Int J Clin Exp Pathol 2016;9(4):4854-4861
www.ijcep.com /ISSN:1936-2625/IJCEP0023639

Original Article

Identification of acetylcholine-related enzymes and the role of acetylcholine and nicotine in human cervical cancer

Kuiran Liu[1], Yang Liu[2], Yanqiu Yu[2], Tianda Feng[3], Shulan Zhang[1]

[1]*Department of Gynecology and Obstetrics, Shengjing Hospital of China Medical University, Shenyang, China;*
[2]*Department of Pathophysiology, College of Basic Medical Science, China Medical University, Shenyang, China;*
[3]*Department of Neurosurgery, Shengjing Hospital of China Medical University, Shenyang, China*

Received January 9, 2016; Accepted March 22, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: Background and aims: Human cervical cancer ranks as the second most common type of female cancer worldwide. Nicotine, a component from tobacco smoke, can substitute for the natural transmitter acetylcholine (ACh) and have carcinogenic effects on non-neuronal cells. In this study, we investigated the role of acetylcholine-related enzymes in cervical cancer and the effect of acetylcholine and nicotine on cervical cancer cells. Methods: 24 clinical cases (normal cervix = 10, cervical squamous cancer = 14) and two kinds of human cervical cancer cell lines (Hela and SKG) were used in our study. Choline acetyltransferase catalyzing enzyme (ChAT), vesicular acetylcholine transporter (VAChT) and acetyl cholinesterase (AChE) were evaluated by immunohistochemistry and immunofluorescence assays. Further, we explored the role of acetylcholine and nicotine on the proliferation of cervical cancer cells, as well as the changes of VEGF expression. Results: ChAT, VAChT and AChE were both expressed in human cervical cancer tissue and cell lines. ChAT and VAChT were significantly up-regulated in cancer tissues than normal cervix. In addition, nicotine promoted the proliferation of Hela cells, accompanied by increasing VEGF expression. Conclusion: Acetylcholine-related enzymes existed in human cervical cancers. Nicotine has a significant positive correlation with cervical cancer cells proliferation, and its mechanism might be related to VEGF's elevation.

Keywords: Acetylcholine, choline acetyltransferase catalyzing enzyme, vesicular acetylcholine transporter, acetyl cholinesterase, nicotine, cervical cancer

Introduction

Human cervical cancer ranks as the second most common type of female cancer worldwide, with an annual global incidence of 530,000 new cases [1]. Despite advances in detection and prevention, there are still approximately 266,000 deaths each year according to the recent National Comprehensive Cancer Network (NCCN) Guidelines [2]. The persistent infection of the genitals with human papilloma viruses (HPVs) has been identified in the etiology of cervical cancer [3]. However, other mechanisms may also be involved in the occurrence of the tumors, because HPV infection is insufficient to account for all cases of cervical cancer. Thus, the etiology of cervical cancer still needs more molecular explanations to provide effective therapeutic strategy.

Nicotine, a components from tobacco smoke, have carcinogenic effects on non-neuronal cells [4]. Nicotine can substitute for the natural transmitter acetylcholine (ACh) to direct activate nicotinic acetylcholine receptors (nAChRs). ACh was identified as a neurotransmitter, and it is best known for its role in the central and peripheral nervous system [5]. According to classic understanding of ACh in nervous system, Ach is synthesized by choline and acetyl coenzyme A within cytoplasm through the limiting choline acetyltransferase catalyzing enzyme (ChAT). Ach could be transported to synaptic vesicles and stored by the action of the vesicular acetylcholine transporter (VAChT), and released into the synaptic cleft when the nerve is stimulated. In addition, it could be hydrolyzed by the acetyl cholinesterase (AChE). Therefore, cholinergic signaling depends on the availability

of ACh, which is modulated by ChAT, VAChT and AChE.

Accumulating evidences showed the non-neuronal functions of ACh in human, and play a specific role in biological characteristics of tumors [4]. The activation of the nAChRs in non-neuronal cells could affect cell biological behaviors, such as cell proliferation, growth and apoptosis [6]. Studies have proved the cholinergic signaling in epithelia of the airway in response to nicotine induced carcinogenesis [7, 8]. Recent study also showed that nicotine promoted the invasion and metastasis of colon cancer cells via the activation of the nAchRs [9], and induced self-renewal of pancreatic cancer stem cells [10]. In addition, numerous epidemiological studies found that tobacco smoking is the most important risk factors for the developing cervical cancer, and is one major risk factor in HPV-mediated cervical cancer [11]. Previous study has shown that nicotine up-regulated epidermal growth factor-receptors (EGFR) in cervical cancer cell lines [12]. However, the underlying molecular mechanism still needs to be well demonstrated.

In this study, we aimed to detect the expression of acetylcholine-related enzymes (ChAT, VAChT and AChE) in human cervical cancer tissues and cell lines. Furthermore, the effects of exogenous acetylcholine and nicotine on the proliferation and VEGF expression of cervical cancer cell lines were also investigated.

Materials and methods

Patients and samples

This study has been approved by ethical committee of the Shengjing Hospital of China Medical University, and informed consent was obtained from all the patients. Paraffin-embedded cervical tissue specimens (normal cervix = 10, cervical squamous cancer = 14) were collected from gynecology and obstetrics department of Shengjing Hospital, and sliced into 4 μm thickness. Normal cervix was obtained from the uterine cervix of patients who underwent a total hysterectomy due to benign uterine diseases. Histologically classified and graded were identified by at least two experienced clinical pathologists, and cervical cancer tissues were squamous cancers. The International Federation of Gynecology and Obstetrics (FIGO) stage for these cancers are from I to IIa which were estimated by at least two clinical gynecologists.

Cell lines and culture

The classic cervical squamous cancer cell line Hela was derived from our laboratory, and SKG-II cell line was a generous gift from Professor Shulan Zhang. Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). All the cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Immunohistochemistry

The immunohistochemistry Ultra SensitiveTM S-P assays KIT-9710, 9790 were purchased from China Maixin Biotechnology limited company. The operations were followed by the manufacturer's protocol. Following being dried, deparaffinized in xylene, dehydrated in gradient ethanol, high pressure epitopes' retrieved, endogenous peroxidases' incubation for 15 minutes, slides were blocked with 5% non-immuno-bovine serum for 30 minutes. They were then incubated with primary antibodies for ChAT (Santa Cruz Biotechnology, Santa Cruz, CA, USA), VAChT (Abcam, Cambridge, MA, USA), AChE (Merck Millipore, Darmstadt, Germany), VEGF (R&D Systems, Minneapolis, MN, USA). These slices were rewashed with PBS and incubated with secondary antibodies for 1.5 hours at 37°C. Diaminobenzidine (DAB) solution was added for 3 minutes. Then hematoxylin was used to restain nuclei and examined by light microscopy. All tests were accompanied by negative control with PBS replacing primary antibodies. IHC analysis were performed by two separate pathologists with scoring 0, 1, 2, 3 respectively stand for none, weak, moderate and strong positive for mean vision of staining slides.

Immunofluoresence assays

The cervical squamous cancer cell lines Hela and SKG-II were cultured on sterile glasses, washed briefly with PBS, fixed with 4% paraformaldehyde for 30 minutes, and permeated with 0.2% Triton X-100 for 20 minutes, followed by incubation in 5% BSA for 60 minutes. Cells were then incubated with the above primary antibodies overnight at 4°C. After washes with PBS, cells were incubated with secondary antibodies for 1 hour and finally were analyzed

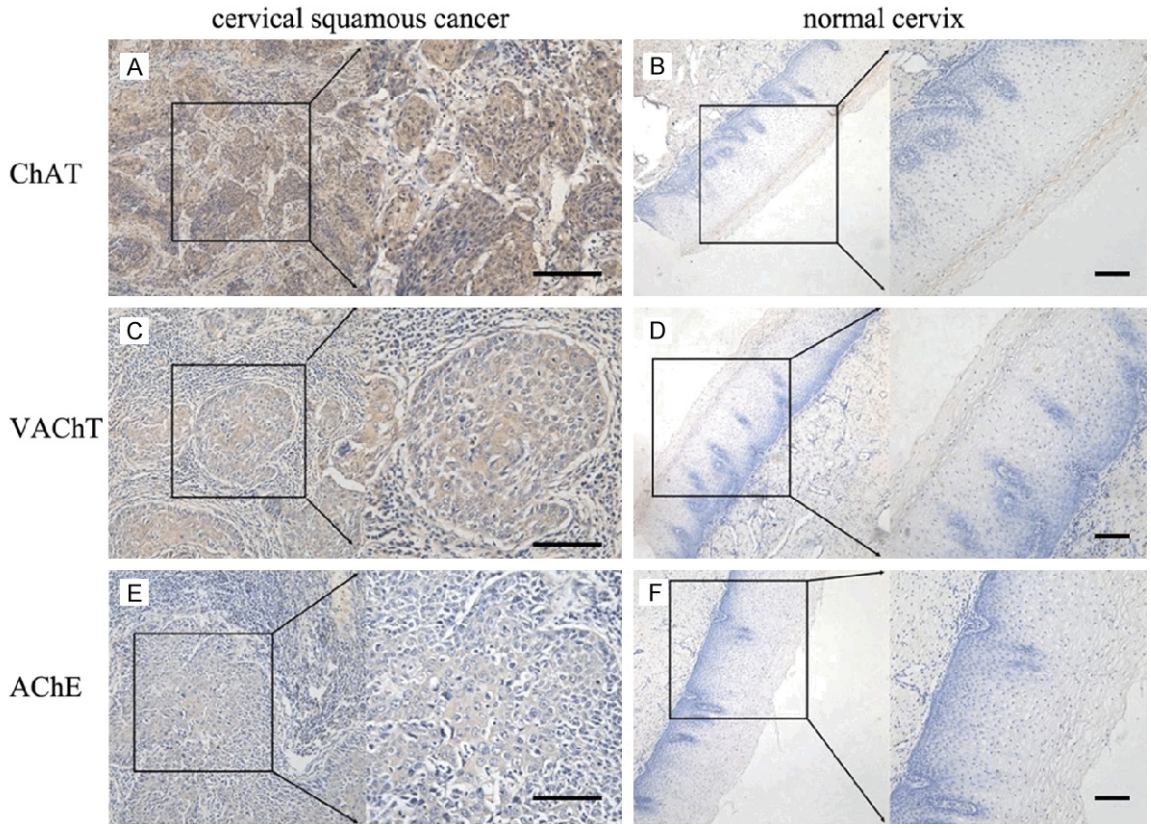

Figure 1. Immunohistochemical staining for the acetylcholine-related enzymes (ChAT, VAChT and AChE) in cervical cancer and normal cervix tissues. A and B. ChAT staining in cervical squamous cancer and normal cervix. C and D. VAChT staining in cervical squamous cancer and normal cervix. E and F. AChE staining in cervical squamous cancer and normal cervix. Scale bars represent 100 μm.

using fluorescence microscopy similarly; all tests were accompanied by negative control with PBS replacing primary antibodies.

Cell proliferation assay

Cell proliferation was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Hela cells and SKG cells were seeded at 1000 per well and 2000 per well in 96-well plates overnight, respectively. Culture medium was replaced with fresh medium supplemented with 2% RPIM 1640 (for Hela cells) or 10% RPIM 1640 (for SKG cells) containing different concentrations of nicotine (0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, 500 μM), acetylcholine chloride (0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, 500 μM), acetylcholine bromide (0.1 μM, 1 μM, 10 μM, 100 μM) and acetylcholine muscarinic receptor blocking agent atropine (0.1 μM, 1 μM, 10 μM) (Sigma-Aldrich, St Louis, MO, USA). Cells were then incubated for 48 hours or 96 hours in 5% CO_2 at 37°C, and the medium were refreshed every two days. After that, 20 μl of CellTiter 96® AQueous One Solution Reagent was added into each well. The plate was incubated at 37°C for 2 hours and records the absorbance at 492 nm.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). For immunohistochemistry scoring between cancer groups and normal epithelium, Fisher exact test (2-tailed) were used. For cell proliferation assay, statistical analysis was performed using One-Way ANOVA. $P < 0.05$ was considered to be significant.

Results

Expression of ChAT, VAChT and AChE in clinical specimens

We first detected the expression of ChAT, VAChT and AchE in 14 cervical squamous cancers and

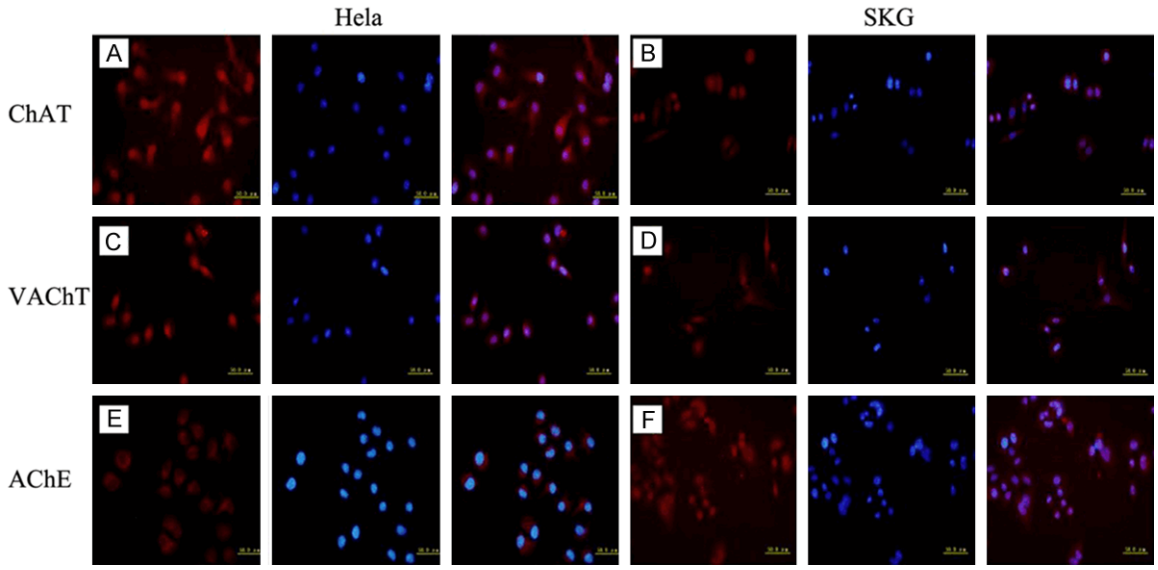

Figure 2. Expression of ChAT, VAChT and AChE in cervical cancer cell lines. A and B. The expression of ChAT in Hela and SKG cells. C and D. The expression of VAChT in Hela and SKG cells. E and F. The expression of AChE in Hela and SKG cells. Scale bars represent 50 μm.

10 normal cervical specimens. Scoring methods have narrated before. With scoring for each group separately, ChAT were moderated positively expressed in cancer group and higher than control group (mean values, 1.43 vs. 0.1, P = 0.002), VAChT were moderated positively expressed in cancer group and higher than control group (mean values, 1.36 vs. 0.2, P = 0.013). However, no significant change in the expression of AChE was detected (P = 0.357) (**Figure 1**).

In addition, other related molecular expressions were also detected. Our results showed that acetylcholine nicotinic receptor α5 and dopamine beta hydroxylse was strongly positive in both groups, acetylcholine muscarinic receptor 3 was negative in both groups, and synaptophysin is almost negative in both groups (data not shown).

Expression of ChAT, VAChT and AChE in cervical cancer cell lines

We next assessed the expression of ChAT, VAChT and AChE in cervical cancer cell lines by immunofluoresence assays. In both cervical cancer Hela cells and in SKG cells, ChAT, VAChT and AChE were all expressed strongly (**Figure 2**).

Nicotine promoted the proliferation of Hela cells and SKG cells in a dose-dependent manner

Different concentrations of nicotine (0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, 500 μM) were added into culture medium of Hela cells and SKG cells and incubation for 48 hours or 96 hours. In both cell lines, the proliferation was promoted in a dose-dependent manner. In addition, OD value of each time point was significantly different compared with control group (**Figure 3**). However, when cells were treated with a wide range of acetylcholine doses, no evidence showed the effects on the proliferation of both cervical cancer cell lines.

VEGF expression was up-regulated in Hela cells by nicotine

After 10 μM nicotine treated with Hela cells for 48 h or 72 h, VEGF expression was detected with immunocytochemistry. Our results showed that VEGF expression was up-regulated by nicotine (**Figure 4**).

Discussion

Classic neurotransmitters are secreted by nerve cells or a small number of neuroendocrine cells, and then interact with specific

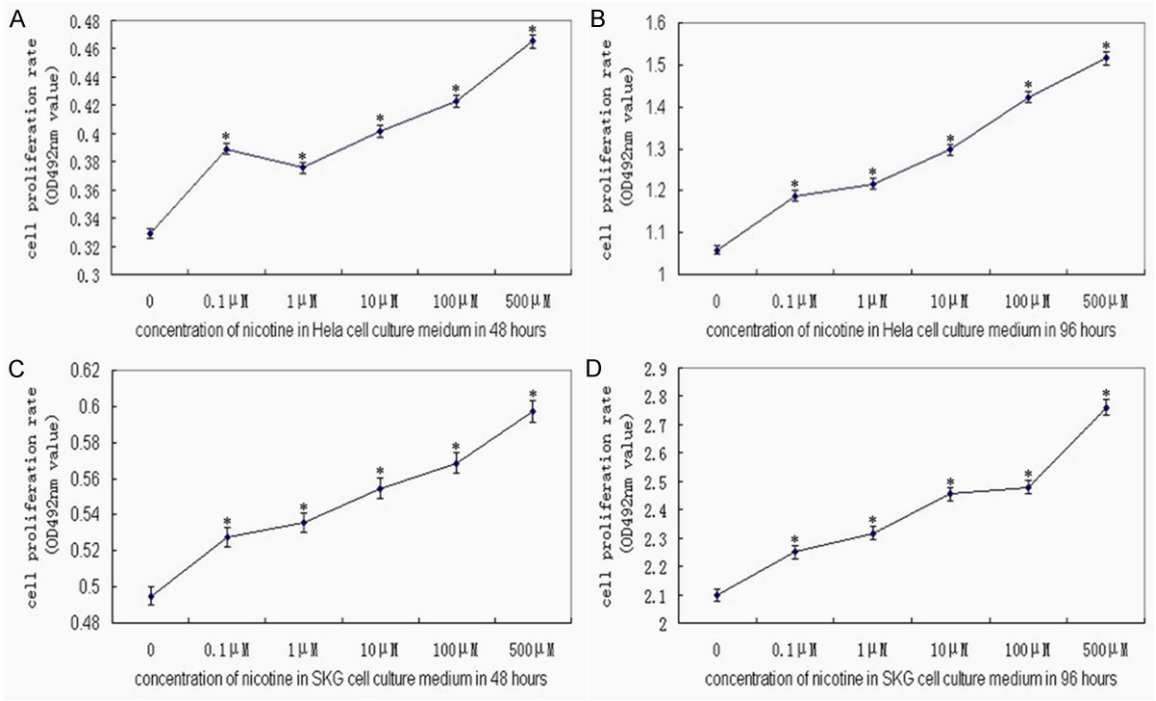

Figure 3. Nicotine promoted the proliferation of cervical cancer cell lines. A. Nicotine promoted Hela cell proliferation in 48 hours. B. Nicotine promoted Hela cell proliferation in 96 hours. C. Nicotine promoted SKG cell proliferation in 48 hours. D. Nicotine promoted SKG cell proliferation in 96 hours. X axis in all images refers to different concentration of nicotine. Y axis refers to the actual OD value at 492 nm by using cell proliferation assay. Data are presented as the mean ± SD (n = 5, each group). $^{*}P < 0.05$ vs. 0 μM group.

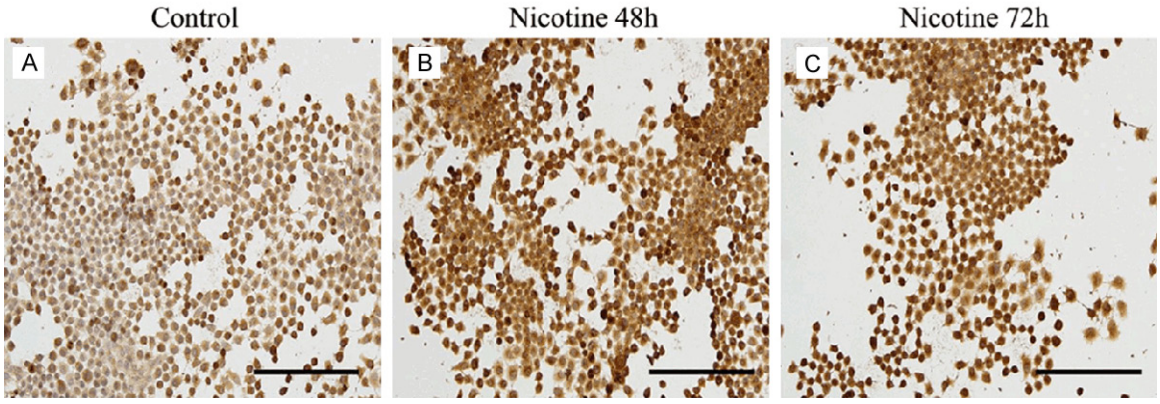

Figure 4. Effect of nicotine on the VEGF expression in Hela cells. A. Hela cells without nicotine treatment. B. VEGF expression after cells treated with 10 μM nicotine in 48 hours. C. VEGF expression after cells treated with 10 μM nicotine in 72 hours. Scale bars represent 200 μm.

receptors to perform biological effects. They also exist in the central and peripheral tissue, and have traditionally been identified that they perform regulative function through the nerves-body fluids immunity network. Carcinogenic factors, such as physical and chemical toxicant, may lead regional cells to mutant and abnormally secrete some hormones, cytokines and neurotransmitters. In turn, deregulated expression of those factors may have malignant effects.

Previously, microbiologists had found acetylcholine in some bacteria [13], and begun to investigate the function of acetylcholine related to non-neural cells in microbiology and bota-

ny. Accumulating reports have gradually demonstrated that acetylcholine did exist in different types of cells in humans until 1978 [14]. Therefore, the understanding of acetylcholine restricted to neuroscience has been broken [15]. Although acetylcholine has been proved exist in several cancer cells and had effects on cell proliferation in recent years, the mechanism is still to be well demonstrated. In order to investigate the connectivity between acetylcholine and cervical cancer, the expression of acetylcholine-related enzymes (ChAT, VAChT and AChE) were detected. Our results showed that ChAT and VAChT was increased in cervical squamous cancer specimens compared with that in normal cervix specimens. However, no significant change in the expression of AChE was detected. Further, we assessed the expression of ChAT, VAChT and AChE in cervical cancer cell lines by immunofluoresence assays, and results showed that ChAT, VAChT and AChE were also expressed in Hela and SKG cell lines. Since these three enzymes are required for acetylcholine metabolism, and our results showed that enzymes exist in both human cervical cancer tissue and cancer cells, the acetylcholine expression in cancer tissues might possibly be related to cervical cancer development. The cholinergic innervations in normal cervix have rarely been explored. R. E. Papka and his group used rapid specimens frozen section accompanied with radioactive immuno-electron microscopy techniques to detect ChAT and VAChT distribution in cholinergic neurons of cervical tissue, and they found both of the two enzymes were related to regulate contract and diastole vessels [16]. In addition, classic acetylcholine release is in the form of vesicles. Synaptophysin is the specific membrane protein of synaptic vesicle, which can affect synaptic vesicle exocytosis [17]. Thus, we detected the synaptophysin expression in cervical cancer tissues and cell lines, and the result was negative. These results suggested that acetylcholine may be not released in the form of synapse in human cervical cancer.

In our study, we added different concentrations of drugs including acetylcholine chloride (0.1 μM, 1 μM, 10 μM, 100 μM, 500 μM), its inhibitor acetylcholine bromide (0.1 μM, 1 μM, 10 μM, 100 μM) and acetylcholine muscarinic receptor blocking agent atropine (0.1 μM, 1 μM, 10 μM) in cultured medium of Hela cells and SKG cells. After culturing 2 days, 4 days, 6 days, 8 days, no significant differences were found between control group and drug-treated groups. These results were not consistent with previous studies in other types of cancer [18-20]. The reason for this might be due to the time of drug treatment was not enough in the proliferation assay of Hela and SKG cells, which need to be further investigated. Nicotine is an activator of nicotinic acetylcholine receptor. The relativity of smoking and cervical cancer has been confirmed [11, 12]. Our study showed: 1. Nicotine promoted the proliferation of Hela cells and SKG cells in dose-dependent manner. 2. Nicotine up-regulated the expression of VEGF in Hela cells. VEGF is an angiogenic factor, these results partly suggested that the carcinogenesis effect of nicotine might be the promotion of cancer angiogenesis.

In cholinergic system there are two classic receptors, M and N receptors. N receptors are located in the cell membrane, constituted by the five subunits of hetero or homo pentamers that enclose a central ion channel. The variety of N receptor subtypes dependent on different cell types in human tumors, normal cells and tissues [6]. However, as to its morphological structure and function of different classic N receptor is not known, its mechanism is still being explored. The distribution of N receptors in cervical cancer cell lines has been demonstrated by PCR, they showed that α5, α7, α9, β1 and ε subunits mRNAs are positive and weak signals from α4, β2, β4, γ and δ subunit mRNAs [21]. Activation of homo pentamers or hetero pentamers could promote the growth of most tumors, this effect involves three aspects. Firstly, the stimulation of VEGF can provide the tumor a better blood and nutrient supply [22, 23]. Secondly, activates adenylate cyclase and in steps activates intracellular signals related to growth and reproduction of cells. Thirdly, GABA and glutamine recruitment which come from hetero pentamers activation and homo pentamers activation separately, can promote the production and secretion of neurotransmitters such as catecholamine. Both of the receptors' activation will lead to the influx of calcium ions. As to calcium ions as a very important second messenger, which can cause the activation of adenylate cyclase, a series of complex

intracellular signal transduction can be followed.

N receptor's activation leading to the acceleration of tumor cell proliferation have been reported in considerable numbers of publication, but the role of acetylcholine in tumors has only been demonstrated in a limited variety of cancers [18-20]. Acetylcholine is one of N receptors activator which can result activation of all kinds of N receptors, while nicotine could just activate N receptor complex containing the α subunit. However, previous study showed that this classic understanding of acetylcholine and nicotine with their spectrum of receptors has broken [24]. A more clear understanding of N receptors from structure to function in malignant tumors is necessary to a great extent. To date, we could not give an explicit explain of differences between acetylcholine activation of N receptors and nicotine's, which needs to be further investigated.

In conclusion, we have shown that ChAT, VAChT and AchE were expressed in human cervical squamous cancer specimens and cell lines, and ChAT and VAChT was increased in cervical squamous cancer specimens. Moreover, nicotine has a significant positive correlation with cervical cancer cells proliferation, and its mechanism might be related to VEGF's elevation. Therefore, the elucidation of molecular mechanism provided potential therapeutic targets for the treatment of human cervical cancer.

Acknowledgements

The work was supported by the National Natural Science Foundation of China (81071738).

Disclosure of conflict of interest

None.

Address correspondence to: Shulan Zhang, Department of Gynecology and Obstetrics, Shengjing Hospital of China Medical University, Shenyang 110004, China. Tel: +86 24 96615-40131; Fax: +86 24 96615-40131; E-mail: zhangslcmu@163.com

References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] Koh WJ, Greer BE, Abu-Rustum NR, Apte SM, Campos SM, Cho KR, Chu C, Cohn D, Crispens MA, Dorigo O, Eifel PJ, Fisher CM, Frederick P, Gaffney DK, Han E, Huh WK, Lurain JR, 3rd, Mutch D, Fader AN, Remmenga SW, Reynolds RK, Teng N, Tillmanns T, Valea FA, Yashar CM, McMillian NR and Scavone JL. Cervical Cancer, Version 2.2015. J Natl Compr Canc Netw 2015; 13: 395-404; quiz 404.

[3] Rodriguez AC, Schiffman M, Herrero R, Hildesheim A, Bratti C, Sherman ME, Solomon D, Guillen D, Alfaro M, Morales J, Hutchinson M, Katki H, Cheung L, Wacholder S and Burk RD. Longitudinal study of human papillomavirus persistence and cervical intraepithelial neoplasia grade 2/3: critical role of duration of infection. J Natl Cancer Inst 2010; 102: 315-324.

[4] Grando SA. Connections of nicotine to cancer. Nat Rev Cancer 2014; 14: 419-429.

[5] Kalamida D, Poulas K, Avramopoulou V, Fostieri E, Lagoumintzis G, Lazaridis K, Sideri A, Zouridakis M and Tzartos SJ. Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity. FEBS J 2007; 274: 3799-3845.

[6] Schuller HM. Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nat Rev Cancer 2009; 9: 195-205.

[7] Improgo MR, Tapper AR and Gardner PD. Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer. Biochem Pharmacol 2011; 82: 1015-1021.

[8] Arredondo J, Nguyen VT, Chernyavsky AI, Jolkovsky DL, Pinkerton KE and Grando SA. A receptor-mediated mechanism of nicotine toxicity in oral keratinocytes. Lab Invest 2001; 81: 1653-1668.

[9] Xiang T, Fei R, Wang Z, Shen Z, Qian J and Chen W. Nicotine enhances invasion and metastasis of human colorectal cancer cells through the nicotinic acetylcholine receptor downstream p38 MAPK signaling pathway. Oncol Rep 2016; 35: 205-210.

[10] Al-Wadei MH, Banerjee J, Al-Wadei HA and Schuller HM. Nicotine induces self-renewal of pancreatic cancer stem cells via neurotransmitter-driven activation of sonic hedgehog signalling. Eur J Cancer 2015; 52: 188-196.

[11] Castellsague X and Munoz N. Chapter 3: Cofactors in human papillomavirus carcinogenesis--role of parity, oral contraceptives, and tobacco smoking. J Natl Cancer Inst Monogr 2003; 20-28.

[12] Mathur RS, Mathur SP and Young RC. Up-regulation of epidermal growth factor-receptors (EGF-R) by nicotine in cervical cancer cell lines: this effect may be mediated by EGF. Am J Reprod Immunol 2000; 44: 114-120.

[13] Ewins AJ. Acetylcholine, a New Active Principle of Ergot. Biochem J 1914; 8: 44-9.
[14] Sastry BV and Sadavongvivad C. Cholinergic systems in non-nervous tissues. Pharmacol Rev 1978; 30: 65-132.
[15] Wessler I and Kirkpatrick CJ. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. Br J Pharmacol 2008; 154: 1558-1571.
[16] Papka RE, Traurig HH, Schemann M, Collins J, Copelin T and Wilson K. Cholinergic neurons of the pelvic autonomic ganglia and uterus of the female rat: distribution of axons and presence of muscarinic receptors. Cell Tissue Res 1999; 296: 293-305.
[17] Thiel G. Synapsin I, synapsin II, and synaptophysin: marker proteins of synaptic vesicles. Brain Pathol 1993; 3: 87-95.
[18] Song P, Sekhon HS, Fu XW, Maier M, Jia Y, Duan J, Proskosil BJ, Gravett C, Lindstrom J, Mark GP, Saha S and Spindel ER. Activated cholinergic signaling provides a target in squamous cell lung carcinoma. Cancer Res 2008; 68: 4693-4700.
[19] Cheng K, Samimi R, Xie G, Shant J, Drachenberg C, Wade M, Davis RJ, Nomikos G and Raufman JP. Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 2008; 295: G591-597.
[20] Zhao Y, Wang X, Wang T, Hu X, Hui X, Yan M, Gao Q, Chen T, Li J, Yao M, Wan D, Gu J, Fan J and He X. Acetylcholinesterase, a key prognostic predictor for hepatocellular carcinoma, suppresses cell growth and induces chemosensitization. Hepatology 2011; 53: 493-503.
[21] Calleja-Macias IE, Kalantari M and Bernard HU. Cholinergic signaling through nicotinic acetylcholine receptors stimulates the proliferation of cervical cancer cells: an explanation for the molecular role of tobacco smoking in cervical carcinogenesis? Int J Cancer 2009; 124: 1090-1096.
[22] Konishi H, Wu J and Cooke JP. Chronic exposure to nicotine impairs cholinergic angiogenesis. Vasc Med 2010; 15: 47-54.
[23] Lane D, Gray EA, Mathur RS and Mathur SP. Up-regulation of vascular endothelial growth factor-C by nicotine in cervical cancer cell lines. Am J Reprod Immunol 2005; 53: 153-158.
[24] Carlisle DL, Liu X, Hopkins TM, Swick MC, Dhir R and Siegfried JM. Nicotine activates cell-signaling pathways through muscle-type and neuronal nicotinic acetylcholine receptors in non-small cell lung cancer cells. Pulm Pharmacol Ther 2007; 20: 629-641.