

β -carotene reverses tobacco smoke-induced gastric EMT via Notch pathway *in vivo*

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Abstract. Tobacco smoke is one of the serious risk factors of gastric cancer. Epithelial-mesenchymal transition (EMT) has been shown to be associated with the initiation and carcinogenesis of gastric cancer. The role of Notch pathway in regulating tobacco smoke-induced EMT has not been investigated. β -carotene, a carotenoid present in fruits, vegetables and rice, suppresses cancer progression. In this investigation, we evaluated the regulatory role of Notch pathway in tobacco smoke-mediated gastric EMT and the preventive effect of β -carotene using a BALB/c mouse smoking model. Exposure of mice to tobacco smoke reduced levels of epithelial markers, while the expression of mesenchymal markers were increased. We further found that Notch pathway modulated tobacco smoke-triggered EMT in the stomach of mice, as evidenced by these findings that tobacco smoke activated Notch activities, and tobacco smoke induced EMT was reversed by blocking Notch activities with FLI-06. Moreover, treatment of β -carotene prevented tobacco smoke-mediated activation of Notch and EMT changes. Our data suggested that Notch regulate tobacco smoke induced gastric EMT and the protective effects of β -carotene *in vivo*. These findings may establish a new mechanism for tobacco smoke-associated gastric tumorigenesis and its chemoprevention.

Introduction

Gastric cancer is an important cancer type occurring in the upper digestive tract, accounting for 8% of cancer cases and

10% of deaths globally (1,2). It is estimated that there are approximately 750,000 new cases diagnosed annually, and 5-year overall survival rates are <25% (3). Due to its highest morbidity and mortality, gastric cancer remains a noticeable public health problem in East Asia (4). Although great efforts have been made, early diagnosis and current treatments for this deadly cancer are still not efficacious. Therefore, it is important to investigate the molecular mechanisms involved in the transformation and progression of gastric cancer.

Many factors are associated with the initiation and progression of gastric cancer: genetic variations, infectious agents, environmental factors, dietary factors, and pathological conditions in the stomach (5). Increasing evidence has showed the positive link between tobacco smoke and gastric cancer (6-11). Many compounds found in tobacco smoke are known to induce free radicals, possess toxic properties and carcinogenic activities, there by contributing to its potential impact on the transformation and progression of many cancers (7,12). Although enormous progress in understanding its molecular pathogenesis leading to gastric cancer has been made, the underlying mechanisms by which tobacco smoke induce gastric cancer are not fully understood.

Epithelial-mesenchymal transition (EMT) is a cell biological processes that is very important in various aspects including embryonic development, cancer progression stem cell biology, wound healing and fibrosis (13). EMT is characterized by downregulation of the intercellular tight junctions and acquire certain properties of mesenchymal cells. Emerging evidence has revealed that, in addition to facilitating invasion and metastasis, EMT is also critically involved in the initiation of tumorigenesis. Exposure of cells to carcinogens, such as tobacco smoke, benzo(a) pyrenediolepoxide, arsenite and methylnitrosourea, induced EMT during cell transformation and tumor formation (14-17). Studies have documented that tobacco smoke promote the occurrence of EMT process (18-20). Tobacco smoke triggered EMT has been found to regulate early events in carcinogenesis: the loss of polarized organization of epithelial tissue, cell-cell junction breakdown, cell-atrix adhesion remodeling, and gain properties of mesenchymal cells with invasive capacity. Nonetheless, the underlying mechanisms by which tobacco smoke induces EMT and the signaling events that underlie EMT are poorly understood.

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Notch signaling regulates a series of cellular processes (21). In addition, the Notch pathway has been reported implicated in a majority of cancers for promoting the malignant phenotype by inducing cell proliferation, drug resistance, resistance to apoptosis, invasion and metastasis (22). Several studies have revealed that Notch pathway is critically involved in the process of EMT (21,23,24). Nonetheless, no studies have been done to examine the role of Notch pathway in tobacco smoke-induced gastric EMT.

Some studies have illustrated that dietary phytochemicals with potent anticancer activity are present in food-based diets. β -carotene is one of the most abundant carotenoids, abundant in carrot, spinach, kale, pink guava yams, palm oil, and sweet potato (25). β -carotene is found to elicit profound effects on the maintenance of human health and disease prevention (26). Numerous studies have consistently demonstrated that β -carotene reduce the risk of developing many diseases such as cardiovascular diseases (27-29), cataract formation (30,31), age-dependent macula degeneration (32), and different types of cancer including stomach, bladder, mouth, pharynx, colon, larynx, esophagus, rectum, and cervix (33,34). However, its effect on tobacco smoke triggered gastric EMT has not been defined.

Herein, we aimed to investigate Notch pathway regulation of tobacco smoke elicited EMT alterations and the preventive effects of β -carotene against tobacco smoke induced EMT in the mouse stomach. We found that Notch regulated tobacco smoke-mediated gastric EMT and the protective effects of β -carotene in tobacco smoke-elicited ERK5 activation and EMT in the stomach tissue by using mouse tobacco smoke exposure models. These findings indicated that Notch pathway play an important regulatory role and the chemopreventive effect of β -carotene in tobacco smoke-associated gastric pathological alterations.

Materials and methods

Reagents and antibodies. Primary antibodies against Notch-1, ZO-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against NICD, Hes-1, E-cadherin, Snail-1, N-cadherin, and vimentin was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-CK5 antibody and anti-GAPDH antibody were from Biogot Technology (Nanjing, China). β -carotene was purchased from Sigma (St. Louis, MO, USA; purity $\geq 97\%$). FLI-06 was purchased from Tocris Bioscience (Bristol, UK). All the primers were synthesized by Invitrogen (Carlsbad, CA, USA) according to published sequences. Sources of other materials are noted in the relevant context.

Experimental animals and treatment. All experiments were performed in accordance with the recommendations in the guidelines of the Laboratory Animal Management Committee of Jiangsu University concerning the care and treatment of experimental animals. The study was approved by the Committee on the Ethics of Animal Experiments of Jiangsu University. A total of 60 male BALB/c mice (6-8 weeks of age) weighing 18-22 g were used in the current experiments. All mice were purchased from the Animal Research Center of Jiangsu University and were fed adaptively for one week.

Six mice were randomly divided into each group. To serve as a control, the BALB/c mice were exposed to filtered air and allowed *ad libitum* access to water until the end of the experiment. Mice in the tobacco smoke exposure group were exposed to tobacco smoke 6 h daily for 12 weeks as described in our previous study. The exposures were monitored and characterized as follows: carbon monoxide (13.23 ± 2.72 mg/m³), total particulate matter (TPM) (0 mg/m³) for the control group; carbon monoxide (157.56 ± 20.12 mg/m³), TPM (79.73 ± 3.92 mg/m³) for the tobacco smoke exposure group.

In vivo delivery of specific Notch inhibitor. Mice were randomly assigned into four groups (n=6 per group): control group, tobacco smoke group, tobacco smoke + DMSO group and tobacco smoke + FLI-06 group. FLI-06, a specific Notch inhibitor was reconstituted in sterile DMSO. The mice in control group and tobacco smoke group were exposed to filtered air or tobacco smoke alone and mice in tobacco smoke + DMSO group and tobacco smoke + FLI-06 group were intraperitoneal injected with FLI-06 (1 mg/kg body weight) or DMSO every other day. After 12 weeks smoke exposure, the mice were sacrificed and stomach tissues were collected and stored at -80°C until analysis.

β -carotene treatment. In a separate study, mice were divided into four groups (n=6 per group): control group, tobacco smoke-exposed group, tobacco smoke + β -carotene 5 mg, tobacco smoke + β -carotene 10 mg. The mice in control group and tobacco smoke group were exposed to filtered air or tobacco smoke and received an intragastric administration of corn oil as a vehicle for 12 weeks. Mice in tobacco smoke + β -carotene groups were treated with 5 or 10 mg/kg body weight (BW)/day β -carotene via intragastric administration dissolved in corn oil and exposed to tobacco smoke throughout the experimental period. Animals were weighed weekly and the administration dosages of β -carotene were based on the measurements of mouse body weight.

Western blot analysis. Frozen stomach tissues were weighed and homogenized in a lysate buffer. Homogenates were centrifuged (12,000 g, 4°C, 20 min) and supernatants were collected. Protein concentrations were measured and 60 μ g of proteins were fractionated by electrophoresis through gradient (7.5-10%) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% skim milk. Membranes were subsequently probed with specific primary antibodies overnight at 4°C, and then incubated with the appropriate secondary antibodies. For densitometric analyses, protein bands on the blots were measured by the use of the ImageJ analysis software.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from stomach tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed by using the Power SYBR Green Master Mix (Takara, Dalian, China) and an Applied Biosystems 7300 real-time PCR detection system (Applied Biosystems, Foster City, CA, USA). The primers used

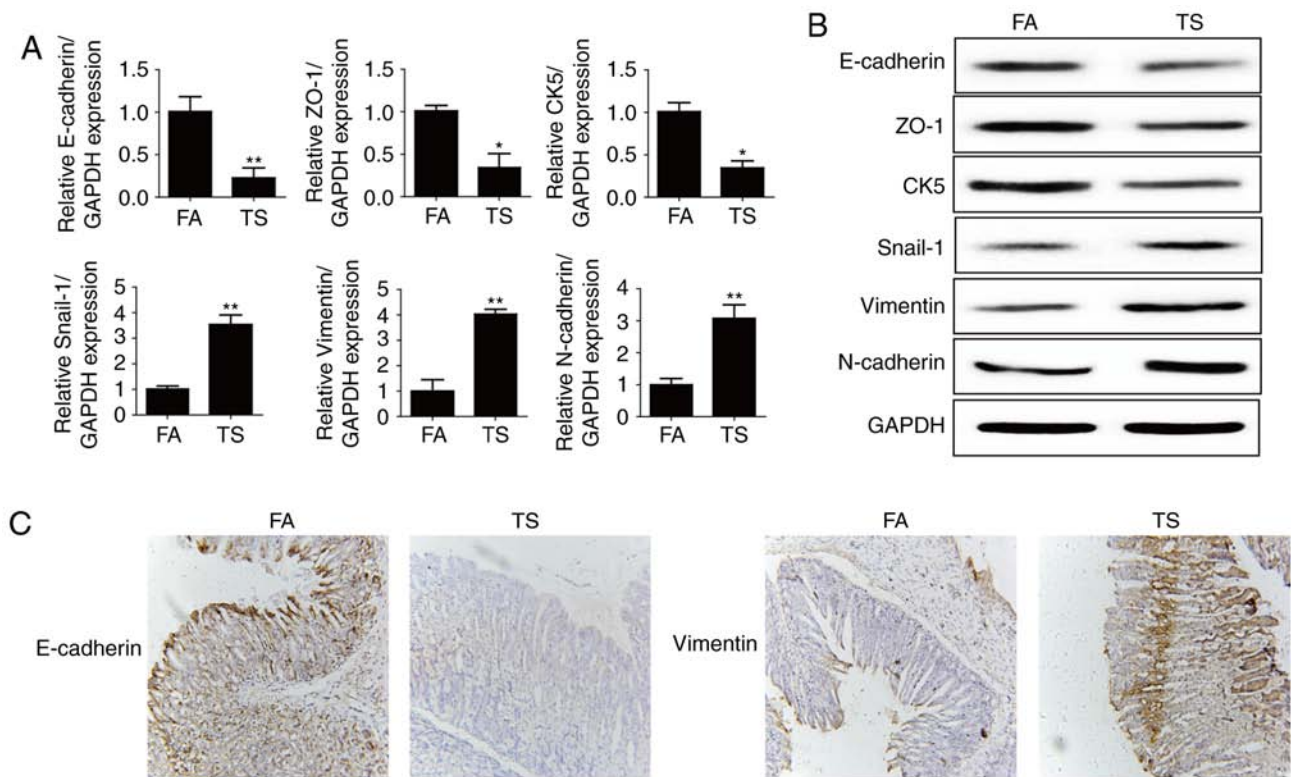


Figure 1. Tobacco smoke induced alterations in the expression of EMT markers. (A) Tobacco smoke reduced mRNA levels of E-cadherin, ZO-1, and CK5, and elevated mRNA levels of Snail-1, vimentin, and N-cadherin in the stomach of mice. (B) Tobacco smoke induced alterations in the protein levels of EMT markers in the stomach of mice. (C) Tobacco smoke decreased E-cadherin protein expression and increased vimentin protein expression shown by immunohistochemical staining. * $P < 0.05$, ** $P < 0.01$, compared with FA. EMT, epithelial-mesenchymal transition; FA, filtered air; TS, tobacco smoke.

were as follows: E-cadherin forward, 5'-TCGACACCCGAT TCAAAGTGG-3' and reverse, 5'-TTCCAGAAACGGAGG CCTGAT-3'; ZO-1 forward, 5'-GCAGCCACAACCAATTCA TAG-3' and reverse, 5'-GCAGACGATGTTTCATAGTTTC-3'; CK5 forward, 5'-CTGGAGAGTAGTCTAGACCAAGCC-3' and reverse, 5'-GTTAGAACCAAAAACAAAATTTGGG-3'; Snail-1 forward, 5'-GACCACTATGCCGCGCTCTT-3' and reverse, 5'-TCGCTGTAGTTAGGCTTCCGATT-3'; vimentin forward, 5'-CCTTGACATTGAGATTGCCA-3' and reverse, 5'-GTATCAACCAGAGGGAGTGA-3'; N-cadherin forward, 5'-ATCAAGTGCCATTAGCCAAG-3' and reverse, 5'-CTG AGCAGTGAATGTTGTCA-3'; GAPDH forward, 5'-GCT GCCCAACGCACCGAATA-3' and reverse, 5'-GAGTCA ACGGATTTGGTCTGT-3'. Fold changes in gene expression were calculated by a comparative threshold cycle (Ct) method using the formula $2^{-\Delta\Delta Ct}$.

Immunohistochemistry. Stomach tissues were fixed in 4% buffered formalin for 24 h then were paraffin embedded. Sections were cut (5 μ m) and de-waxed in xylene and rehydrated in graded alcohol, after which endogenous peroxidase activity was quenched by incubating the sections in 3% (v/v) H_2O_2 in methanol. Antigen retrieval was carried out in citrate buffer (pH 6.0) for 20 min in a microwave. Sections were subsequently incubated with a protein-blocking solution for 30 min, then incubated with the primary antibody (E-cadherin and vimentin) at 4°C overnight. Sections were subsequently washed with phosphate-buffered saline (PBS) before incubation for 1 h with peroxidase-conjugated secondary antibodies. After

1 h, sections were stained with 3,3'-diaminobenzidinetetrahydrochloride (DAB) and counterstained with hematoxylin. Images were collected using a Nikon eclipse Ti-S microscope (Nikon Corporation, Tokyo, Japan) at a x200 magnification.

Statistical analysis. Statistical analyses were performed with SPSS 18.0. All data are expressed as mean \pm standard deviation. One-way ANOVA was used for comparison of statistical differences among multiple groups, followed by the LSD significant difference test. Unpaired Student's t-test was also used for the comparison between two groups. A value of $P < 0.05$ was considered significantly different.

Results

Tobacco smoke-elicited EMT-like changes in mice gastric. Our previous study showed that tobacco smoke induced EMT changes in gastric tissues of mice. In the present study we also found that 12-week tobacco smoke exposure decreased the expression of E-cadherin, ZO-1 and CK5, and elevated the mRNA expression levels of Snail-1, vimentin and N-cadherin (Fig. 1A and B). Immunohistochemical staining also showed that TS decreased E-cadherin protein expression and increased vimentin protein expression in the mouse stomach (Fig. 1C).

Tobacco smoke-induced gastric EMT is associated with Notch pathway. Notch pathway has been reported to be implicated in a majority of cancers for promoting the EMT

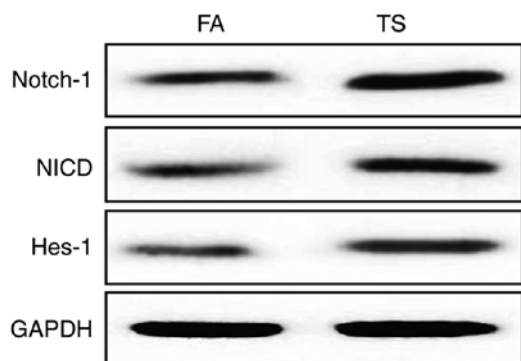


Figure 2. Tobacco smoke increases Notch pathway activation in the mouse stomach. Western blot analyses of Notch-1, NICD, Hes-1 in the tissues of mice exposed to tobacco smoke for 12 weeks. FA, filtered air; TS, tobacco smoke.

process. To determine whether tobacco smoke-triggered gastric EMT is associated with Notch pathway, the expression level of Notch-1, NICD, and Hes-1 were measured. The results revealed that long-term tobacco smoke exposure increased the levels of Notch, NICD and Hes-1 in the gastric tissues of mice (Fig. 2).

Notch inhibition reverses tobacco smoke-mediated gastric EMT. Since the above results revealed that tobacco smoke-mediated gastric EMT was associated with Notch pathway, we further determine the role of Notch pathway in tobacco smoke-induced gastric EMT regulation. Mice were treated with FLI-06 (1 mg/kg body weight), a specific Notch

pathway inhibitor. Results showed that FLI-06 downregulated Notch, NICD and Hes-1 expression levels (Fig. 3A). Western blot analyses showed that treated mice with FLI-06 attenuated tobacco smoke-induced alterations of E-cadherin, ZO-1, CK5, Snail-1, vimentin and N-cadherin (Fig. 3B). We also found that the changes in mRNA expression of E-cadherin, ZO-1, CK5, Snail-1, vimentin and N-cadherin were effectively reversed by FLI-06 (Fig. 3C).

β -carotene prevented tobacco smoke-induced EMT in the mouse stomach. To determine the effects of β -carotene on tobacco smoke-mediated gastric EMT, the BALB/c mice were administered β -carotene (5 or 10 mg/kg BW/day) and exposed to tobacco smoke for 12 weeks. Our results showed that these alterations in expressions of the EMT markers induced by tobacco smoke (Fig. 4), including decreases of the epithelial markers E-cadherin, ZO-1, CK5 and increases of the mesenchymal markers Snail-1, vimentin, N-cadherin, were significantly attenuated by β -carotene (10 mg/kg BW/day). These results indicated that β -carotene prevent tobacco smoke-induced gastric EMT in the BALB/c mouse model.

β -carotene attenuated tobacco smoke-activated Notch pathway. In order to explore the effects of β -carotene on tobacco smoke-activated Notch pathway, we further examined the changes of Notch, NICD and Hes-1 expression following β -carotene treatment in the mouse stomach tissues. Western blot analyses and immunohistochemical staining showed that 10 mg/kg BW/day β -carotene obviously inhibited tobacco smoke-induced Notch, NICD and Hes-1 expression levels (Fig. 5).

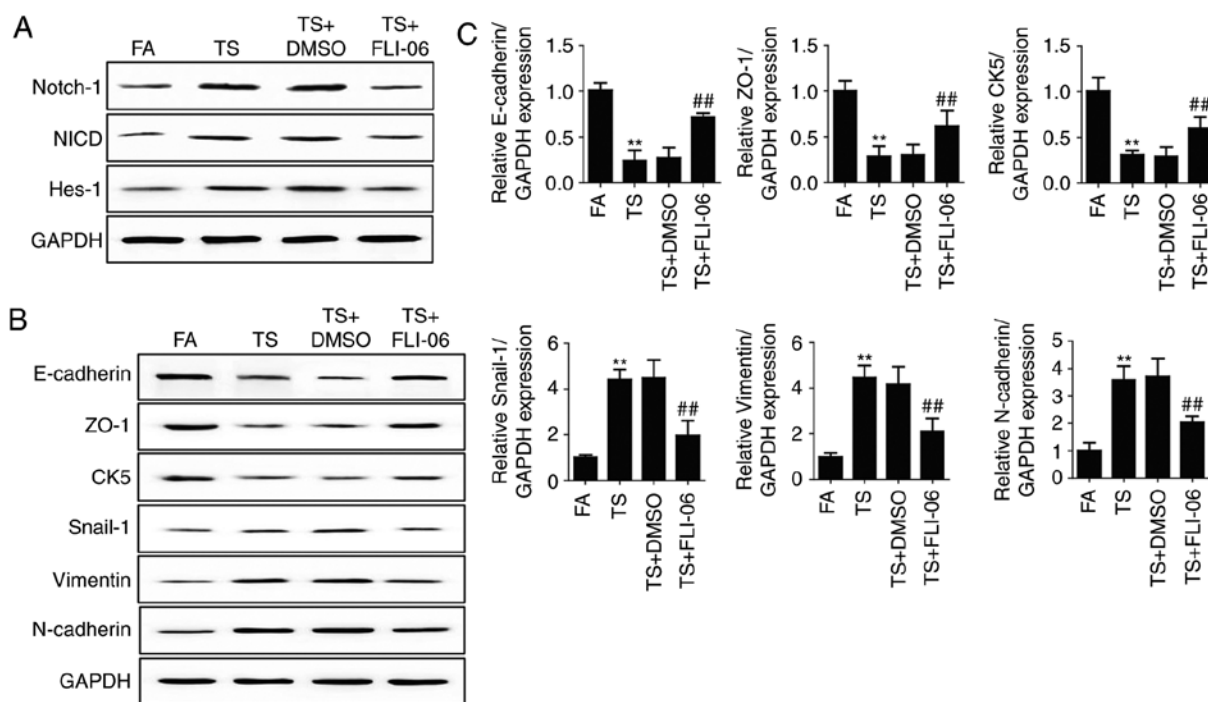


Figure 3. Notch inhibition reverses tobacco smoke-mediated gastric EMT in the stomach of mice. (A) Western blot analyses of Notch, NICD and Hes-1 expression levels after treatment of mice with FLI-06. (B) Western blot analyses of E-cadherin, ZO-1, CK5, Snail-1, vimentin, and N-cadherin proteins after treated mice with FLI-06. (C) qRT-PCR analyses of EMT markers. ** $P < 0.01$, compared with FA; ## $P < 0.01$, compared with TS. EMT, epithelial-mesenchymal transition; FA, filtered air; TS, tobacco smoke.

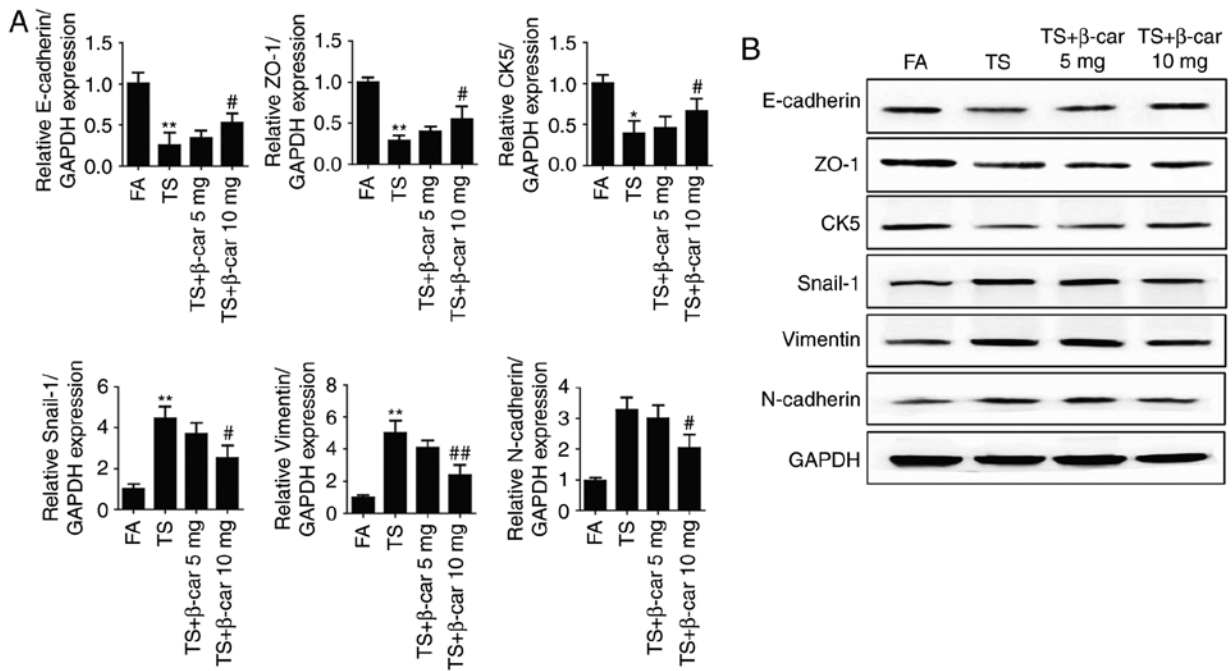


Figure 4. β-carotene prevents tobacco smoke-induced EMT in the mouse stomach. (A) qRT-PCR analyses of E-cadherin, ZO-1, CK5, Snail-1, vimentin, and N-cadherin mRNAs. (B) Western blot analyses of EMT marker proteins. *P<0.05, **P<0.01, compared with FA; #P<0.05, ##P<0.01, compared with TS. EMT, epithelial-mesenchymal transition; FA, filtered air; TS, tobacco smoke.

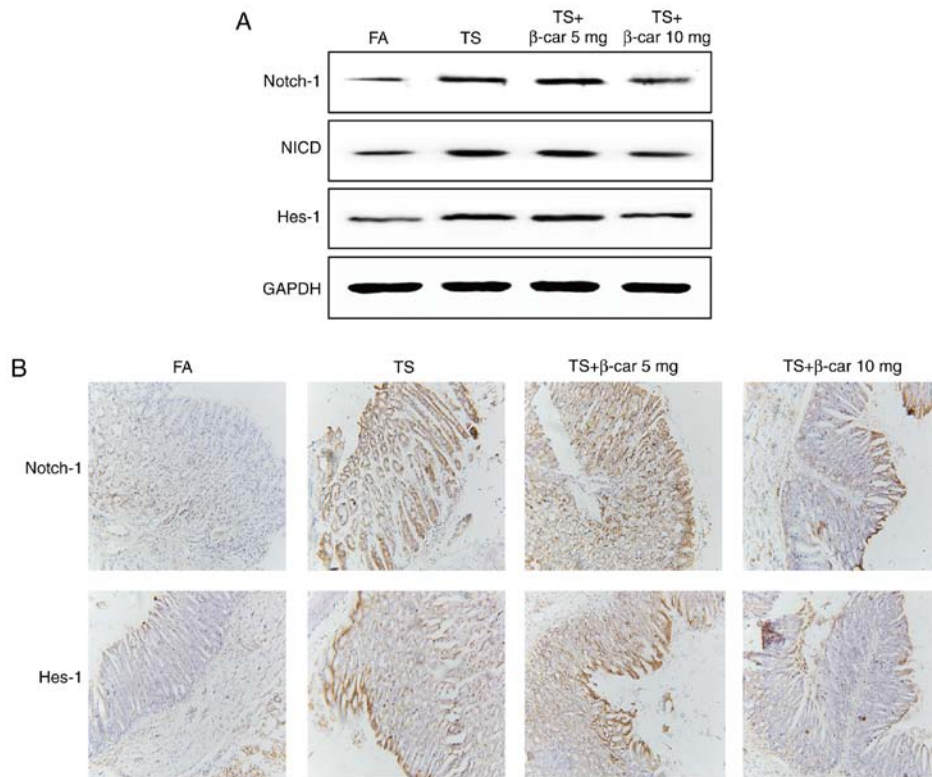


Figure 5. β-carotene attenuates tobacco smoke-activated Notch pathway in mouse stomach. (A) Western blot analyses of Notch, NICD and Hes-1. (B) The expression changes of Notch-1 and Hes-1 after tobacco induction and β-carotene treatment are shown by immunohistochemical staining. FA, filtered air; TS, tobacco smoke.

Discussion

Gastric cancer remains the leading cause of cancer-related deaths. It is critical to explore the novel molecular mechanisms

and the chemoprevention of gastric cancer. Tobacco smoke is an important risk factor for gastric cancer, which promotes the initiation and progression of gastric tumorigenesis. However, the underlying molecular mechanisms by which tobacco

smoke causes gastric cancer still has not been well defined. The present study demonstrated for the first time that Notch regulated tobacco smoke-mediated gastric EMT in BALB/c mice. Moreover, our data indicated that β -carotene effectively attenuated tobacco smoke-triggered Notch pathway activation and gastric EMT. These findings provide new insights into the pathogenesis and the chemoprevention of tobacco smoke-associated gastric cancer.

Characterized by downregulation of the intercellular tight junctions, changes in migration and invasion capacity, as well as the expression of epithelial and mesenchymal markers, EMT is a crucial process in initiation of tumorigenesis (35,36). Studies have documented that tobacco smoke promotes the EMT process, resulting in loss of cellular polarity, downregulation of epithelial cadherin and the intercellular tight junctions, acquire certain properties of mesenchymal cells, and increased mobility of cells (19,20,37). In the present study, we also found tobacco smoke altered the expression of EMT markers, including decreased E-cadherin, ZO-1, and CK5, and increased Snail-1, vimentin, and N-cadherin. These results revealed that tobacco smoke exposure induced gastric EMT in BALB/c mice.

Nonetheless, the underlying mechanisms by which tobacco smoke induces EMT and the signaling events that underlie EMT are poorly understood. Notch signaling regulates a series of cellular processes (21). Several studies have revealed that Notch pathway is critically involved in the process of EMT (21,23,24). To examine the role of Notch pathway in tobacco smoke-induced EMT, we addressed the relationship between Notch and gastric EMT induced by tobacco smoke. The mice were treated with a specific Notch inhibitor FLI-06 (1 mg/kg body weight) or DMSO every other day. Results have shown that FLI-06 downregulated Notch, NICD and Hes-1 expression levels. Furthermore, inhibition of Notch pathway attenuated tobacco smoke-induced EMT, as indicated by decreased E-cadherin, ZO-1, and CK5, and increased Snail-1, vimentin, and N-cadherin. These data indicated that Notch pathway positively regulates tobacco smoke-induced EMT in the gastric tissues of mice.

It has been reported that approximately one third of cancers can be prevented by controlling diet and regular physical activities. Dietary phytochemicals have been shown to be a very promising approach to the prevention of cancer development. β -carotene is one of carotenoids, abundant in carrot, spinach, kale, pink guava yams, palm oil, and sweet potato (25). Studies have demonstrated the safety of β -carotene as well as its anticancer activities in different types of cancer (33). The doses of β -carotene used in our present study were 5 or 10 mg/kg BW per day. After treatment with β -carotene and tobacco smoke for 12 weeks, the effect of β -carotene on tobacco smoke-induced alterations in the expression of the EMT markers were examined. As shown in Fig. 4 tobacco smoke-triggered EMT were significantly attenuated by 10 mg/kg BW/day dose of β -carotene. In order to explore the effects of β -carotene on tobacco smoke-activated Notch pathway, we further examine the changes of Notch pathway following β -carotene treatment. Tobacco smoke-elevated expression levels of Notch, NICD and Hes-1 were obviously inhibited following the 12-week β -carotene treatment (Fig. 5).

The present study demonstrated for the first time that Notch pathway positively regulates tobacco smoke-induced gastric

EMT and the protective effects of β -carotene in tobacco smoke-induced Notch pathway activation and EMT *in vivo*. These findings provide new insights into the mechanisms and the chemoprevention of tobacco smoke-associated gastric tumorigenesis.

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Competing interests

The authors declare that they have no competing interests.

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