

Original Article

Analysis of human papillomavirus 16 E6/E7 and L1 in the bronchial brushing cells of patients with squamous cell carcinoma of the lungs

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Abstract: A type of high-risk human papillomavirus (HPV), HPV16 takes part in lung carcinogenesis. E6 and E7 are the major oncoproteins of high-risk HPV, and L1 is the major capsid protein. In this study, we detected their mRNA expressions and analyzed their relationship in the bronchial brushing cells of 211 patients with malignant lesions (squamous cell carcinoma of the lungs) and benign lesions (pneumonia and tuberculosis) by quantitative real-time PCR. HPV16 E6, E7, and L1 mRNA expressions in the malignant group were statistically higher than in benign group ($P < 0.05$), and their mRNA expressions in the squamous cell carcinoma of the lung group were statistically higher than in pneumonia group ($P < 0.05$). There was a negative correlation between L1 and E6 expression in the squamous cell carcinoma of the lungs group (Spearman correlation coefficient $r = -0.498$, $P = 0.000$). An ROC curve shows that the combination of L1 and E6 is a significant predictor for the diagnosis of squamous cell carcinoma of the lungs (AUC: 0.878; Sensitivity: 96.00%; Specificity: 77.91%), which could make up for the deficiency of cytologic testing. The combined detection of HPV16 E6 and L1 mRNA expressions in bronchial brushing cells by quantitative real-time PCR has a great significance for the diagnosis of squamous cell carcinoma of the lungs, providing new therapeutic targets for the clinical treatment of squamous cell carcinoma of the lungs.

Keywords: HPV16 E6, HPV16 L1, bronchial brushing cells, squamous cell carcinoma of the lungs

Introduction

Lung cancer is one of the common malignancies in the world [1]. Squamous cell carcinoma is a common histological subtype of non-small-cell lung cancer [2]. However, there are no specific targeting genes for the treatment of squamous cell carcinoma of the lungs. A recent study indicates that human papillomavirus (HPV) infection may be an important role in the etiology of squamous cell carcinoma of the lungs [3].

As a kind of double-stranded circle DNA virus, HPV is associated with a variety of cancers [4], particularly a persistent infection of high-risk HPV [5]. Syrjanen firstly proposed the hypothesis that HPV infection might play an important role in lung cancer [6]. With the rapid development of molecular biology, a large number of epidemiological studies show that HPV16 (the

high-risk type) infection plays a key role in lung cancer [7, 8], and we have found that HPV16 expression in malignant lung lesions is significantly higher than in benign lung lesions [9].

The HPV genome is composed of an early region and a late region. The early region encodes the viral regulatory proteins (E1, E2, E3, E4, E5, E6, E7, and E8), and late region encodes the viral structural proteins (L1 and L2). E6 and E7 are multifunctional proteins, playing important roles in the transformation of HPV-infected cells. They can enhance the proliferation of HPV-infected cells [10], resulting in the inactivation of tumor suppressor genes p53 and Rb [11, 12]. E6 and E7 are constantly expressed in HPV-infected cells [13], acting as tumor-specific antigens [14]. L1 expression is correlated with the severity of cervical intraepithelial neoplasia (the precursor of cervical cancer) [15]. A previous study indicated that the L1 protein could be

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Table 1. The primer used in quantitative real-time PCR

Gene	Primer sequences	Amplicon size (bp)
L1	Forward 5'-GAGTAGGTGTTGAGGTAGGTCGT-3'	108
	Reverse 5'-TTGCTGCATAAGCACTAGCATT-3'	
E6	Forward 5'-GTATGGAACAACATTAGAACAGCAA-3'	80
	Reverse 5'-GTGGCTTTTGACAGTTAATACACC-3'	
E7	Forward 5'-GCATGGAGATACACCTACATTG-3'	292
	Reverse 5'-TGGTTTCTGAGTAACAGATGG-3'	
GAPDH	Forward 5'-TGCTGAGTAACCTTCGAACC-3'	136
	Reverse 5'-ACTGTCTCTCCCGCAAAG-3'	

Preparation of specimens

Two experienced bronchoscopists performed the bronchoscopies. The area of suspected malignancy was brushed three times; two smears were fixed in 95% alcohol and stained by Papanicolaou's method, and the third brushing was transferred to a small vial containing SurePath™ pre-

detected in HPV infection lesions and cancer cells [16, 17]. L1 has the ability to stimulate an effective cellular immune response [18]. A specific cellular immunity against HPV16 L1 inhibits and clears tumor cells expressing the antigen [19]. However, the regulatory mechanism among E6, E7, and L1 in HPV16-infected squamous cell carcinoma of the lungs is still uncertain.

In this study, we used quantitative real-time PCR to detect the mRNA expressions of HPV16 E6, E7, and L1 in the bronchial brushing cells of 211 patients with squamous cell carcinoma of the lungs, pneumonia, and tuberculosis. And we also analyzed the correlation among their mRNA expressions.

Materials and methods

Patient data

The samples used in this study were obtained in accordance with the Declaration of Helsinki and the Human Subject Research Protocols approved by the ethics committees of the First Affiliated Hospital of China Medical University (No. AF-SOP-07-1.0-01). We obtained written informed consent from the patients. A total of 211 cases of bronchial brushing cells from outpatients and hospitalized patients in the First Affiliated Hospital of China Medical University from June 2013 to June 2015 were collected, including 125 malignant samples (squamous cell carcinoma of the lungs) and 86 benign samples (pneumonia: 65 cases; tuberculosis: 21 cases). The cases of lung squamous cell carcinoma were selected as study subjects, and the cases of pneumonia and tuberculosis were selected as control subjects. All cases were diagnosed by cytologic testing and bronchial biopsy or confirmed by postoperative pathology.

servative fluid (BD Tripath, Burlington NC, USA) for the cytological diagnosis of the liquid-based Pap test. The remaining specimens in the small vial were tested for quantitative real-time PCR. The detailed procedures are shown in our recent research [20].

RNA extraction and cDNA synthesis

The remaining specimens in the small vial were added into centrifuge tubes and centrifuged at 2000 rpm for 5 min. Next the supernatant was discarded, and the sediment was mixed with Trizol, incubating for 5 min at room temperature. After adding chloroform, we centrifuged it at 12000 rpm for 15 min at 4°C. We transferred the upper layer to a new tube and added an equal amount of isopropanol. After centrifuging at 12000 rpm for 10 min, we discarded the supernatant and added 75% ethanol. After centrifuging at 8000 rpm for 5 min, we discarded the supernatant and put it in a vacuum for drying at room temperature for 5 min. Then the extracted RNA was incubated with DNase I (RNase-free DNase set, Qiagen) to eliminate any contamination by DNA. We used the PrimeScript™ 1st strand cDNA Synthesis Kit (TaKaRa, Dalian, China) for cDNA synthesis according to the manufacturer's instructions.

Quantitative real-time PCR

We used SYBR Premix Ex Taq (Tli RNase H Plus, TaKaRa, Dalian, China) for quantitative real-time PCR (7900HT Fast Real-Time PCR) according to the manufacturer's instructions. The results were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. We analyzed the amplicon from the PCR reaction by its melting temperature in the dissociation curve. The relative quantification of the target genes (HPV16 E6, E7 and L1) was carried out

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Table 2. The detection of L1, E6 and E7 mRNA expressions in bronchial brushing cells of patients with malignant and benign lung lesions (Mean \pm SEM) by quantitative real-time PCR

Group	n	L1	E6	E7
Malignant (SCC)	125	0.0070 \pm 0.0006 ^{§,†}	0.0166 \pm 0.0010 ^{§,†,*}	0.0079 \pm 0.0006 ^{§,†}
Benign	86	0.0021 \pm 0.0003 [§]	0.0076 \pm 0.0013 [§]	0.0055 \pm 0.0005 [§]
Pneu	65	0.0012 \pm 0.0002 [†]	0.0069 \pm 0.0015 [†]	0.0049 \pm 0.0005 [†]
Tuber	21	0.0048 \pm 0.0010	0.0097 \pm 0.0029 [*]	0.0073 \pm 0.0008

SCC, squamous cell carcinoma; Pneu, pneumonia; Tuber, tuberculosis; [§]there was a statistical difference between the malignant group and the benign group (P<0.05); [†]there was a statistical difference between the SCC group and the pneumonia group (P<0.05); ^{*}there was a statistical difference between the SCC group and the tuberculosis group (P<0.05).

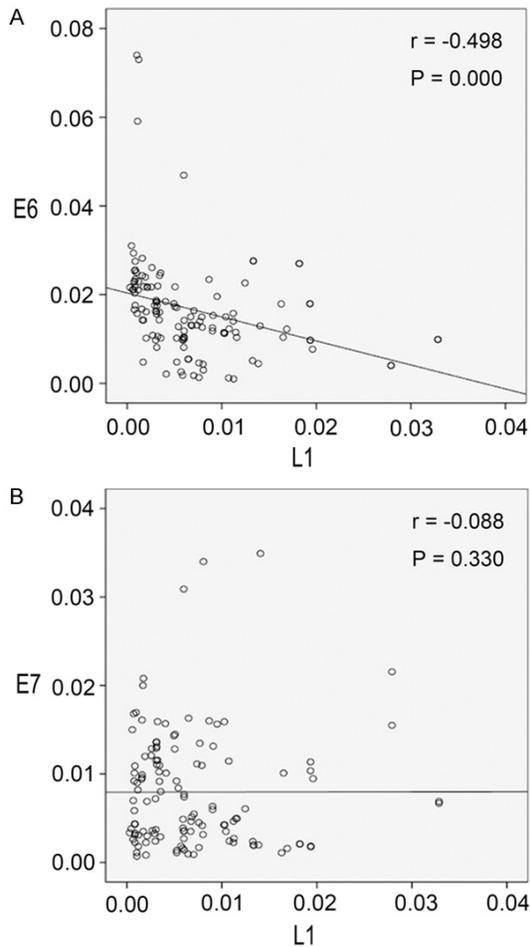


Figure 1. The correlation of HPV16 E6/E7 and L1 in the bronchial brushing cells of patients with squamous cell carcinoma of the lungs. A. Scatter plot of E6 and L1 in the bronchial brushing cells of patients with squamous cell carcinoma of the lungs (R Sq Linear =0.101); B. Scatter plot of E7 and L1 in bronchial brushing cells of patients with squamous cell carcinoma of the lungs (R Sq Linear =2.227E-6).

using the $\Delta\Delta CT$ (threshold cycle) method [21]. GAPDH was selected as an internal reference for normalizing the relative gene expression.

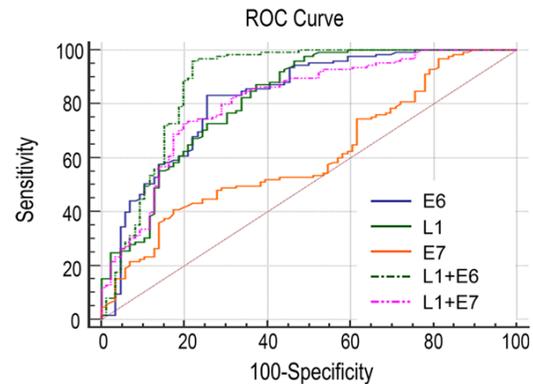


Figure 2. ROC curve analysis.

We repeated the experiments in triplicate. The sequences of primers are shown in **Table 1**.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS Inc, Chicago, IL). When the P value was less than 0.05, it was considered statistically significant. The results are expressed as mean \pm SEM and statistically analyzed based on Student's *t*-test. A Spearman correlation test was used to assay the correlations among the expression of these markers. The receiver operating characteristic (ROC) curve was used to evaluate the area under the curve (AUC), the sensitivity, and the specificity of the predictors, which was made and analyzed by MedCalc.

Results

The mRNA expressions of E6, E7, and L1 in bronchial brushing cells

The bronchial brushing cells of 211 patients were divided into squamous cell carcinoma of the lungs, tuberculosis, and pneumonia. We

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Table 3. Diagnostic value of E6, E7, and L1 in the diagnosis of bronchial brushing cells of patients with squamous cell carcinoma of the lungs

Variable	AUC	95% CI	Youden index	Sensitivity (%)	Specificity (%)
L1	0.817	0.758-0.867	0.4999	87.20	62.79
E6	0.822	0.764-0.871	0.5762	83.20	75.38
E7	0.606	0.537-0.673	0.2336	40.80	82.56
L1+E6	0.878	0.827-0.919	0.7391	96.00	77.91
L1+E7	0.809	0.750-0.860	0.5303	72.80	80.23

used quantitative real-time PCR to detect the mRNA expressions of HPV16 E6, E7, and L1 and then compared their $\Delta\Delta CT$ mean values. Their mRNA expressions in malignant bronchial brushing cells are significantly higher than in the benign bronchial brushing cells ($P < 0.05$). Compared with the pneumonia group, the mRNA expressions of HPV16 E6, E7, and L1 in the malignant group (squamous cell carcinoma of the lungs) are statistically elevated ($P < 0.05$). Compared with the tuberculosis group, the mRNA expression levels of HPV16 E6 in the squamous cell carcinoma of the lungs group are statistically elevated ($P < 0.05$) (Table 2).

The correlation of HPV16 E6/E7 and L1 in the bronchial brushing cells of patients with squamous cell carcinoma of the lungs

There were 125 cases of bronchial brushing cells of patients with squamous cell carcinoma of the lungs. We analyzed the mRNA expressions of L1, E6 and E7 in this group. There is a significantly negative correlation between the mRNA expression of L1 and E6 (Spearman correlation coefficient $r = -0.498$, $P = 0.000$; Figure 1A), but not between the mRNA expression of L1 and E7 (Spearman correlation coefficient $r = -0.088$, $P = 0.330$; Figure 1B).

Diagnostic value of E6, E7, L1, and cytology in the diagnosis of bronchial brushing cells of patients with squamous cell carcinoma of the lungs

An ROC curve was used to determine the predictive values of E6, E7, and L1 for the diagnostic potential of bronchial brushing cells in squamous cell carcinoma of the lungs. The combined detection of E6 and L1 (AUC = 0.878) was a better predictor than any one value alone or the combined detection of E7 and L1 ($P < 0.05$, Figure 2; Table 3). Compared with the result of

cytologic testing (Sensitivity: 60.80%; Specificity: 100%), the combined detection of E6 and L1 has a good sensitivity. They could complement each other.

Discussion

In recent years, lung cancer has had the highest morbidity and mortality among all malignant tumors. Many studies have shown that HPV infection is closely related to lung cancer [3]. HPV16, a high-risk type of HPV, is associated with the progression of lung cancer [8, 9]. Our results show that the mRNA expressions of HPV16 E6/E7 and L1 in the bronchial brushing cells of malignant lesions are higher than in benign lesions. Therefore, infection control of the high-risk HPV virus may be a new treatment for lung cancer.

The carcinogenic effect of HPV is associated with the integration of HPV DNA. After integration, the E6 and E7 oncogenes take part in carcinogenesis by altering the cell cycle regulation so as to cause an unlimited proliferation of cancer cells [22-26]. The major late protein L1 is an important constituent part of the viral capsid. The L1 protein is detected only in its viral productive phase, reflecting the state of HPV replication. This indicates that the body is infected by HPV and the virus is at the stage of replication. L1 is the major target of cellular immunologic response. After the HPV genes are integrated into the host genes, the L1 protein is not expressed [27]. When L1 expression is lost, the body's immune system cannot effectively recognize and eliminate the HPV infected cells, resulting in the progression of malignant transformation [28]. In this study, we found that there was a significant negative correlation between E6 and L1 in bronchial brushing cells of squamous cell carcinoma of the lungs. However, there is no significant correlation between the expression of HPV 16 E6/E7 and L1 in the group of patients who were diagnosed with squamous cell carcinoma of the lungs by histological biopsy with carcinoma cells not being found in the bronchial brushing specimens. It is probably because the brushed area of the bronchoscopic examination is paracancerous tissue in these patients. The ROC curve also showed that the combined detection of L1 and E6 was a good predictor. Therefore, the combined detection of L1 and E6 by quantitative

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real-time PCR is an effective auxiliary method for the diagnosis of squamous cell carcinoma of the lungs, which could make up for the deficiency of cytologic testing. We could speculate that L1 has an effect on E6 in squamous cell carcinoma of the lungs.

The detection of bronchial brushing specimens is generally useful in squamous cell carcinoma of the lungs [29, 30]. In this study, we found that a significantly negative correlation between L1 and E6 mRNA expressions was present in lung squamous cell carcinoma. The number of lung adenocarcinoma cases detected by bronchial brushing was very limited and didn't achieve statistical significance. Further research in this area is needed.

In this study, we found that the detection of HPV16 E6 and L1 mRNA expressions by quantitative real-time PCR had a great significance for cancer diagnosis in the bronchial brushing cells of patients with squamous cell carcinoma of the lungs, which provides new therapeutic targets for the clinical treatment of squamous cell carcinoma of the lungs.

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Disclosure of conflict of interest

None.

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