

Overexpression of Human Papillomavirus Type 16 Oncoproteins Enhances Epithelial–Mesenchymal Transition via STAT3 Signaling Pathway in Non-Small Cell Lung Cancer Cells

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The human papillomavirus (HPV) infection may be associated with the development and progression of non-small cell lung cancer (NSCLC). However, the role of HPV-16 oncoproteins in the development and progression of NSCLC is not completely clear. Epithelial–mesenchymal transition (EMT), a crucial step for invasion and metastasis, plays a key role in the development and progression of NSCLC. Here we explored the effect of HPV-16 oncoproteins on EMT and the underlying mechanisms. NSCLC cell lines, A549 and NCI-H460, were transiently transfected with the EGFP-N1-HPV-16 E6 or E7 plasmid. Real-time PCR and Western blot analysis were performed to analyze the expression of EMT markers. A protein microarray was used to screen the involved signaling pathway. Our results showed that overexpression of HPV-16 E6 and E7 oncoproteins in NSCLC cells significantly promoted EMT-like morphologic changes, downregulated the mRNA and protein levels of EMT epithelial markers (E-cadherin and ZO-1), and upregulated the mRNA and protein levels of EMT mesenchymal markers (N-cadherin and vimentin) and transcription factors (ZEB-1 and Snail-1). Furthermore, the HPV-16 E6 oncoprotein promoted STAT3 activation. Moreover, WP1066, a specific signal transducer and activator of transcription 3 (STAT3) inhibitor, reversed the effect of HPV-16 E6 on the expression of ZO-1, vimentin, and ZEB-1 in transfected NSCLC cells. Taken together, our results suggest that overexpression of HPV-16 E6 and E7 oncoproteins enhances EMT, and the STAT3 signaling pathway may be involved in HPV-16 E6-induced EMT in NSCLC cells.

Key words: Human papillomavirus (HPV); Epithelial–mesenchymal transition (EMT); Signal transducer and activator of transcription 3 (STAT3); Non-small cell lung cancer (NSCLC)

INTRODUCTION

Non-small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer cases. It is well known that NSCLC is predominantly caused by cigarette smoking. However, global statistics in 2002 estimated that about 25% of patients with lung cancer all over the world were never-smokers¹. Recently, more and more patients with NSCLC, especially women, have been found to be never-smokers^{2–5}. Therefore, the role of nonsmoking factors in never-smokers with NSCLC should be investigated.

Human papillomavirus (HPV), a group of small non-enveloped DNA viruses, was first suggested to play a role in the development and progression of lung cancer in

1979⁶. Recently, accumulating epidemiological evidence and meta-analyses have shown that the infection of high-risk HPV types, especially HPV types 16/18, may have an association with NSCLC^{7–11}. The HPV 16/18 infection was found to increase the risk of squamous cell lung carcinoma⁷, and a higher frequency and increased significance of oncogenic HPV 16/18 were observed in never-smokers and women with lung cancer^{8–11}. Specifically, HPV type 16 (HPV-16) was demonstrated to be the most frequent genotype of HPV in NSCLC^{12–14}. Most recently, the HPV-16 infection was reported to act synergistically with environmental exposure to induce lung tumorigenesis in nonsmokers¹⁵. These reports indicated that the

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HPV-16 infection might contribute to NSCLC. However, different results have also been reported^{16,17}, and the role of HPV-16 E6 and E7 oncoproteins in the development and progression of NSCLC is not completely clear. Therefore, more studies are essential to further explore the role of HPV-16 oncoproteins in the development and progression of NSCLC.

Invasion and metastasis play key roles in the development and progression of NSCLC. Epithelial–mesenchymal transition (EMT), a process by which epithelial cells lose the epithelial phenotype and gain the mesenchymal phenotype, is a crucial step for invasion and metastasis¹⁸. EMT is characterized by the downregulation of epithelial molecular markers such as E-cadherin and ZO-1, the upregulation of mesenchymal molecular proteins such as N-cadherin and vimentin, and the induction of EMT-related transcription factors such as ZEB-1 and Snail. Recently, Shen et al. found that EMT contributed to docetaxel resistance in human NSCLC¹⁹. Zhao et al. demonstrated that E-cadherin expression in pleural effusion cells was associated with EGFR mutation status and patient prognosis in lung adenocarcinoma patients in first-line chemotherapy²⁰. Atmaca et al. reported that SNAI2/SLUG was prognostic of the outcome of NSCLC patients, and SNAI2/SLUG and estrogen receptor mRNA levels were inversely correlated²¹. These reports indicated that EMT played a crucial role in the development and progression of NSCLC. However, whether HPV-16 oncoproteins can promote the development and progression of NSCLC by interfering with EMT still remains unclear.

The signal transducers and activators of transcription (STATs) are a family of seven proteins with high homology including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6²². STAT3, the most ubiquitous of the STATs, has been demonstrated to enhance tumorigenesis by mediating the expression of various target genes, including cell cycle regulators, antiapoptotic genes, and angiogenic factors²². Recently, STAT3 has been found to regulate the EMT program in various cancer cells^{23–25}.

In this study, we analyzed the effect of HPV-16 oncoproteins on EMT in NSCLC cells and the role of STAT3 in that effect. To our knowledge, we found, for the first time, that HPV-16 E6 and E7 oncoproteins promoted EMT, and the STAT3 signaling pathway was involved in HPV-16 E6-induced EMT in both A549 and NCI-H460 NSCLC cells.

MATERIALS AND METHODS

Reagents

WP1066, a STAT3 inhibitor, was purchased from Merck China Ltd. (Shanghai, P.R. China). WP1066 was dissolved at a concentration of 50 mmol/L in 100%

dimethyl sulfoxide (DMSO) as a stock solution and stored at -20°C. The final DMSO concentration did not exceed 0.1% throughout the study. Transfection reagent (Lipofectamine™ 2000) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). TRIzol® reagent was purchased from Invitrogen. Reverse transcription and real-time PCR (SYBR Green) kits were purchased from Tiangen Biotech (Beijing, P.R. China). Lysis and blocking buffers were purchased from Beyotime Biotechnology Corporation (Shanghai, P.R. China). Rabbit anti-human E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-human β-actin antibody was purchased from Beyotime Biotechnology Corporation. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling Technology. The Human Phospho-Kinase Array Kit (No. ARY003) containing 46 kinase phosphorylation sites was obtained from RD Systems China Co. Ltd. (Shanghai, P.R. China).

Cell Lines and Cell Cultures

Human NSCLC cell lines, A549 and NCI-H460, were purchased from the American Type Culture Collection (Rockville, MD, USA) and the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, P.R. China), respectively. All NSCLC cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified condition with 5% CO₂.

Transient Transfection

EGFP plasmid vectors harboring HPV-16 E6, E7, or mutant cDNA were constructed by our lab. A549 and NCI-H460 cells at 70% to 80% confluence were transiently transfected for 4 h with pEGFP-N1-HPV-16 E6 or E7 plasmid using Lipofectamine™ 2000 transfection reagent. The cells transfected with empty vector or mutant plasmid were used as negative controls, and the cells exposed to Lipofectamine™ 2000 alone acted as mock transfection controls. Twenty-four hours after transfection, the transfected cells were harvested for further analysis. The green fluorescence signals in the transfected cells were observed under a fluorescence microscope, and the transfection efficiency was analyzed by flow cytometry (Epics-XL; Beckman Coulter, Brea, CA, USA). The expression of HPV-16 E6 and E7 oncoproteins in transfected cells was confirmed in a previous study²⁶. E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 mRNA levels were analyzed by real-time PCR. The expression of E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 proteins in transfected cells was detected by Western blot analysis.

Real-Time PCR

Total RNA was extracted from transfected- and mock-transfected cells using the TRIzol® reagent. E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 mRNA relative levels were determined using a reverse transcription kit and real-time PCR (SYBR Green) kit according to the manufacturer's instructions (Tiangen Biotech). The sequences of the primers were as follows: human E-cadherin, 5'-ttgctactggaacaggcac-3' (forward) and 5'-cccggtgttagtctgcgt-3' (reverse) (Genbank: NM_001317184.1); ZO-1, 5'-ggatgttatcgtcgattgt-3' (forward) and 5'-aagagccagttccattgt-3' (reverse) (Genbank: NM_001301025.1); N-cadherin, 5'-ttatcctgtgctgatgttg-3' (forward) and 5'-tcttccttcacccatcttc-3' (reverse) (Genbank: NM_001792.3); vimentin, 5'-tggcacgtttgacc-3' (forward) and 5'-ggcatcgatgctgagaa-3' (reverse) (Genbank: NM_003380.3); ZEB-1, 5'-tccccatcaccttaaac-3' (forward) and 5'-ccctgtgcttggtagtcaa-3' (reverse) (Genbank: NM_001174096.1); Snail-1, 5'-tcctcgtccctct-3' (forward) and 5'-tgtgcagtattgcagtgt-3' (reverse)

(reverse) (Genbank: NM_005985.3); β -actin, 5'-tgacggacatccgcaaag-3' (forward) and 5'-ctgaaagggtggacagcga-3' (reverse) (Genbank: NM_001101.3). All the primers were synthesized by Sangon Biotech (Shanghai, P.R. China). The reverse transcription conditions were as follows: 25°C for 10 min, 55°C for 30 min, and 85°C for 5 min. The real-time PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. The size of the PCR product of E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, Snail-1, and β -actin was 179, 158, 139, 176, 122, 155, and 186 bp, respectively. All relative mRNA levels were normalized to β -actin.

Western Blot Analysis

The method was described in our previous studies²⁶⁻²⁸. Briefly, transfected and mock-transfected cells were lysed with lysis buffer (Beyotime Biotechnology Corporation) and complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The proteins were run on

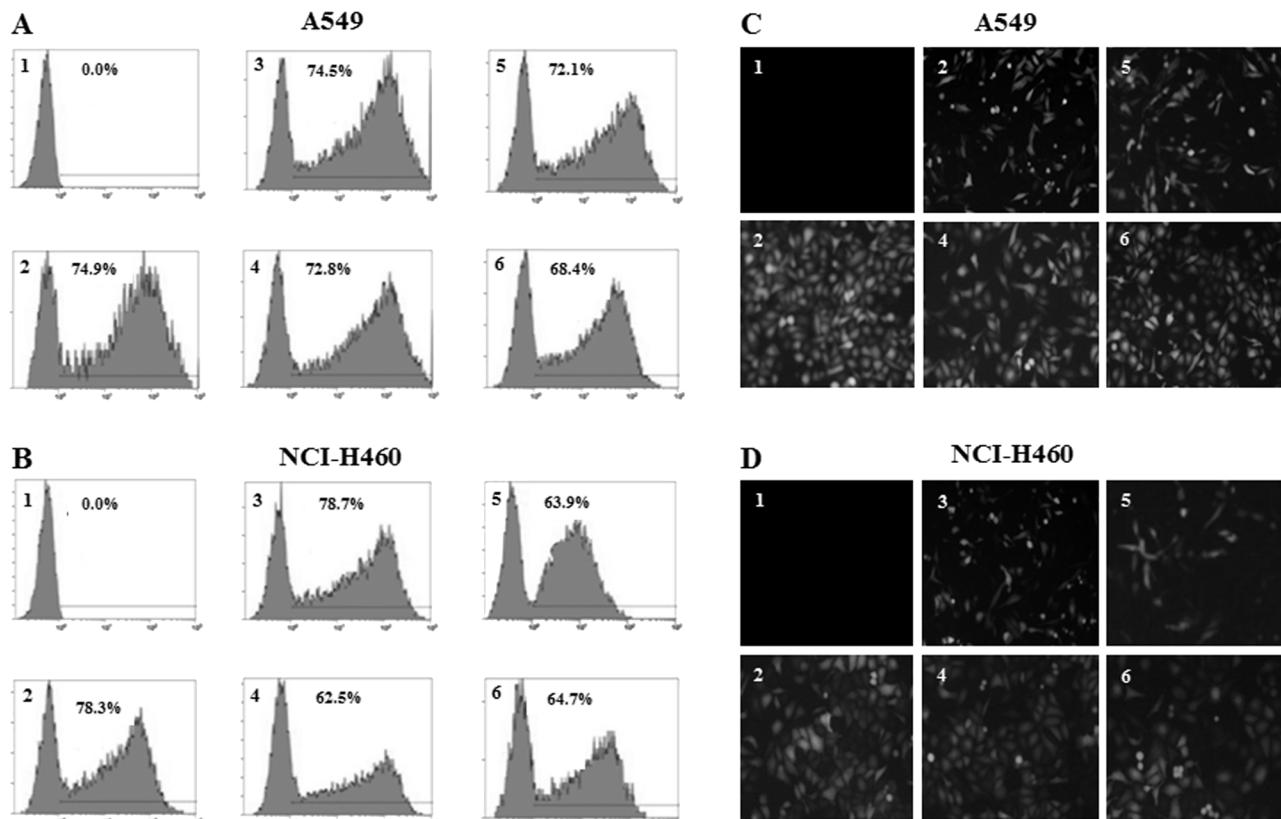


Figure 1. Analysis of transfection efficiency and morphologic changes. A549 and NCI-H460 cells were transiently transfected with EGFP plasmid vectors harboring HPV-16 E6, E7, or mutant cDNA. (A, B) The transfection efficiency was analyzed by flow cytometry (A: A549; B: NCI-H460). (C, D) The morphologic changes were observed under a fluorescence microscope (C: A549; D: NCI-H460). Original magnification: 200 \times . 1, Mock transfection control; 2, empty vector control; 3, HPV-16 E6; 4, HPV-16 E6 mutant (E6-mut) control; 5, HPV-16 E7; and 6, HPV-16 E7 mutant (E7-mut) control. The results are representative of three independent experiments.

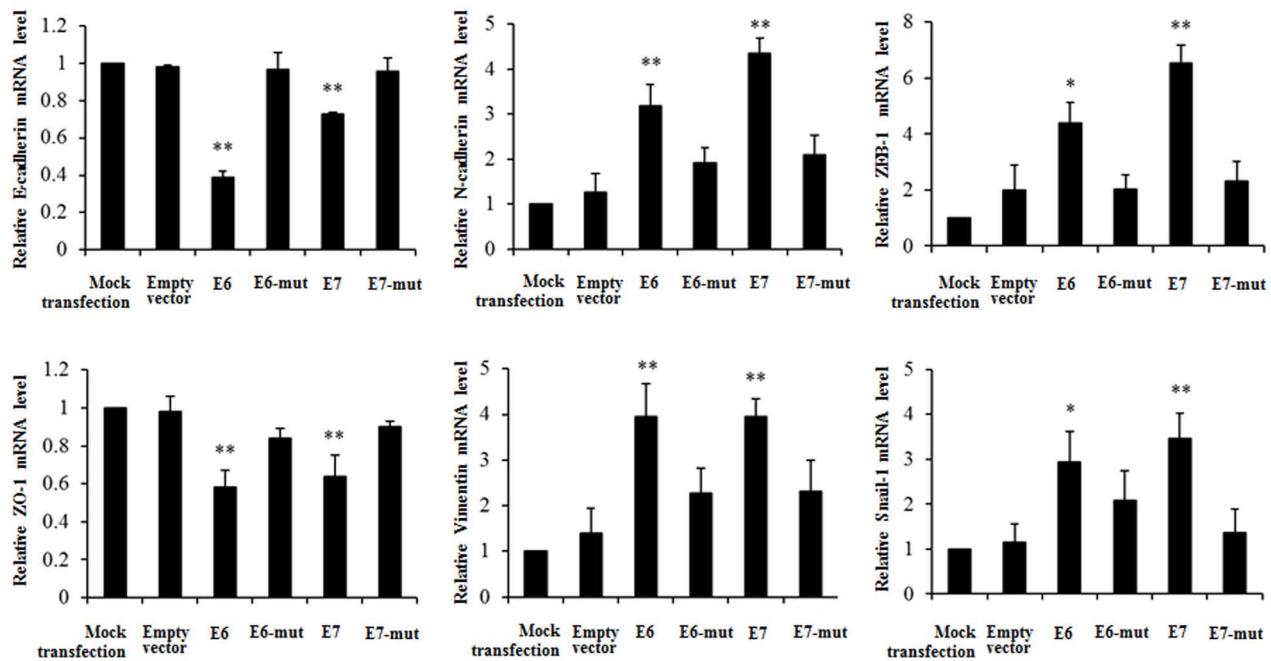


Figure 2. Effects of HPV-16 oncoproteins on the mRNA levels of EMT markers in A549 cells. Real-time PCR was performed to analyze the mRNA levels of E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 in transfected A549 cells. All data were expressed as mean \pm SD of three independent experiments. * p <0.05, ** p <0.01, compared with empty vector control or mutant controls (E6-mut and E7-mut).

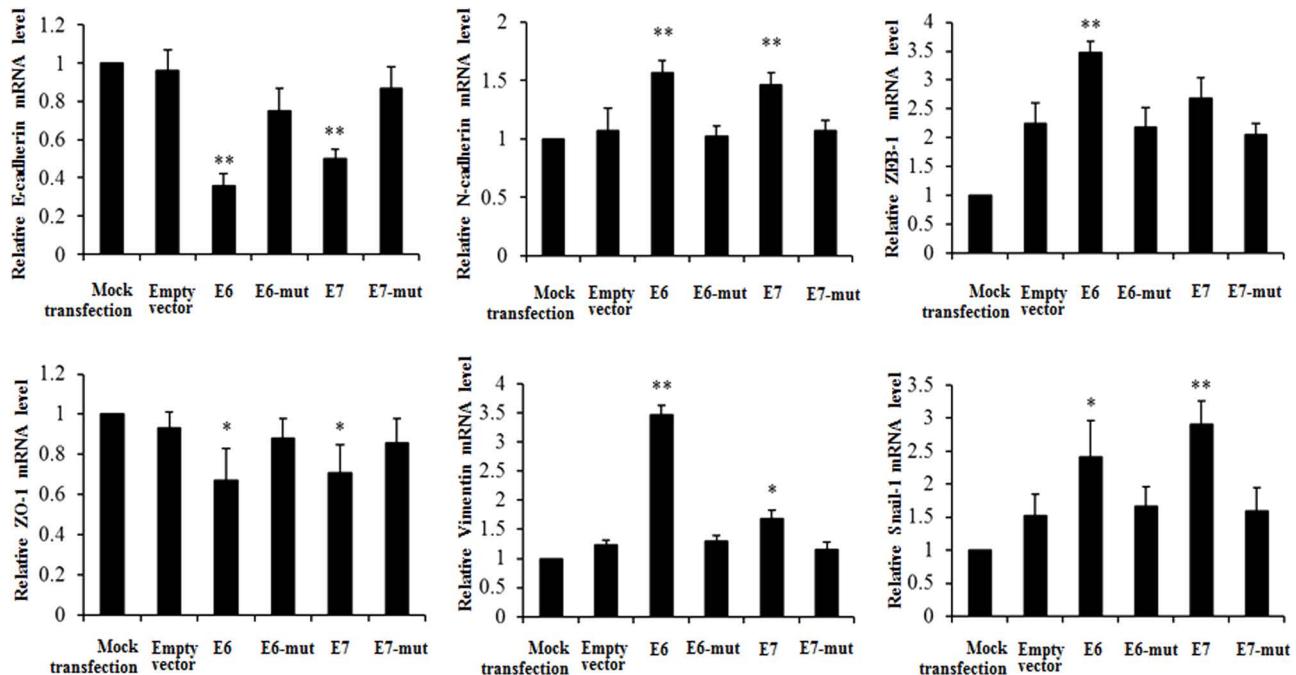


Figure 3. Effects of HPV-16 oncoproteins on the mRNA levels of EMT markers in NCI-H460 cells. Real-time PCR was performed to analyze the mRNA levels of E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 in transfected NCI-H460 cells. All data were expressed as mean \pm SD of three independent experiments. * p <0.05, ** p <0.01, compared with empty vector control or mutant controls (E6-mut and E7-mut).

a 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. After blocking with blocking buffer (Beyotime Biotechnology Corporation), the membranes were incubated with specific primary antibodies and HRP-conjugated secondary antibodies, respectively. Signals were visualized using enhanced chemiluminescence (ECL). The analysis of β -actin protein expression was used as a loading control.

Protein Microarray for Human Phospho-Kinase

The levels of 46 kinase phosphorylation sites were analyzed using the Human Phospho-Kinase Array Kit (RD systems China Co. Ltd.) according to the manufacturer's instructions. Briefly, two membranes spotting with capture and control antibodies were put into a well of the eight-well multidish and blocked with the blocking buffer on a rocking platform for 1 h. The total protein with reconstituted detection antibody cocktail was added into each well and incubated overnight at 4°C on a rocking platform. Afterward, the membranes were incubated with diluted streptavidin-HRP for 30 min at room temperature, followed by incubation with ECL. The signals were visualized after exposure to X-ray film.

Statistical Analysis

All data in this study were expressed as mean \pm SD for three independent experiments. One-way ANOVA and LSD methods were used as statistical analysis by SPSS 19.0 software. A value of $p < 0.05$ indicated the difference between two groups was statistically significant.

RESULTS

HPV-16 Oncoproteins Promoted EMT-Like Morphologic Changes in NSCLC Cells

A549 and NCI-H460 NSCLC cells were transfected with EGFP plasmid vectors harboring HPV-16 E6, E7, or mutant cDNA. The transfection rates ranged from 62.5% to 78.7% (Fig. 1A and B). Moreover, 24 h after transfection, obvious EMT-like morphologic changes such as polarity were observed in HPV-16 E6- and E7-transfected NSCLC cells compared with empty vector or mutant controls (Fig. 1C and D).

HPV-16 Oncoproteins Regulated the mRNA Levels of EMT Markers in NSCLC Cells

To study the effect of HPV-16 oncoproteins on EMT in NSCLC cells, A549 and NCI-H460 NSCLC cells were transfected with pEGFP-N1-HPV-16 E6 or E7 plasmid. Twenty-four hours after transfection, real-time PCR was performed to determine the mRNA levels of EMT epithelial markers (E-cadherin and ZO-1), mesenchymal markers (N-cadherin and vimentin), and transcription factors (ZEB-1 and Snail-1) in transfected NSCLC cells. We demonstrated that HPV-16 E6 and E7 oncoproteins significantly downregulated E-cadherin and ZO-1 mRNA levels and upregulated N-cadherin, vimentin, ZEB-1, and Snail-1 mRNA levels compared to the empty vector and mutant controls in A549 cells (Fig. 2). Similar results were found in NCI-H460 NSCLC cells (Fig. 3).

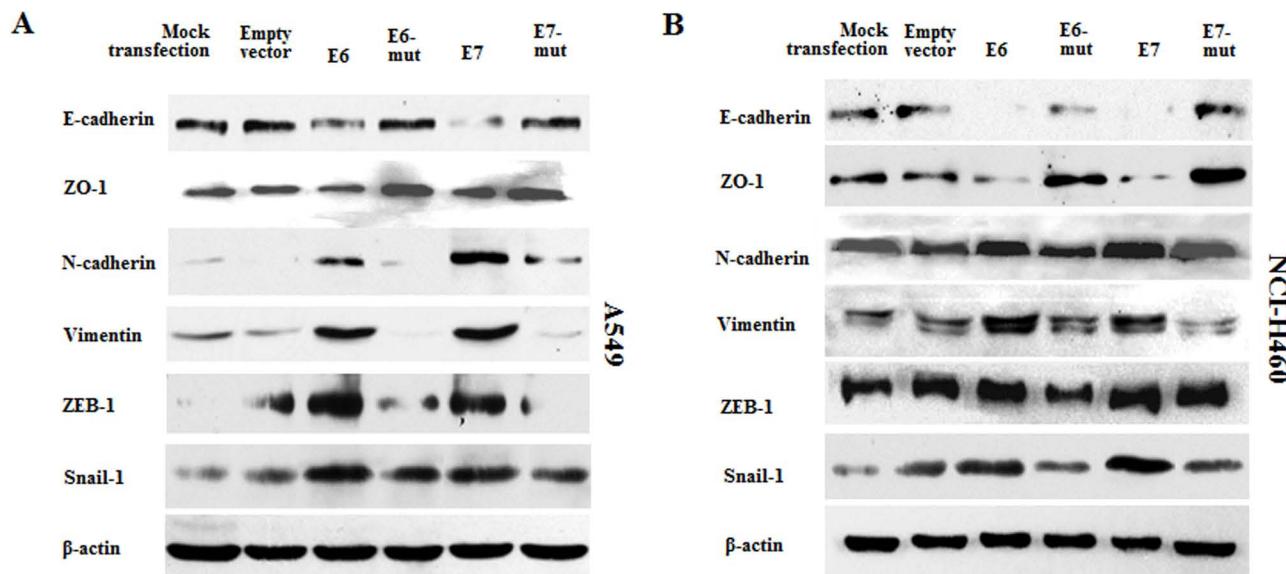


Figure 4. Effects of HPV-16 oncoproteins on the protein expression of EMT markers in NSCLC cells. Western blot analysis was performed to analyze the expression of E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 proteins in transfected A549 (A) and NCI-H460 (B) cells. The results are representative of three independent experiments.

HPV-16 Oncoproteins Regulated the Protein Levels of EMT Markers in NSCLC Cells

To further observe the effect of HPV-16 oncoproteins on the translational levels of EMT markers in NSCLC cells, Western blot analysis was performed to analyze the expression of E-cadherin, ZO-1, N-cadherin, vimentin, ZEB-1, and Snail-1 proteins in transfected NSCLC cells. Our results showed that the protein expression of E-cadherin and ZO-1 was decreased, while the protein expression of N-cadherin, vimentin, ZEB-1, and Snail-1 was increased by overexpression of HPV-16 E6 and E7.

oncoproteins in A549 cells (Fig. 4A). Similar results were found in NCI-H460 NSCLC cells (Fig. 4B).

STAT3 Signaling Pathway Was Involved in HPV-16 E6-Induced EMT

Multiple signaling pathways have been found to mediate EMT. To investigate signaling pathways that can regulate HPV-16 oncoprotein-induced EMT, we analyzed the phosphorylation levels of 46 proteins using protein microarray. Our results showed that the HPV-16 E6 oncoprotein interfered with the phosphorylation levels of 16 proteins,

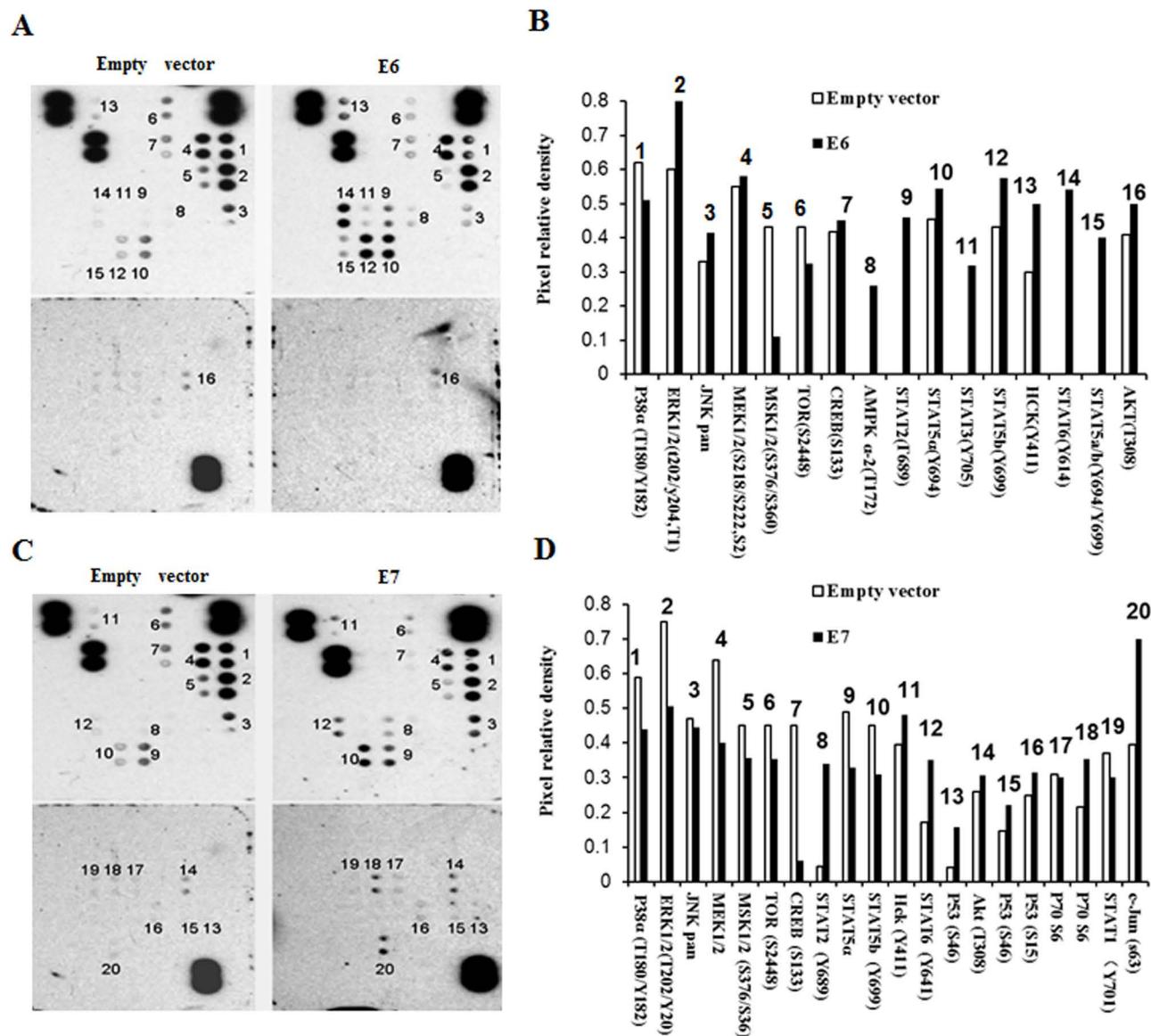


Figure 5. Effects of HPV-16 oncoproteins on the phosphorylation levels of 46 proteins. Protein microarray was performed to analyze the effects of HPV-16 oncoproteins on the phosphorylation levels of 46 proteins. (A: E6; C: E7) and (B, D) density results (B: E6; D: E7).

especially the HPV-16 E6 oncoprotein upregulated the phosphorylation levels of STAT3, STAT5a, STAT5b, and STAT6 (Fig. 5A and B). The HPV-16 E7 oncoprotein regulated the phosphorylation levels of 20 proteins. The HPV-16 E7 oncoprotein especially upregulated the phosphorylation levels of STAT1, STAT2, STAT5a, STAT5b, STAT6, and c-Jun (Fig. 5C and D). These results indicated that HPV-16 oncoproteins activated STAT signaling.

Previous studies have demonstrated that STAT3 can mediate EMT²³⁻²⁵, so we further analyzed the role of STAT3 signaling in HPV-16 E6 oncoprotein-induced EMT in NSCLC cells. The results from the Western blot analysis further confirmed that HPV-16 E6 upregulated the phosphorylated STAT3 (p-STAT3) protein level in both A549 (Fig. 6A) and NCI-H460 cells (Fig. 6B), indicating that HPV-16 E6 promoted the activation of STAT3 in two types of NSCLC cells. Next, NSCLC cells were pretreated with different concentrations of WP1066, a specific STAT3 inhibitor, followed by transfection with the pEGFP-N1-HPV-16 E6 plasmid. Our results showed that p-STAT3 protein expression was inhibited by WP1066. As expected, the inhibition of p-STAT3 expression reversed the effect of HPV-16 E6 on ZO-1, vimentin, and ZEB-1

protein expression in A549 (Fig. 6C) and NCI-H460 (Fig. 6D) cells. Moreover, the inhibition of p-STAT3 expression dramatically abrogated the effect of HPV-16 E6 on ZO-1, vimentin, and ZEB-1 mRNA expression in two types of NSCLC cells (Fig. 7). Taken together, our results suggest that STAT3 signaling is involved in HPV-16 E6 oncoprotein-induced EMT in NSCLC.

DISCUSSION

EMT is accompanied by degradation of the extracellular matrix and gain of mesenchymal cytoskeletal proteins¹⁸. HPV-16 E6 and E7 oncoproteins were found to induce EMT-like processes in the epithelial MDCK cell line via induction of the EMT transcription factors including Slug, Twist, ZEB-1, and ZEB-2, especially ZEBs²⁹. HPV-16 E5 expression was also reported to cause switching from fibroblast growth factor receptor (FGFR) 2b to FGFR2c and EMT in cell models of transfected human keratinocytes as well as in cervical epithelial cells³⁰. E5 and E6/E7 of high-risk HPVs were reported to cooperate in enhancing cancer progression through initiation of EMT³¹. Most recently, HPV-16 E6/E7 was found to promote cell migration and invasion in cervical cancer

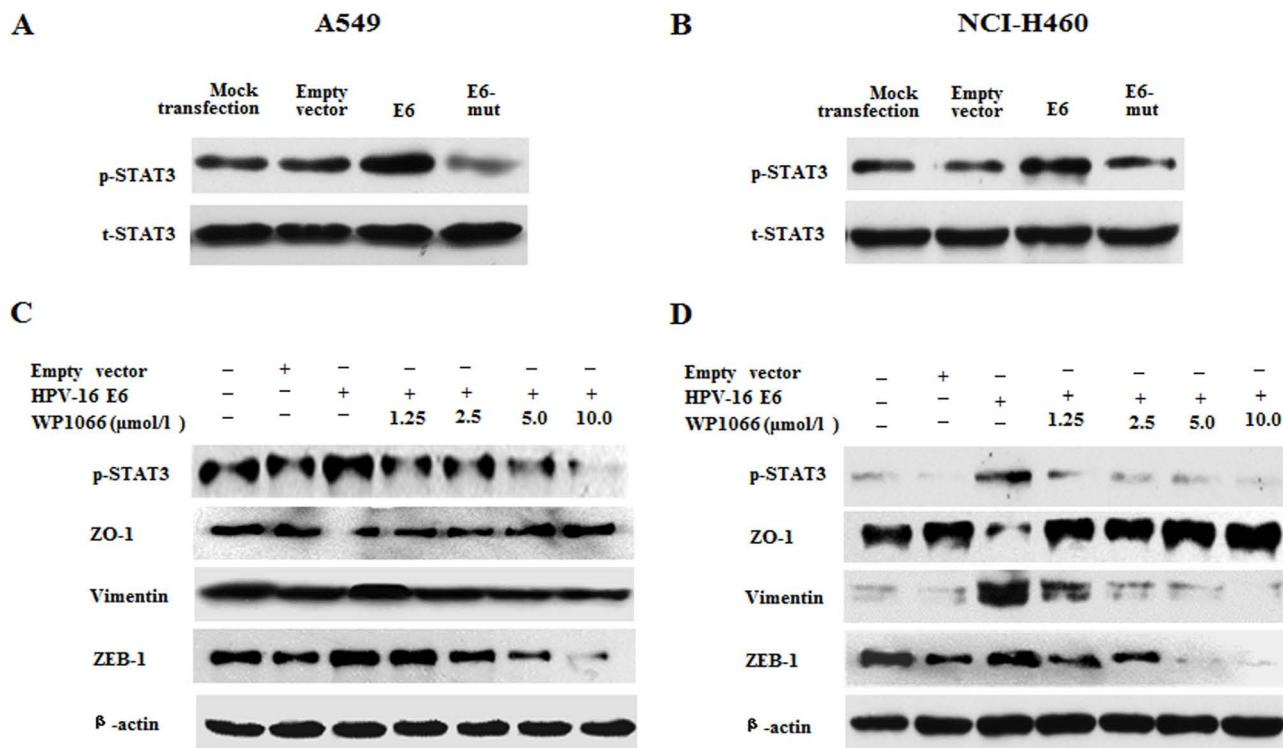


Figure 6. WP1066 reversed the effect of HPV-16 E6 on the expression of ZO-1, vimentin, and ZEB-1 proteins in NSCLC cells. (A, B) Western blot analysis was performed to analyze the expression of total-STAT 3 (t-STAT) and phosphorylated-STAT3 (p-STAT3) in transfected A549 (A) and NCI-H460 (B) cells. (C, D) Transfected A549 (C) and NCI-H460 (D) cells were pretreated with different concentrations of WP1066 for 24 h, followed by Western blot analysis for the expression of STAT3, ZO-1, vimentin, and ZEB-1 proteins.

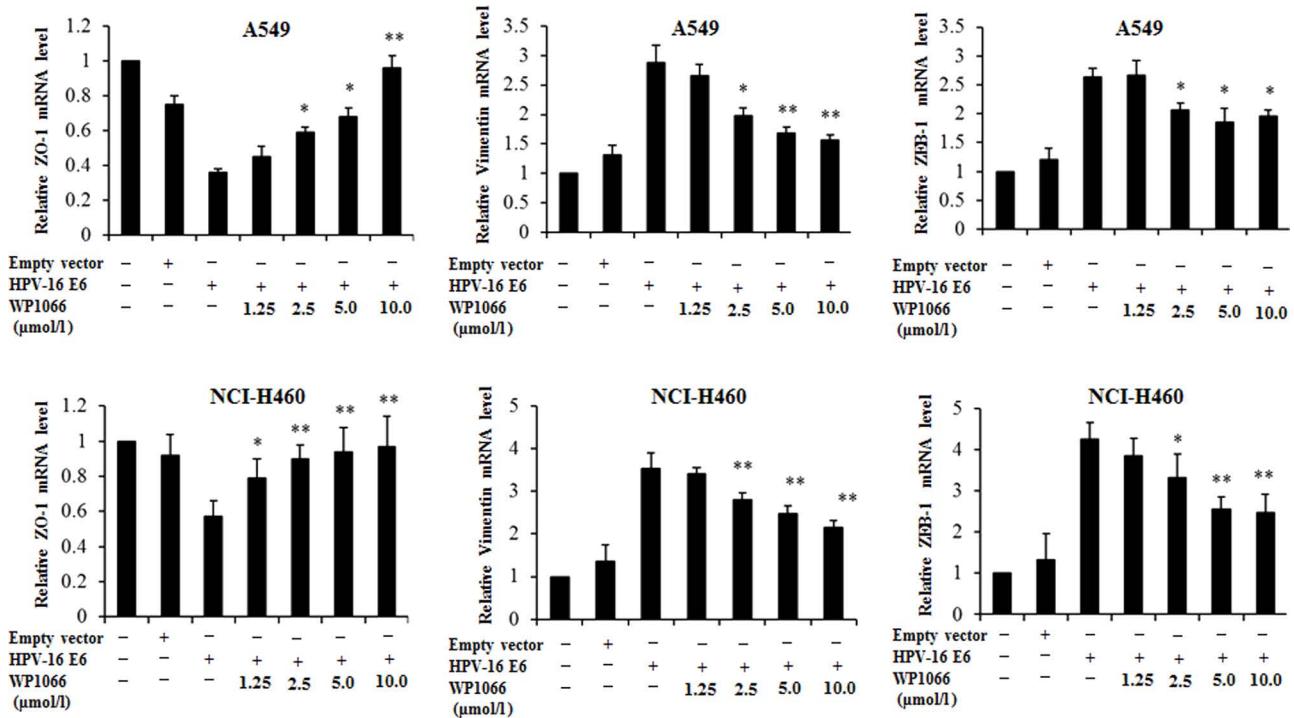


Figure 7. WP1066 reversed the effect of HPV-16 E6 on ZO-1, vimentin, and ZEB-1 mRNA expression in NSCLC cells. Transfected A549 and NCI-H460 cells were pretreated with different concentrations of WP1066 for 24 h, followed by real-time PCR analysis for STAT3, ZO-1, vimentin, and ZEB-1 mRNA levels. All data were expressed as mean \pm SD of three independent experiments. * $p<0.05$, ** $p<0.01$, compared with HPV-16 E6-transfected cells.

via regulating cadherin switch in vitro and in vivo³². However, HPV⁺ vulvar cancers did not exhibit EMT-like events and had a better prognosis³³. These reports indicate that the relationship between HPV and EMT is not completely clear. Moreover, the effect of HPV-16 oncoproteins on EMT in NSCLC has not been reported.

Our previous studies demonstrated that overexpression of HPV-16 E6 and E7 oncoproteins promoted angiogenesis in A549 and NCI-H460 NSCLC cells via enhancing the expression of hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF)²⁶, and PI3K/Akt and c-Jun signaling pathways were involved in HPV-16 oncoprotein-induced HIF-1 α , VEGF, and IL-8 expression and in vitro angiogenesis²⁷. Accumulating evidence has demonstrated that HIF-1 α , VEGF, and IL-8, the important angiogenic factors, can promote EMT³⁴⁻³⁷. HIF-1 α was found to promote EMT and metastasis through the direct regulation of ZEB-1 in colorectal cancer³⁴ and mediate hypoxia-induced EMT in peritoneal mesothelial cells³⁵. HIF-1 α and HIF-2 α were reported to enhance the migratory and neoplastic capacities of hepatocellular carcinoma cells by promoting EMT³⁶. Additionally, increased expression of VEGF was demonstrated to lead to EMT in prostate intraepithelial neoplasia-like cells through an autocrine loop³⁷. IL-8 was also found to inhibit E-cadherin

expression in nasopharyngeal carcinoma cells by enhancing E-cadherin promoter DNA methylation³⁸. Moreover, overexpression of HPV-16 E6 and E7 oncoproteins was demonstrated to mediate HIF-1 α upregulation of GLUT1 expression in lung cancer cells³⁹ and enhance the abilities of migration and invasion by promoting the expression of MMP-2 and MMP-9 in NSCLC cells^{40,41}. A growing body of evidence has demonstrated that EMT plays a crucial role in invasion and metastasis^{18,42}. Therefore, in this study, we further investigated the effect of HPV-16 oncoproteins on EMT in NSCLC cells. Interestingly, we found that overexpression of HPV-16 E6 and E7 oncoproteins in A549 and NCI-H460 NSCLC cells significantly promoted EMT-like morphologic changes, inhibited the expression of EMT epithelial markers (E-cadherin and ZO-1), and enhanced the expression of EMT mesenchymal markers (N-cadherin and vimentin) and transcription factors (ZEB-1 and Snail-1) at both transcriptional and translational levels, indicating that HPV-16 oncoproteins can promote EMT in NSCLC cells. EMT and angiogenesis play a key role in the development and progression. Therefore, taken together, our previous^{26,27} and present studies suggest that HPV-16 oncoproteins can promote NSCLC development and progression by enhancing angiogenesis and EMT.

STAT3, a key transcriptional factor, is involved in a wide variety of essential cellular functions related to proliferation, survival, and angiogenesis⁴³. Recently, STAT3 has been demonstrated to mediate EMT^{23–25}. STAT3 was found to play an important role in Notch1-induced EMT in breast cancer cells²³ and cooperate with Twist to mediate EMT in human hepatocellular carcinoma cells²⁴. Recently, it was reported that NANOG mediated EMT and drug resistance through the activation of the STAT3 pathway in epithelial ovarian cancer²⁵. In the present study, we found that HPV-16 E6 enhanced the activation of STAT3 in NSCLC cells, and WP1066, a specific STAT3 inhibitor, significantly reversed the effects of HPV-16 E6 on the expression of EMT markers including ZO-1, vimentin, and ZEB-1 at both mRNA and protein levels in NSCLC cells. Taken together, our results indicate that STAT3 is involved in HPV-16 E6-induced EMT in NSCLC cells, suggesting that STAT3 may play a role in HPV-16 E6-mediated development and progression of NSCLC.

To summarize, in this study we first demonstrated that overexpression of HPV-16 E6 and E7 oncoproteins enhanced EMT in NSCLC cells, contributing to the development and progression of NSCLC. Furthermore, the STAT3 signaling pathway is involved in HPV-16 E6-induced EMT in NSCLC cells.

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