection (LCM) on clinical breast cancer tissues, we found at least three nAChRs ($\alpha 5$, $\alpha 9$ and $\alpha 10$) exist in human female breast cells for both normal and tumor parts in Taiwan. A confirmed data also demonstrated with several nAChRs combinations express in human mammary adenocarcinoma breast cancer cell lines (MDA MB 231, MCF 7 and MCF 10A). And furthermore, the tumor stages with its nAChRs $\alpha 9$ expression seem to have an interesting positive correlation. The human breast cancer staged at more tumor malignancy, we observed a significant increase on the expression of nAChR $\alpha 9$ in breast cancer cells than in normals. In cell culture system, breast cancer cell lines MCF-7 and MDA-MB-231 both express nAChRs $\alpha 9$ which likely to activate PI3K/AKT (PKB) pathway by nicotine stimulation causing increasing cell growth curve protein such as cyclin D1. Activation of nAChR $\alpha 9$ also has ability to promote tumor formation and malignancy in nude mice, increase anchorage independent ability and tumor growth in cell culture by introducing nicotine. However, these tumor progressions can be reversed by using nAChR-specific $\alpha 9$ siRNA and LY 294002, a PI3K/AKT inhibitor. Luciferase reporter and Chromatin immunoprecipitation assays showed both nicotine and estrogen can specific bind the DNA promoter region of nAChRs $\alpha 9$ to promote mRNA transcription and protein expression. This consequence provides more nicotine could bind with nAChRs $\alpha 9$ and finally circulating a positive feedback result in breast cancer cells progression. This might to explain why some previous studies indicated that

smoking could increase the risks of getting breast cancer, especially smoking during pregnancy period. We believe that nAChRs $\alpha 9$ is not only functions as simply as a calcium channel but also it might acts as a nicotine receptor mediate signal transduction which more likely a novel growth factor receptor in human breast cell. Therefore, blocking nAChR $\alpha 9$ expression or downstream signal pathway such as PI3K/AKT could be a new strategy to inhibit breast cancer progression in lab or clinic.

Neural tissue, so far has been reported to have most abundant nAChRs subunits expression which are composed of heteropentamers derived from subunits $\alpha 1$ - $\alpha 6$ or homopentamers subunits $\alpha 7$ - $\alpha 10$ and $\beta 2$ - $\beta 4$ subunits. However, previous studies have shown some nAChRs have their tissue specificity and biological function such as subunits $\alpha 3$ - $\alpha 4$ in neural cells 35 and $\alpha 7$ subunit nAChRs in human bronchial epithelial and endothelial cells 36.

三、研究報告內容

Identification of the nAChR subunits expressed in human breast and normal cancer cells. The profile of nAChR subunits expressed in human breast normal and cancer cells were determined in a standard reverse transcription PCR (RT-PCR) assay using primer sets for human $\alpha 2$ - $\alpha 7$, $\alpha 9$, $\alpha 10$ and $\beta 2$ - $\beta 4$ nAChR subunits and amplification conditions described elsewhere 27.

Cell cultures and Nitrosamine-induced transformation of MCF-10A cells.

Human mammary gland epithelial adenocarcinoma MCF-7, MDA-MB-231, AU-565, human mammary gland epithelial fibrocystic cell MCF-10A, and HBL-100 cell lines were obtained from the American Tissue Cell Culture collection (ATCC number HTB 22, HTB 26, CRL-2531, CRL 10317, and HTB124 respectively). Non-cancerous human breast epithelial cell line (MCF-10A) was maintained in

complete MCF-10A culture medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12, [DMEM/Ham's F12], supplemented with 100 ng/ml cholera enterotoxin, 10µg/ml insulin, 0.5µg/ml hydrocortisol, 20 ng/ml epidermal growth factor, and 5% horse serum) (Life Technologies, Rockville, MD)¹². MCF-7, MDA-MB-231, and HBL-100 cells were maintained in DMEM supplemented with 10% heat inactivated fetal calf serum. AU-565 cells were maintained in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum. All cultures were maintained in medium in the presence of 100 units/ml penicillin and 100µg/ml streptomycin in 5% CO₂ at 37°C. Morphological features of cultured cells were monitored microscopically. A stock aqueous solution of 5mM NNK (Chemsyn, Lenexa, KS) was prepared in dimethyl sulfoxide and used diluted with culture medium to a final concentration of 100 pM. Sodium orthovanadate was prepared in H₂O and used at a final concentration of 4 mM.

Soft-agar cloning assay

The base layer consisted of 2% low gelling SeaPlaque agarose (Sigma, St. Louis, MO) in complete MCF10A culture medium. Soft-agar consisting of 0.4% SeaPlaque agarose in complete MCF10A culture medium was mixed with 1 × 10⁴ cells and plated on top of the base layer in 60mm diameter culture dishes. Soft-agar cultures were maintained at 37°C and observed microscopically for the appearance of colonies. Cell colonies were isolated from soft-agar and incubated with 0.5% trypsin for 10min. Cells were dispersed into complete MCF10A culture medium, maintained at 37°C, and developed into cell lines.

RT-PCR. Total-RNA extraction and PCR reaction mixtures were as described previously²⁷. Subunit-specific primers for nAChRs were synthesized by Sigma-Genosys (The Woodlands, Texas, USA) with the following sequences: α1: 5'-CGTCTGGTGGCAAAGCT-3' (sense), 5'-CCGCTCTCCATGAAGTT-3' (antisense); α2:

5'-CCGGTGGCTTCTGATGA-3' (sense), 5'-CAGATCATTCCAGCTAGG-3'(antisense) ; α3: 5'-CCATGTCTCAGCTGGTG-3' (sense), 5'-GTCCTTGAGGTTTCATGGA-3' (antisense); α4: 5'-CTCTCGAACACCCACTC-3' (sense), 5'-AGCAGGCTCCCG-GTCCCT-3' (antisense); α5: 5'-TCATGTAGACAGGTAATTC-3' (sense), 5'-ATTTGCCCATTATATAAATAA-3'(antisense); α6: 5'-GGCCTCTGGACAAGACAA-3' (sense), 5'-AAGATTTTCCTGTGTTCCT-3' (antisense); α7: 5'-CACAGTGGCCCTGCAGACCGATGGTACGGA-3' (sense), 5'-CTCAGTGGCCCTGCTGACCGATGGTACGGA-3' (antisense); α9: 5'-GTCCAGGGTCTTGTGTTTGT-3' (sense), 5'-ATCCGCTCTTGCTATGAT-3' (antisense); α10: 5'-CTGTTCCGTGACCTCTTT-3' (sense), 5'-GGAAGGCTGCTACATCCA-3' (antisense); β2: 5'-CAGCTCATCAGTGTGCA-3' (sense), 5'-GTGCGGTCGTAGGTCCA-3' (antisense); β3: 5'-AGAGGCTCTTTCTGCAGA-3' (sense), 5'-GCCACATCTTCAAAGCAG-3' (antisense); β4: 5'-CTGAAACAGGAATGGACT-3' (sense), 5'-CCATGTCTATCTCCGTGT-3' (antisense); and β-actin primers were 5'-GTGGGGCGCCCCAGGCACCA-3' (sense) and 5'-CTCCTTAAGTCACGCACGATTTC-3' (antisense) (Sigma-Genosys). nAChR primers generated predicted products of 505 bp (α1), 466 bp (α2), 401 bp (α3), 371 bp (α4), 265 bp (α5), 413 bp (α6), 598 bp (α7), 403 bp (α9), 388 bp (α10), 347 bp (β2), 354 bp (β3), and 310 bp (β4).

Human α9 nAChR cDNA (1440 and 1890 bp), cloned into an expression vector driven by a minimal cytomegalovirus promoter fused to the tet operator sequence, was transfected into an normal human breast epithelial (MCF 10A) cells expressing tTA, a tetracycline-controlled transactivator.

Results:

nAChR expression in human breast tumor tissues and breast cell lines

Previous studies demonstrated that nicotine and its metabolic activate compounds such as

4-(methylnitrosamino)-1-(3-pyridyl)-1-butan one (NNK) were bound to nAChRs subunits which mediate the carcinogenic effects of these tobacco components 1,27,37,38. For these reasons, we characterized the expression of nAChR subunits in human breast normal (MCF-10A and HBL-100) and cancer (MDAMB-231, AU545, and MCF-7) cells. nAChRs belong to the superfamily of ligandgated ion channels that are primarily identified in neural tissue, but they have recently been reported to be expressed in other human tissues 27,39,40. Functional nAChRs are composed of homopentamers derived from subunits $\alpha 7$ – $\alpha 10$ or heteropentamers derived from six α subunits ($\alpha 1$ – $\alpha 6$) and three β subunits ($\beta 2$ – $\beta 4$) 27. nAChRs containing $\alpha 3$ or $\alpha 4$ are most abundant in neural tissue³⁵, and $\alpha 7$ -containing nAChRs have been described in human bronchial epithelial and endothelial cells^{27,36}. We performed nAChR subunit-specific RT-PCR analysis of subunits $\alpha 1$ – $\alpha 10$ and $\beta 2$ – $\beta 4$ in human breast cancer and normal cell lines and were summarized in Fig. 1A (see supplement data). Our results demonstrated that nAChRs subunits are expressed with different profiles in MCF-7 ($\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$, $\alpha 10$ and $\beta 4$), MDA MB-231 ($\alpha 5$ and $\alpha 9$), AU565 ($\alpha 7$ and $\alpha 9$), MCF-10A ($\alpha 5$, $\alpha 9$ and $\alpha 10$), and HBL-100 ($\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 9$ and $\alpha 10$) cells. Our results imply that all these cells express similar homopentamer nAChR because they containing $\alpha 7$ – $\alpha 9$ subunits but likely express some heteropentamers: such as $\alpha 1\beta 4$ in MCF-7 cells 27. Interestingly, the $\alpha 9$ subunit was detected in all of these cells (Fig. 1A).

To ascertain the profiles of nAChRs occurrences in breast cancer, human breast tumor with surrounding normal tissues were dissected separately as a paired sample from Taiwanese patients ($n = 150$). The phenotypes of the nAChRs subunits in human tumor and normal tissues were detected by RT-PCR analysis (Fig. 1B,

$n=50$). Our results shown that the $\alpha 5$, $\alpha 9$, and $\alpha 10$ were the major nAChRs subunits detected in human tissues (Fig. 1B). However, the mRNA level of $\alpha 9$ nAChR were higher in tumor tissues than in normal tissues ($n=50$, data not show). The mRNA level of $\alpha 9$ nAChR subunits in 250 paired tissues were then detected by quantitative real-time PCR (Q-PCR) analysis (Fig. 1C). As shown in Fig. 1C, the PCR profiles of $\alpha 9$ nAChR in tumor tissues (red lines) were significantly “left-shifted” than in normal tissues (green lines) that indicates the higher expression level of $\alpha 9$ nAChR mRNA in tumor tissues. The results of Q-PCR were calculated and divided into two groups according to the level of $\alpha 9$ nAChR expression (Fig. 1D). In the first group, of which the average level of $\alpha 9$ nAChR expression was detected with a high level (> 8 folds) containing 2/3 (96/150) tumor tissues (Fig. 1D, bars 1 and 2). In these populations ($T>N$), more than 40% (42/96) of the tumor tissues which expressed higher $\alpha 9$ (> 6 folds) nAChR subunits were detected (Fig. 1E, bars 3 and 4). However, in another group ($N>T$, $n=54$), the expression level of $\alpha 9$ nAChR in normal tissues was detected as merely as less than 1-5 folds (Fig. 1E, bars 5 and 6).

$\alpha 9$ nAChR expression in differentiation stage of breast tumor tissues

As shown in Fig. 2A, $\alpha 9$ nAChR mRNA levels in different differentiation stages of tumor tissues were detected. Our results demonstrated that the mRNA expression level of $\alpha 9$ nAChR was detected higher in the terminal-differentiated stage tumors (Fig. 2A, bars 3 and 4). To confirm such observations, laser-capture-microdissected (LCM) tumor and normal cells were harvested separately from 4 differentiation stages of tumor samples (one case for each stage, Fig. 2B). The $\alpha 9$ nAChR mRNA expression levels in the LCM-captured cells were detected by Q-PCR analysis (Fig. 2C). Our results demonstrated that the $\alpha 9$ nAChR mRNA expression levels were increased in a stage-dependent manner (Fig. 2C). The protein localization of $\alpha 9$ nAChR were detected by immunohistochemical (IHC) staining method in frozen tumor

sections (Fig. 2D). The results shown here demonstrated that no significant expression of $\alpha 9$ nAChR was detected in normal tissues (Fig. 2D, green square, arrow head). In contrast, significant increased of $\alpha 9$ nAChR protein level was detected in tumor tissues (Fig. 2D, red and blue squares, brown-stained cells indicated as arrowheads). The morphology of the tumor cells were confirmed by H.E. staining method according to the clinical criteria (Fig. 2D, black square). The intracellular location of

$\alpha 9$ nAChR was detected as a membranous protein in human MCF-7 breast cancer cells by confocal microscopic immunostaining methods (Fig. 2E, arrowhead).

$\alpha 9$ nAChR affect the cell growth in human breast cancer cells

To investigate whether the endogenous $\alpha 5$ and $\alpha 9$ nAChR subunits affect on tumor cells growth, the MDAMB 231 cells which merely expressed $\alpha 5$ and $\alpha 9$ nAChRs were selected as a research model (Fig. 1A). It was found that $\alpha 5$ - or $\alpha 9$ -nAChR protein expression was depleted at 24 h after treatment with small-interfering (si) RNA (data not ready). The mRNA level in the $\alpha 5$ - and $\alpha 9$ -nAChR knock-downed cells was down regulated by 77 and 89% respectively (Fig. 3A, right). The $\alpha 5$ - and $\alpha 9$ -nAChR double knock-downed cells can not be established due to its requisite role for cell survival. Furthermore, it was found that the cell proliferation in wild-type or scrambled-vector transfected cells was significantly increased by treated with NNK or nicotine (Fig. 3B). Such effects were significantly inhibited in the $\alpha 9$ nAChR SiRNA cells (Fig. 3B). This result implied that the endogenous $\alpha 9$ nAChR was essential for the NNK or nicotine-induced breast cancer cell proliferation. To test these suggestions, the MDAMB 231 cells were treated with 3H-nicotine for determination of ligand-receptor binding activity. Our results demonstrated that the dissociation constants (Kd) of 3H-nicotine binding was 3 nM (Fig. 3C, left panel) and reached a maximum binding activity at 30 minutes (Fig. 3C, right panel) in MDAMB 231 cell. The 3H-nicotine binding activity were inhibited drastically in the $\alpha 9$ nAChR SiRNA

cells (Fig. 3C, right panel).

$\alpha 9$ nAChR-mediated PI3K/Akt signal pathway activation

Previous studies demonstrated that nicotine-mediated lung cancer cell proliferation was induced through activation of AKT-signaling pathway. As shown in Fig. 4A, phosphorylation of Akt (Ser 473) was induced in human breast cancer cells (MDAMB231) treated with NNK or nicotine (1-100 M, for 30 min). The phosphorylated Akt (Ser 473) was detected initially at 10 minutes and reached the maximum level at 30-60 minutes after 1

M nicotine treatment. In order to confirm the PI3K-regulated signals involved in Akt phosphorylations, MDAMB231 cells were pretreated with LY294002 (PI3K inhibitor, 10 M for 30 minutes) and then exposed to nicotine (1 M) for an additional 30-60 minutes. Our results revealed that the p85 but not p110 subunit of the PI3K kinase was phosphorylated at 30-60 minutes after exposed to nicotine. Similarly, the maximum effects of nicotine on Akt (Ser 473) phosphorylation and cyclin D1 induction were detected at 30-60 minutes. All these effects described above were attenuated by LY294002 (Fig. 4C). Such results postulated that the nicotine-induced PI3K/Akt signaling pathway was involved in cyclin D1 induction in human breast cancer cells. However, the role of $\alpha 9$ nAChR involved in nicotine-mediated PI3K/Akt signaling pathways activation was still uncertain. The established $\alpha 9$ nAChR SiRNA, vector control, and wild type MDAMB231 cells lines were treated with nicotine (1 M) for 60 minutes and immunoblotting was then performed (Fig. 4D, lanes 1-6). The cells pretreated with LY294002 in the presence or absence of nicotine was also adapted as a positive control group (Fig. 4D, lanes 7-8). Our results demonstrated that the nicotine-induced Akt (Ser 473) phosphorylation and cyclin D1 induction were blocked in the $\alpha 9$ nAChR SiRNA cells (Fig. 4D, lane 6). Our results suggested that the $\alpha 9$ nAChR subunit was act as an important receptor which participate the signaling pathways of nicotine-mediated

carcinogenic effects in human breast cancer cells.

Estrogen receptor was involved in nicotine-induced $\alpha 9$ nAChR signaling pathway

Steroid hormone such as estrogen has been reported by playing important roles in breast cancer formation through estrogen receptors (ER α and β). Accordingly, human breast cancer cell lines MCF7 (ER+) or MDAMB231 (ER-) were selected to investigate the role of estrogen and its receptor (ER) which involved in nicotine-induced PI3K/Akt activation. The MDAMB 231 cells were treated with nicotine (1 μ M) in a time-dependent manner (Fig. 5A, lanes 1-9). Our results demonstrated that significant $\alpha 9$ nAChR mRNA induction was detected initially at 2 hrs and reached the maximum level at 72 hrs after nicotine treatment whereas the $\alpha 5$ nAChR remained unchanged. The protein level of the $\alpha 9$ nAChR was induced in MDAMB231 cells by treated with nicotine (0.1-10 μ M) for 24 hrs (Fig. 5B).

Previous studies demonstrated that ER was a transcriptional factor which regulates genes expression through its specific binding sequences. To test whether the estradiol (E2) could affect on nAChRs mRNA expression, human MCF-7 (ER+) cells treated with E2 (10 nM), nicotine (1 μ M), or combination treatment for 12 hrs were harvested for determination of the $\alpha 5$ and $\alpha 9$ nAChR mRNA expression level (Fig. 5C). Our results revealed that $\alpha 9$ nAChR mRNA expression was significantly induced by E2 (Fig. 5C, lane 3). Combine treatment of nicotine enhance the E2-induced $\alpha 9$ nAChR mRNA induction was less profound (Fig. 5C, lane 4). However, the direct transcriptional binding activity of ER on $\alpha 9$ nAChR promoter was demonstrated by ChIP analysis by using ER specific antibody in two ER+ (MCF-7 and MCF-10A) cells (Fig. 5D). Our results demonstrated that increase binding of ER to $\alpha 9$ nAChR promoter was detected in cells treated either with nicotine (> 1 μ M), E2 (> 10 nM), or both (Fig. 5E). All these results postulated that ER was involved in

nicotine- and E2-mediated $\alpha 9$ nAChR gene activation.

Nicotine or E2-induced ER was a transcription factor involved in $\alpha 9$ nAChR gene activation through different binding sites

As described above (Fig. 5E), ER could be act as a transcriptional factor that regulate the nicotine- and E2-induced $\alpha 9$ nAChR promoter activation. To clearly define the ER transcriptional binding sites, serially deleted $\alpha 9$ nAChR gene promoter constructs were transient-transfected in MDAMB 231 and MCF-7 cells and luciferase activity was then determined (Fig. 5F). In this study, luciferase activity in the full-length PGL3Luc (-1000) transfected cells MCF-7 without nicotine or E2 treatment was defined as 100% (supplement results, Figs. 5F, G, lane 1). Transfect of 2 μ g RLTK plasmid DNA into MCF-7 produce more than 10 folds of luciferase activity (supplement results, Fig. 5F, lane 4). The transient transfected MCF-7 cells were then treated either with nicotine or E2 dose-dependently (supplement results, Fig. 5F and G). Accordingly, the PGL3 plasmids-transfected MDAMB231 and MCF-7 cells were treated with nicotine (10 μ M) for 24 hrs for determination of luciferase activities (Fig. 5F). As seen in both cell lines, the nicotine-induced luciferase activity was preserved when the promoter construct was deleted from -1000 to -44. In contrast, the responsiveness was abolished when the promoter was deleted from -44 to basic (Fig. 5F). Accordingly, we postulated that the nicotine-induced ER responsive element was located at the SP-1 site (-30 to -39, sequenced as ggggagtttt). To determine whether the SP-1 binding activity is correlated with its transactivation potential, cells were transfected with pGL3(SP1)5, a luciferase reporter plasmid with a SP1 DNA binding site from the $\alpha 9$ nAChR gene promoter (-30 to -39) that is repeated 5 times. Our results showed that nicotine dose-dependently increased SP1-linked luciferase activity (Fig. 5H), and this effect could be significantly abrogated in the MDAMB231 cells transfected with

PGL3(mut SP1)5 with mutated SP1 DNA binding sequences (Fig. 5H). As shown in Fig. 5H, the nicotine-induced SP-1 transcriptional activity was reduced for ???% in the 9 nAChR SiRNA cells (data not ready).

On the other hand, the E2 (5 nM, for 24 hrs)-induced ER responsive element seen in MCF-7 cells was postulated at the region between -250 and -125. Accordingly, we postulated that the E2-induced ER responsive element was located at the xxx site (-xxx to -xxx, sequenced as xxxx). To determine whether the xxx binding activity is correlated with its transactivation potential, the promoter sequence containing a mutated version of the xxxx site (-xxx mutLuc) were constructed. As shown in Fig. 5H, the xxx transcriptional activity was reduced approximately ???% in the mutated construct as compared with the wild-type PGL3Luc (data not ready). The results demonstrated that nicotine- or E2-induced ER was involved in the transcriptional regulation of 9-nAChR promoter through different binding sites. Such observations explained the results shown in Fig. 5C indicated that combine treatment of E2 does not enhance the nicotine-induced 9 nAChR mRNA expression.

Inhibition of $\alpha 9$ nAChR expression in MDAMB231-xenografted tumor cells

In this study, the MDAMB231 9 nAChR Si RNA cells were established as a stable cell line by G418 selection method (Fig. 3A). Soft-agar assay was performed and the transformed colonies numbers were significantly reduced in the 9 nAChR knock-downed cells (Fig. 6A, bars 7-9). Increased of the transformed colonies numbers were seen in the NNK- or nicotine-treated control cells when compared to the SiRNA knock-downed cells (Fig. 6A, bars 8-9). We next examined the cell growth cessation effects of 9 nAChR knock-downed cells in vivo by treating SCID mice bearing MDAMB231 tumor xenografts, using concentrations of either nicotine (50 mg/ml) or NNK (5 mg/ml) in drinking water. After 6 weeks, the 9 nAChR SiRNA knock-downed MDAMB231

tumor volumes in mice treated either with nicotine or NNK was significant inhibited in comparison with wild type and scrambled controls (Figs. 6B-D). No gross signs of toxicity were observed in mice receiving these treatment regimens (body weight, visible inspection of general appearance and microscopic examination of individual organs). The tumor tissues were dissected from mice at 6th weeks after tumor cells transplantation. RT-PCR analysis was performed and found significant inhibit of the 9 nAChR mRNA level in the SiRNA knock-downed tumors. In contrast, the mRNA expression level of 5 nAChR was not changed in the same tumors.

Overexpression of $\alpha 9$ nAChR expression in MCF-10A-xenografted tumor cells

In order to investigate whether $\alpha 9$ nAChR is involved in the smoking-induced transformation process of normal human breast epithelial cells, we established MCF-10A (DOX) cells in which $\alpha 9$ nAChR expression is inducible under the doxycycline (Dox+), a tetracycline analogue, control (BD Adeno-X Tet-off system). Real-time PCR quantitative methods revealed that the 9 nAChR mRNA expression level in the MCF-10A (DOX+) cells was maximally induced 9-12 hr (> 200 folds) after remove of doxycycline (Fig. 7A). The $\alpha 9$ nAChR protein level in the MCF-10A (DOX-) cells was greatly increased as compared to MCF-10A (DOX+) control cells (Fig. 7B, lanes 1-4). In the MCF-10A (DOX-) cells, the $\alpha 9$ nAChR protein expression level in 1440 and 1890 clones was similar (Fig. 7B, lanes 1-4). Induction of $\alpha 9$ nAChR expression consistently results in a marked increase in intracellular phosphorylation of AKT and cyclin D1 expression (Fig. 7B). The 9 nAChR overexpressed MCF-10A (DOX-) cells upon doxycycline removal significantly enhance the cell proliferation when compared to the control (DOX+) cells (Fig. 7C, solid v.s. empty symbol). Nicotine or NNK treatment increased the cell proliferation in the MCF-10A (DOX+) but not in the MCF-10A (DOX-) cells. Previous studies revealed that normal human breast

epithelial (MCF-10A) cells can be transformed by NNK in an in vivo animal model 12. In order to mimic the long-term carcinogenic effect of nicotine/receptor binding on normal human breast epithelial cell transformation, the MCF-10A (DOX+ or DOX-) cells were treated with NNK (1 M) or nicotine (10 M) and replaced medium every 2 days for at least 60 days. The NNK- or nicotine-treated MCF-10A (DOX+ or DOX-) cells were cultured in soft-agar for additional 21 days and colony formation was counted microscopically. The colony numbers were significantly higher in the NNK-treated MCF-10A (DOX-, n = 127) cells when compared to the relevant control MCF-10A (DOX+, n = 46) cells (Fig. 7D, bars 3 versus 6). Interestingly, 6 colonies were also formed in the nicotine treated MCF-10A (DOX-) cells (Fig. 7D, bar 5) indicated that long-term exposure of lower concentration nicotine could induce transformation of normal breast epithelial cells in which the over expressed $\alpha 9$ nAChR play some important role.

Two cell lines were developed from colonies and designated as MCF-10A-Nic (Fig. 7E, lower middle) and MCF-10A-NNK (Fig. 7E, lower right). As shown in Fig. 7E, both cells in culture exhibited subtle changes in their morphology including slightly increased rounded morphology and aggregation in comparison with parental MCF-10A culture. To investigate the role of acquired $\alpha 9$ nAChR over expression associate with colony formation effects, the MCF-10A-Nic and MCF-10A-NNK transformed cells were seeded in soft-agar with or without DOX (Fig. 7F). The colony formation efficiency of MCF-10A-NNK cells was 2 fold higher than MCF10A-Nic cells after NNK or nicotine treatment (Fig. 7F). The colony numbers were significantly increased in MCF-10A-NNK (DOX-) when compared to the relevant MCF-10A-NNK (DOX+) cells (Fig. 7F, bars 5-6). The parental MCF-10A cells which transiently transfected with $\alpha 9$ nAChR adenovirus were not transformed into colonies in soft-agar (Fig. 7F, bars 1-2). These results demonstrated that over expression of $\alpha 9$ nAChR in MCF-10A cells without NNK or nicotine treatment can not

induced malignant cells transformation.

We thought to determine whether induction of $\alpha 9$ nAChR in vivo in the presence or absence of nicotine stimulation would effectively promote tumor growth. Nude mice were injected subcutaneously with MCF-10A-Nic (DOX) and vector control cells. Mice with established tumors were treated with doxycycline in the drinking water. The MCF-10A-Nic (DOX)-xenografted tumor growth was significantly increased in response to nicotine treatment ($p < 0.01$) (Fig. 7G). The tumor growth induction in the nicotine-treated mice was potentiated upon withdraw of doxycycline ($p < 0.01$) (Fig. 7G). Doxycycline treatment had no significant effects on tumor growth in vector control mice ($p > 0.05$). The results recapitulate the tissue culture data and show that $\alpha 9$ nAChR over expression in human normal breast epithelial cells sensitize cells to transformation in response to nicotine exposure.

Discussion:

The activation of Akt and the promotion of breast cancer cell proliferation by tobacco components recapitulate biologic processes that may underlie the aggressive clinical outcomes for patients with tobacco-related cancers who continue to smoke. Moreover, these studies advance prior studies of nicotine and NNK by identifying nAChR $\alpha 9$ as the key cellular target of these tobacco components in established breast cancer cells. Activation of AKT through nAChRs by tobacco components has been observed in many bronchial or small airway epithelial cell systems relevant to lung cancer. The dose- and time-dependent activation of Akt by nicotine or NNK in breast cancer cells is similar to that observed in human lung epithelia cells. Likewise, activation of Akt by nicotine or NNK has also been observed with human breast cells at intermediate steps in the transformation process. Taken together, human breast cells throughout the phenotypic spectrum activate Akt in response to tobacco components in vitro. Inhibiting the ligand binding with nAChR $\alpha 9$ by tobacco components might therefore be a

valuable approach to mitigate the effects of smoking in smokers at risk for the development of breast cancer, and/or in breast cancer patients who continue to smoke.

Such approaches would need to consider the consequences of inhibiting nicotinic induction of nAChRs in other cell types, especially in neuronal tissues. In neuronal tissues, activation nAChRs by tobacco components might ironically confer health benefits, which could hypothetically complicate interventions suggested above. Many epidemiologic studies have shown that smokers are at decreased risk for the development of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. In vitro model systems of Alzheimer's disease have shown that nicotine-mediated protection of neuronal cells against α -amyloid-induced cytotoxicity is dependent upon Akt activation. Thus, would a therapeutic strategy against Akt tailored for breast cancer increase neuronal degeneration? Or down-regulation nAChR α 9 can be an alternative treatment for breast cancer therapy? Animal models may be useful to address this question. Activation of Akt by nicotine or NNK clearly propagated the Akt pathway in breast mammary cells because increased phosphorylation of multiple identified and unidentified downstream substrates of Akt was observed in a time-dependent manner. Which substrate(s) might be most important for determining cellular responses to tobacco components? The patterns of phosphorylation of downstream substrates were mostly similar for nicotine or NNK, but two potentially important differences were that nicotine, but not NNK, increased phosphorylation of MDM2 and mTOR. Phosphorylation of MDM2 by Akt can promote nuclear translocation of MDM2 and degradation of p53, which can clearly affect apoptosis. mTOR can also promote cellular proliferation and cellular survival, especially in cells with high levels of Akt activity. Thus, propagation of the Akt signal to MDM2 or mTOR might be responsible for nicotine-induced survival.

The identification of nAChR subunits in breast mammary cell lines and human breast tissues provides a mechanistic basis for Akt activation in response to tobacco components, and fills a gap in the analysis of nAChR subunit expression in cells derived from breast tissues. Once thought to be restricted to neuronal cells, nAChR subunit expression has now been demonstrated in human breast cells, MCF 7 cells and MDA MB 231 cells. In addition, nAChR have been described in keratinocytes and endothelial cells. This wide distribution of nAChR may underlie the systemic physiological responses to smoking.

Although other studies have shown that nicotine or other agonists of nAChR such as acetylcholine can promote the growth of SCLC cells, our study is the first to demonstrate that nicotine or NNK stimulates breast proliferation that is dependent upon nAChR α 9. The fact that expression of siRNA to nAChR α 9 inhibited nicotine- or NNK-induced proliferation of MDA MB 231 cells, suggests that other signaling pathways that might be increased by nicotine stimulation such as the AKT pathway. In addition to nAChR, Nicotine-induced Akt activation and proliferation of breast cells is probably due to stimulation of nAChR rather than b-adrenergic receptors. Regardless of the relative roles of different receptors at the cell surface, nicotine- or NNK-induced signaling converged on Akt, and proliferation was dependent upon Akt activation.

In addition to the neuronal cell systems discussed above, nicotine can promote survival of many cell types including breast cancer cells.

Finally, these studies highlight the complex biology of nicotine and tobacco. Nicotine, originally thought only to be responsible for tobacco addiction, is now recognized for modulation of key cellular proteins, such as Akt, cyclin D1, NFkB and bcl-2, and key cellular processes, such as increased proliferation and survival, two components of the transformed phenotype. If the conditions used in these studies were to be replicated in vivo, it is hypothetically possible that increased growth of nascent,

undiagnosed cancers and/or increased resistance to cytotoxic therapy could occur with exposure to nicotine. Because of the widespread availability and use of nicotine supplements in current or former smokers, further investigation on the sustained biological activities of nicotine is warranted.

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Figure 1.

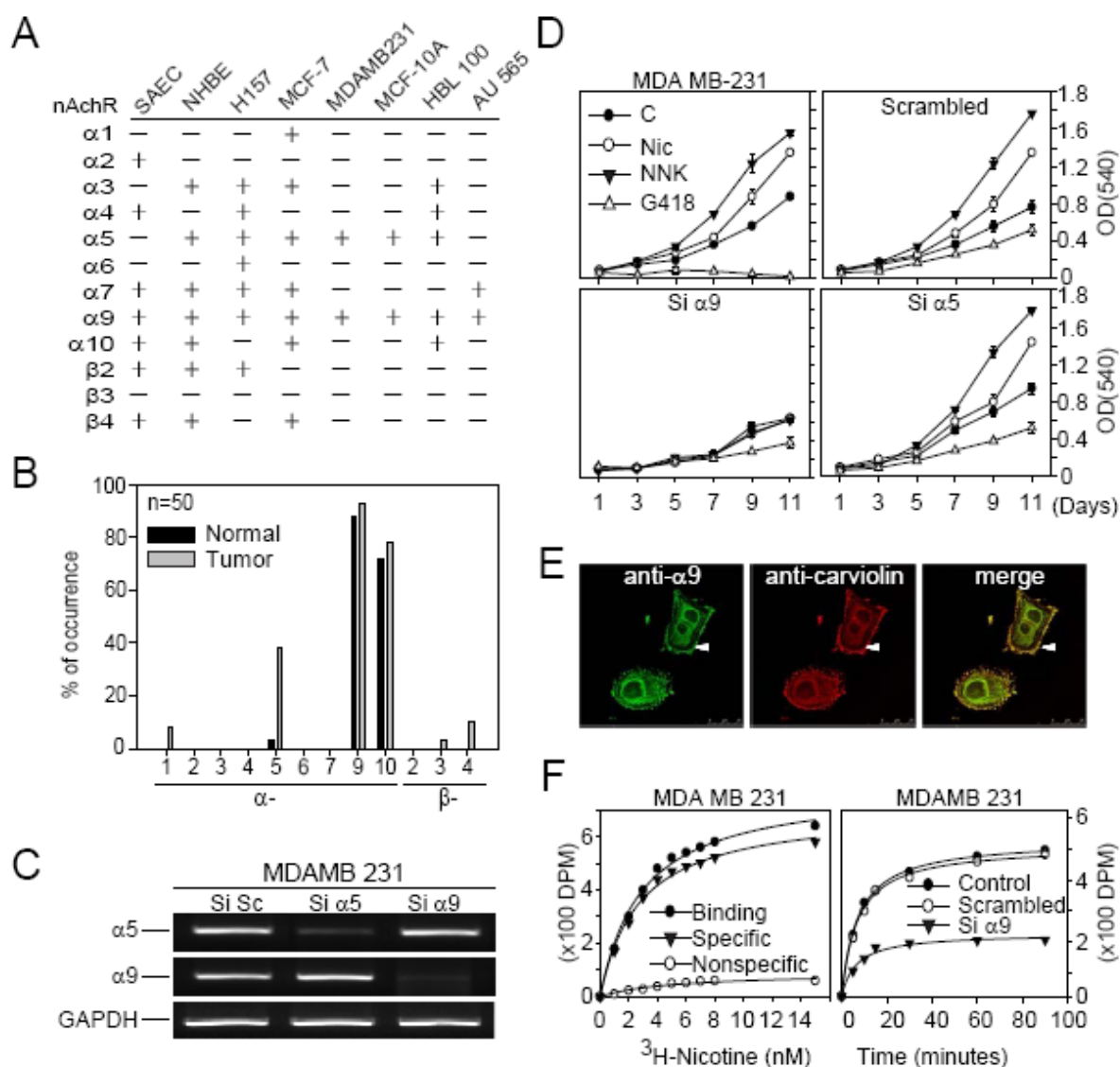


Figure 2.

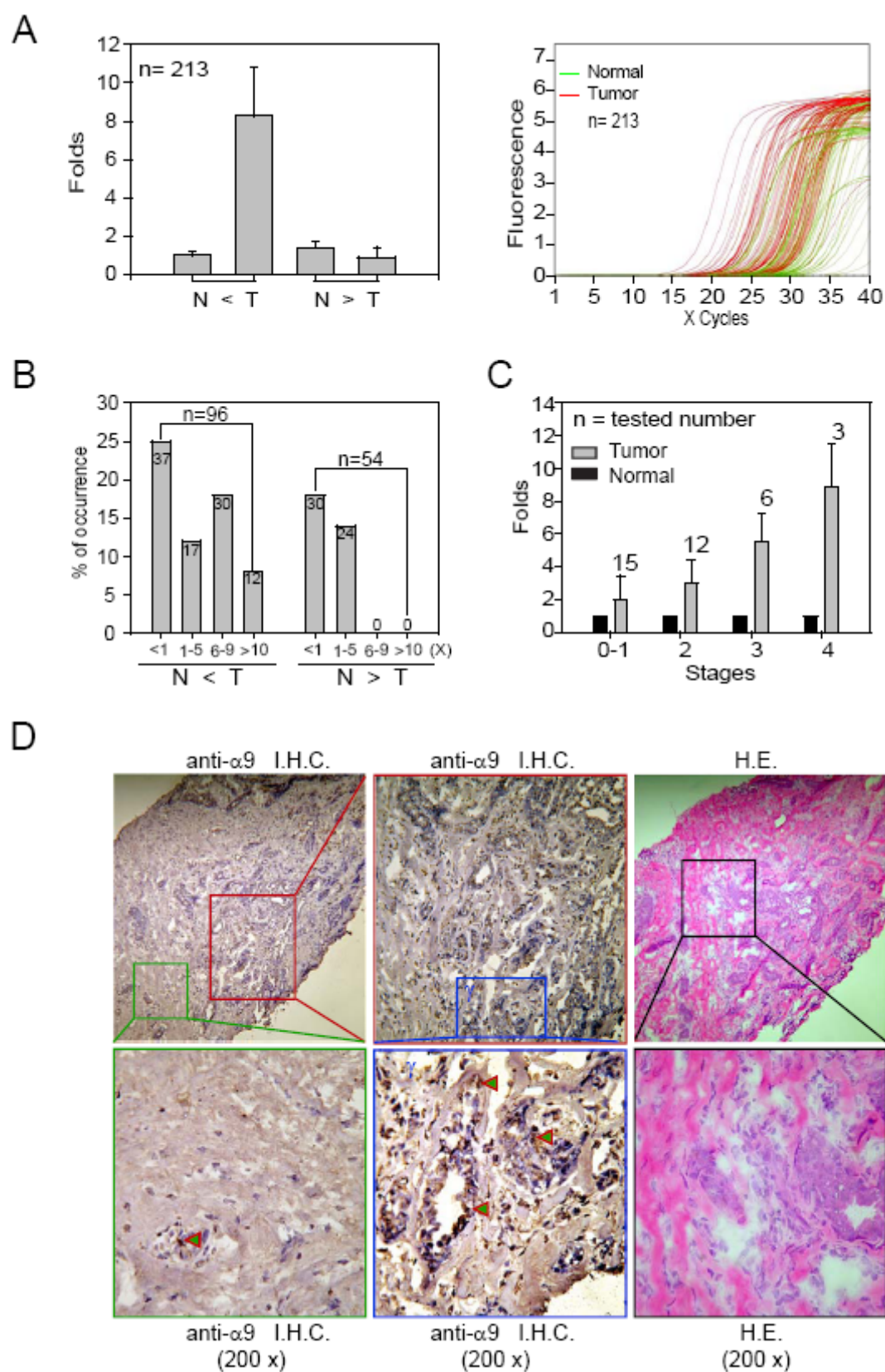


Figure 3.

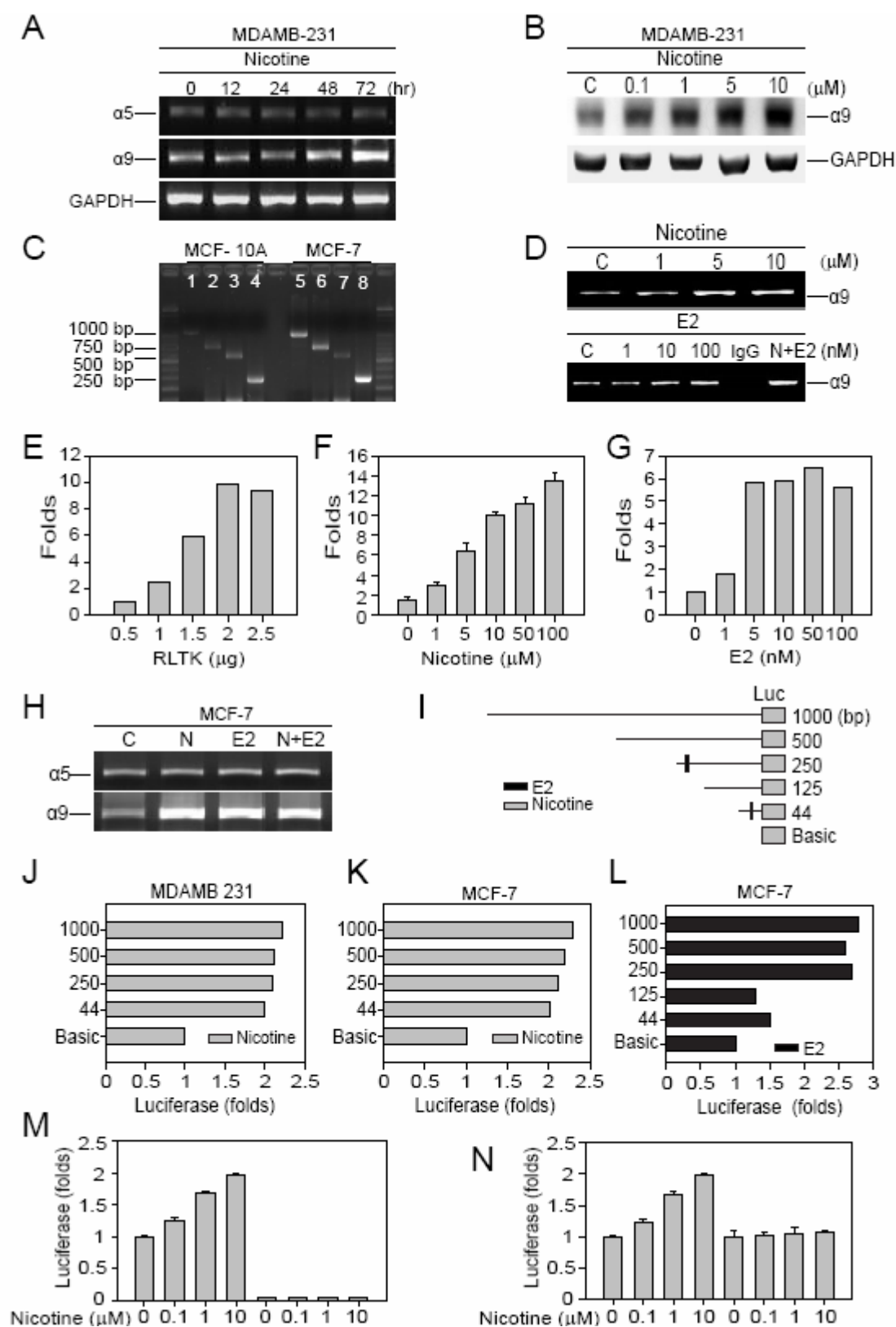


Figure 4.

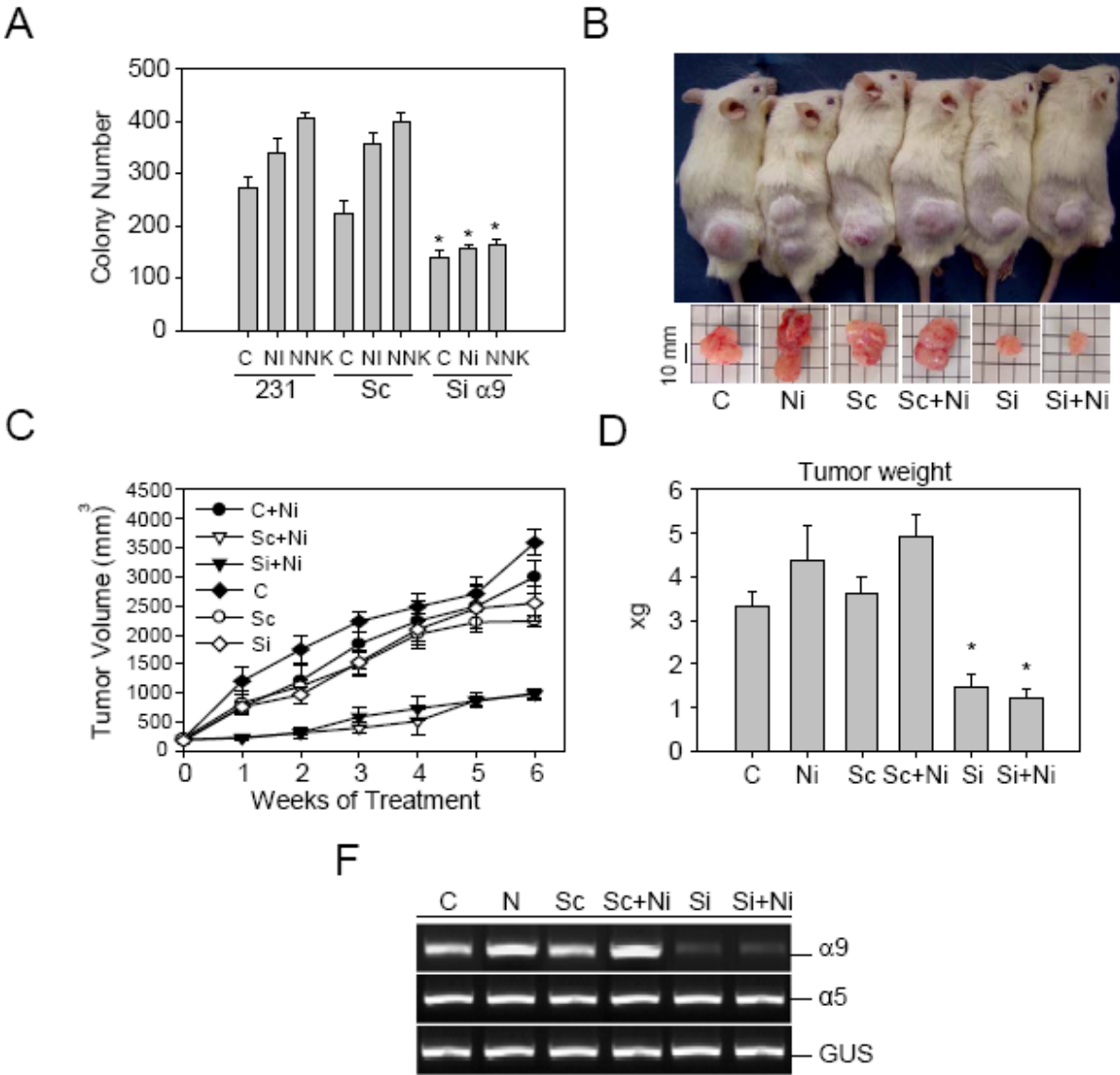


Figure 5.

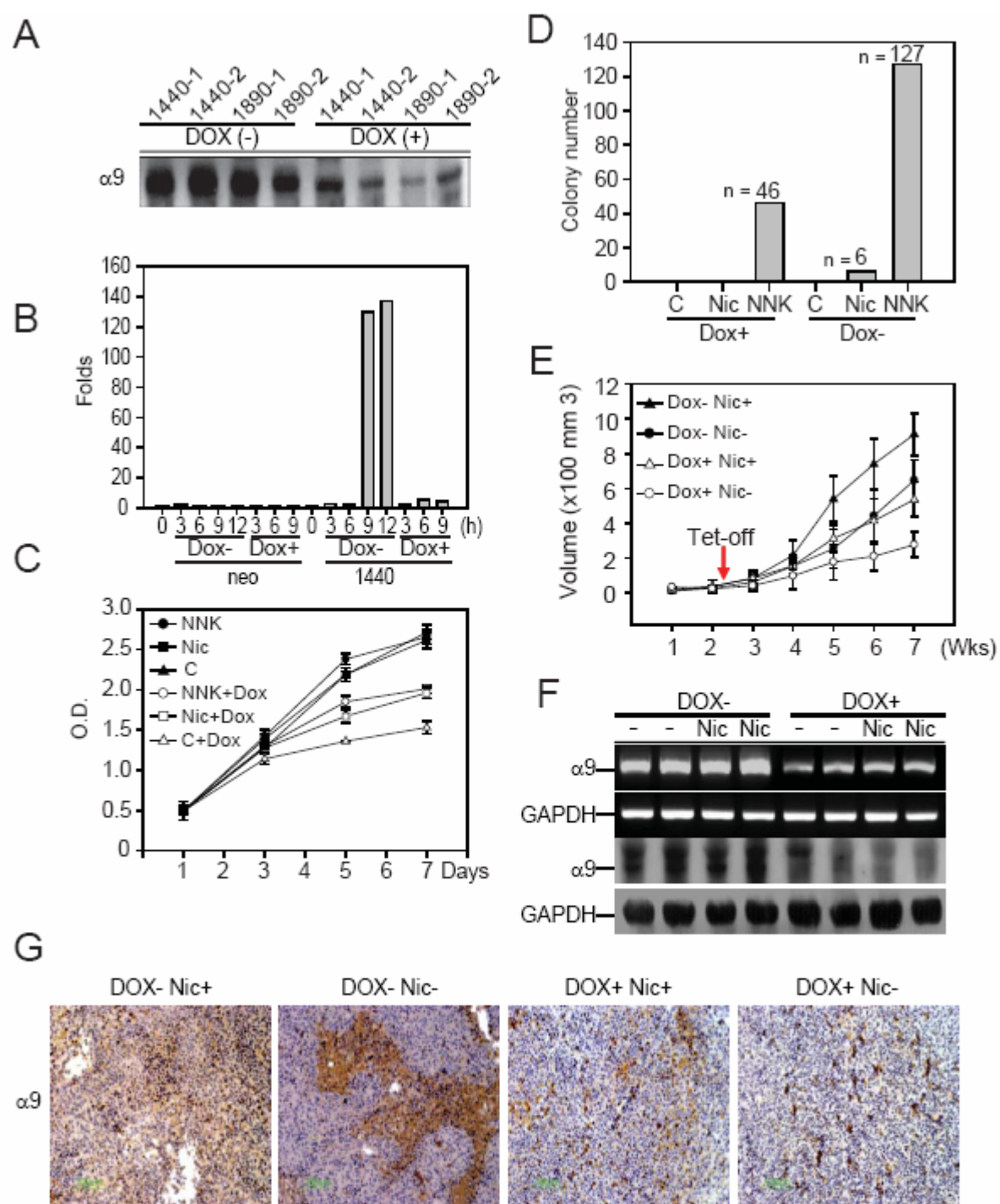


Figure 1:

(A) Agarose gel electrophoresis of the products from 2 ug from MDA MB-231, MCF-7 and MCF-10A RNA obtained by RT-PCR procedure designed to amplify nAChR $\alpha 1$ - $\alpha 7$, $\alpha 9$ - $\alpha 10$ and B2-B4. (B) 50 clinical breast cancer tissues were collected with their normal and tumor regions and analysis their nAChR $\alpha 5$ and nAChR $\alpha 9$ expression, respectively. (C) The distribution of nAChR subunits in 50 breast cancer cases. (D) The breast cancer tissues were analyzed their nAChR $\alpha 9$ gene expression by using Real time PCR. The green curves represent normal region of nAChR $\alpha 9$ expression, red color curves represent tumor parts. n=150. (E) A further analysis of nAChR $\alpha 9$ expression by dividing total cases into two groups, tumor over normal and normal over tumor. (F) More detailed analysis was using nAChR $\alpha 9$ expression folds between tumor and normal regions. Almost half of cases have at least 6 folds nAChR $\alpha 9$ expression in tumor parts than in normal parts. However, in the section of normal over tumor, none of a single case has found with over 6 folds nAChR $\alpha 9$ expression in normals than in tumors.

Figure 2:

Overexpression of nAChR $\alpha 9$ is presented on breast cancer tissue LCM and IHC. Increasing nAChR $\alpha 9$ mRNA expression in both total tissue region (A) and LCM (C) section assessed by real-time PCR. Clinical Breast cancer tissues were staged by American Journal of Critical Care (AJCC). (B) A breast adenocarcinoma section (upper panel represents normal selection, lower section was selected for tumor cells) photos are taken from HE staining, before, selected and after laser capture. Captured normal and cancer cells are in the last line and represent in green arrows. (D) NACHR $\alpha 9$ is over expressed in breast cancer cells than in normal cells. 10X nAChR $\alpha 9$ IHC (top corner on the left) has strong expression around cancer cells. Higher power field 20X (top middle) and 40X (second column in the middle) have more significant comparison then in 40X normal cells (second column on

the left). Confocal immunolabeling of MCF-7 cells. nAChR $\alpha 9$ antibody is labeled with FITC (lowest on the left- green color), and the caveolin-1 is labeled with Rhodamine (lowest in the middle- red color). Overlapping distribution yields the yellow color (lowest on the right).

Figure 3

Si scrambled, si nAChR $\alpha 5$ and si nAChR $\alpha 9$ was constructed into silencer vector and transfected into MB-231 cells. Confirm experiment was performed with RT-PCR (right panel). Stabled expression siRNA cell lines were treated with medium, nicotine or NNK for 11 days and counted with MTT substrate (B). Saturation binding of [3 H]-Nicotine to MB-231 cells (C, left panel). Non-specific binding activity added 10uM nicotine before [3 H]-Nicotine was adding. Specific-binding = Total binding – Non-specific binding. Wild type MB 231, si scrambled, and si nAChR $\alpha 9$ stable express cells were also confirmed by nicotine binding activities (C, right panel).

Figure 4:

Phosphorylation of Akt by nicotine or NNK in breast cancer cells and effect on downstream substrates. (A) We measured Akt kinase activity in MCF7 by immunoblotting active Akt and assessing phosphorylation of an exogenous peptide, after administration of nicotine for 0, 1, 10, 100 uM (left panels) or NNK for 0, 1, 10, 100uM (right panels). (B) Nicotine increased Akt phosphorylation was also in a time-dependent manner in MCF-7 cells, as assessed by immunoblotting with anti-phospho-S473 and anti-Akt antibodies. (C) Phosphorylation of substrates upstream and downstream of Akt was increased after administration of nicotine with time dependently increasing. LY294002 significantly inhibited nicotinic induction of Akt kinase activity. (D) Wildtype MDA MB-231, scrambled, nAChR $\alpha 9$ siRNA and LY294002 treatment cells were also examined with Akt kinase activity for nicotine treatment or not.

Figure 5:

nAChR $\alpha 9$ is induced by Nicotine and E2 via promoter derived regulation. (A) MDA MB-231 cells were treated with 10 μ M nicotine and performed RT-PCR to observe nAChR $\alpha 5$, $\alpha 9$ and GAPDH mRNA change. (B) nAChR $\alpha 9$ is induced by both nicotine and E2 after 24 hours incubation, whereas nAChR $\alpha 5$ remain unchanged. Both MCF-10A and MCF-7 can detect ER bound nAChR $\alpha 9$ promoter with 250, 500, 750, and 1000bps via CHIP assay (C). Treatment with nicotine (upper panel) and E2 (middle panel) dose dependent found increasing ER bound nAChR $\alpha 9$ promoter. Western Blotting also observed the same result with nicotine increasing nAChR $\alpha 9$ protein expression at 24 hours (lower panel). A full length of nAChR $\alpha 9$ (1000 bps) in PGL3 plasmid had the highest transfection efficiency with 2 μ g in a 30mm well (E). Both nicotine (F) and E2 (G) induced nAChR $\alpha 9$ promoter binding activities with peak at 10 μ M and 5 nM. (H) Serial deletion of 1000bps nAChR $\alpha 9$ promoter into 500, 250, 44 and basic in PGL3 plasmid were transfected into MB 231 (the middle panel) or MCF-7 cells (the right panel) with nicotine or E2. The firefly

luciferase was counted and normalized with renilla luciferase activity.

Figure 6:

Tumorigenicity and histological study of nicotine or NNK treatment on nAChR $\alpha 9$ knockdown MDA MB 231 cells. (A) Medium, nicotine or NNK were treated on wild type MDA MB 231, scrambled and nAChR $\alpha 9$ siRNA MDA MB 231 cells in colony formation assay. After two weeks, the colony number was counted with crystal violet staining under 10X microscopy observation. (B) Mice were injected with 1×10^7 cells of wild type MDA MB 231, scrambled and nAChR $\alpha 9$ siRNA MDA MB 231 cells mixed with Matrigel. Photograph of the animals bearing tumors 100 days after implantation of cells. During 100 days, every mice was measured with tumor volume every week (C). Xenografts removed from mice implanted with mentioned cells were placed on quadrille paper. The mice and tumors were weighted, respectively (D) (E) (F). (G) The tumors were finally examined nAChR $\alpha 9$ mRNA expression to ensure siRNA effects still remaining in the cells.

成果自評:

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研究人力訓練成果

本計劃相關參與人員均已獲得完整之訓練，其中完成碩士訓練並進修博士或出國者有數人：曾嘉珍(台大生化科學所博士畢業)，陳容甄(成大環醫所博士生)，翁孟仕(台大生化所博士畢業)，賴彥鎔(中研院生醫所博士生)，鄒瑋玲(陽明大學神經細胞生物所博士生)，李嘉華(台北醫學大學細胞分子生物學所博士生)，鄭自君(台北醫學大學醫學科學研究所博士生)，許文建(台北醫學大學醫學科學研究所博士生)其餘皆在研究單位或醫療機構擔任醫檢師或研究助理，另外本計劃所訓練之大學部學生眾多，無法一一系列也分別進入過內外研究所攻讀碩博士學位。