Expression and Significance of HPV16 E6/E7 mRNAs in the Bronchial Brush and TBNA Cells of Patients With Small Cell Lung Cancer

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Abstract
Background and Objective: Small cell lung cancer (SCLC) is characterized by rapid growth, strong invasion, and early metastasis. However, the cause of its occurrence remains unclear. High-risk HPV infection is closely related to the occurrence of non-small cell lung cancer and cervical small cell neuroendocrine carcinoma. Methods: The expression levels of E6 mRNA and E7 mRNA in HPV16 were detected by qRT-PCR in the bronchial brushing and transbronchial needle aspiration (TBNA) of 310 patients with lung cancer and with benign lung diseases. To make the design of this experiment scientific and reasonable, the expression levels in lung squamous cell carcinoma were taken as positive controls, while those in benign cells were taken as negative controls. Results: The expression levels of E6 mRNA and E7 mRNA in SCLC group were significantly higher than those in benign cell group and slight higher than those in squamous cell carcinoma group. The expression levels of E6 mRNA and E7 mRNA in the central type of SCLC were significantly higher than those in the peripheral type of SCLC. Conclusions: We speculate that the occurrence of some small cell carcinoma is the same as that of some squamous cell carcinoma, which is closely related to HPV16 infection. The overexpression of E6 mRNA and E7 mRNA is in some benign lesion cells, which may be related to HPV transient infection.

Keywords
human papillomavirus, bronchial brushing, transbronchial needle aspiration, lung cancer, mRNA

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Introduction
Small cell lung cancer (SCLC) is the third most common subtype of lung cancer after squamous cell lung cancer and adenocarcinoma. SCLC has the characteristics of rapid growth, high invasion and early metastasis. Its 5-year survival rate is only 5%. In recent years, the amount of researches on high-risk human papillomavirus and tumor pathogenesis has increased significantly. The relationship between human papillomavirus infection and cervical cancer or oropharyngeal squamous cell carcinoma has been discussed in detail, and the relationship between human papillomavirus infection and cervical small cell neuroendocrine carcinoma has been demonstrated.

Human papillomavirus is a kind of circular double-stranded DNA virus. Its infection is closely related to many benign and malignant diseases of human beings. With the rapid development of molecular biology, more and more evidences show that HPV infection plays an important role in the occurrence of non-small cell lung cancer. High-risk HPV16 is considered to be the main type of infection. E6 and E7 proteins in HPV16 are the main oncogenes in the occurrence of...
lung cancer.\textsuperscript{10} Although it has not been confirmed that HPV infection is related to the occurrence of lung cancer in the western population so far,\textsuperscript{11} a considerable number of infection rates (37.22\%) in Asia, especially in China and Japan, are closely related to the occurrence of lung cancer.\textsuperscript{9,12-14} Recently, the research on this aspect has also been confirmed in our research group.\textsuperscript{15-17} However, it is not clear whether high-risk human papillomavirus infection is related to the occurrence of SCLC.

In this study, we used qRT-PCR to detect E6 mRNA and E7 mRNA in bronchial brushing and TBNA cells of 310 patients with lung diseases. We used benign lung lesions as negative control and squamous cell carcinoma as positive control to explore whether high-risk HPV16 infection is related to the occurrence of small cell lung cancer.

**Methods**

**Patient Recruitment and Sample Collection**

This study was approved by the Ethics Committee of the First Hospital of China Medical University, and informed consent was obtained from each patient. This study also included the pathological diagnosis of both histology and cytology from 310 patients who attended the department of pathology at the First Hospital of China Medical University during the period January-December 2018. The cytological features of small cell lung cancer, squamous cell carcinoma, adenocarcinoma and benign epithelial lesions were shown in Figure 1. The study group comprised 177 men and 133 women, ranging in age from 22 to 77 years.

All bronchoscopies were performed by 2 experienced bronchoscopists using a standard video bronchoscope (1T260, Olympus, Tokyo, Japan), a flexible long biopsy forceps (FB21C-1, Olympus, Tokyo, Japan) and straight brushes (BC-202D-2010, Olympus, Tokyo, Japan). To obtain sufficient numbers of cells from the brushings, the brushings were performed before the forceps biopsies, this point had been confirmed in our previous experiments.\textsuperscript{18} All subjects subjected forceps biopsies and brushings, except for some special lesions. The special lesions of hilar or mediastinal masses, lesions outside the bronchus, and enlarged lymph nodes were used with TBNA to obtain abundant cell components. The suspected malignant tumor site was brushed for 2-3 times,

![Figure 1](image)

**Figure 1.** The cell morphological characteristics of small cell lung cancer (SCLC), squamous cell carcinoma (SCC), adenocarcinoma (AC), and benign epithelial lesions (Benign) were in bronchial brushing or TBNA (Papanicolaou stain, $\times 400$). A, SCLC A cluster of cancer cells arranged in a mosaic pattern with obvious crowding. The cancer cells are different in size, various in shape and naked in nucleus. B, SCC In the lower left corner and the center of the image, there is a large cancer cell, respectively. The nucleus is large, irregular in shape, and the cytoplasm is keratinized obviously. C, AC A cluster of cancer cells showed atypical adenoid arrangement, with obvious 3-dimensional sense. The nuclei were ovoid in shape with various sizes, and mucous vacuoles were found in the cytoplasm. D, Benign A cluster of epithelial cells was arranged in sheets with similar size and shape, and cilia were seen on the top of the cells.
each brushing was performed the smear for one slide, the smears were fixed in 95% alcohol and stained by Papanicolaou’s method. After each smear, the remaining cells in the brush were rinsed into a small vial containing SurePath preservative fluid (BD Tripath, Burlington, NC, USA) for qRT-PCR detection. Then, the same brushing site was biopsied with forceps for histological examination, and the sections were stained with H&E and/or immunohistochemistry. TBNA specimens were smeared for 2 slides first, and the remaining specimens were used to prepare wax blocks for histological diagnosis. The remaining cells in the needle tube were rinsed into a small vial containing SurePath preservative fluid for qRT-PCR detection.

**Quantitative Reverse Transcriptase PCR**

Cells were subjected to total RNA extraction using Trizol solution (TaKaRa, Dalian, China) after treatment. qRT-PCR was performed using SYBR® Premix Ex Taq II (TaKaRa, Dalian, China) according to manufacturer’s guide. For detailed operation steps described in reference with PMID 26508030. The primer sequences for the assay are given in Table 1.

**Statistical Analysis**

The SPSS 16.0 statistical software package (SPSS, Inc. Chicago, IL, USA) was used for all analyses. The McNemar’s test was used to compare the expression levels of both E6 and E7 mRNA in SCLC, SCC, AC, and Benign. Analysis of variance and the least significant difference test were employed for statistical analysis. The level of statistical significance was set at P < 0.05.

**Results**

The expression levels of E6 mRNA and E7 mRNA in small cell carcinoma and squamous cell carcinoma were significantly higher than those in benign cells.

The expression levels of E6 mRNA and E7 mRNA in small cell carcinoma and squamous cell carcinoma were significantly higher than those in benign cells (P < 0.01 or P < 0.05), while only E6 mRNA in adenocarcinoma cells was higher than that in benign cells (P < 0.05). When the small cell carcinoma and the squamous cell carcinoma were compared with each other, although the difference between the 2 groups was not significant, the expression levels of both E6 mRNA and E7 mRNA in the small cell carcinoma were slightly higher than those in the squamous cell carcinoma (P > 0.05). The results were shown in Figure 2 and Table 2.

The occurrence of small cell lung cancer may be the same as that of squamous cell lung cancer. At least some cases are closely related to HPV16 infection.

According to the results of Table 2, the expression levels of E6 mRNA and E7 mRNA in lung squamous cell carcinoma were taken as positive controls, while those in benign cells were taken as negative controls. It could be found that the expression levels of E6 mRNA and E7 mRNA in small cell lung cancer group were significantly higher than those in benign cell group and slight higher than those in squamous cell carcinoma group. According to a large number of epidemiological reports and our previous experimental results, the occurrence of some squamous cell carcinoma of the lung is closely related to HPV infection. Therefore, from the results of this experiment, it is inferred that HPV16 infection may also be closely related to the occurrence of some small cell carcinoma.

In small cell lung cancer, the expression levels of E6 mRNA and E7 mRNA in central type were significantly higher than those in peripheral type.

In 44 cases of small cell lung cancer, the expression levels of E6 mRNA and E7 mRNA in 38 cases of central type were higher than those in 6 cases of peripheral type (P < 0.01). The results are shown in Table 3.

The overexpression of E6 mRNA and E7 mRNA in some benign lesion cells. In 126 cases of benign lesion cells, we also selected the cutoff values of E6 mRNA and E7 mRNA as 3.5. The results showed that there were 2 cases of E6 mRNA with high expression, and 5 cases of E7 mRNA with high expression.

**Discussion**

Bronchial brushing and TBNA are the minimally invasive diagnostic techniques, which are suitable for all types of lesions. Therefore, in the diagnosis of lung cancer, assisted forceps biopsy is of great significance. In the previous studies, we used RT-PCR to detect the cancer cells in the bronchial brush cells of patients with lung cancer, proving that this method was more sensitive than conventional cytological diagnosis. In this study, the expression levels of E6 mRNA and
E7 mRNA in HPV16 were detected by qRT-PCR in 310 patients with lung diseases. The results showed that the expression levels of E6 mRNA and E7 mRNA in small cell lung cancer were significantly higher than those in benign cells, and slightly higher than those in squamous lung cancer cells. Therefore, we speculate that the high-risk HPV infection may be closely related to the occurrence of small cell lung cancer. At present, this finding has not been reported.

In 44 cases of small cell lung cancer, we found that the expression levels of E6 mRNA and E7 mRNA in 38 cases of central type SCLC and 6 cases of peripheral type SCLC were significantly different. The expression levels of E6 mRNA and E7 mRNA in central type SCLC were significantly higher than those in peripheral type SCLC. Therefore, we think that this may be related to that central type small cell lung cancer is more susceptible to HPV infection. More and more clinical and epidemiological data show that HPV infection is closely related to the tumorigenesis of organs directly connected with the outside body. These organs are more susceptible to HPV infection.

High risk HPV infection plays an important role in the occurrence and progress of cervical cancer, head and neck cancer, esophageal cancer, gastric cancer and other malignant tumors. All these evidences indicate that HPV infection may have a causal relationship with organs directly connected outside the body, and these organs are more likely to be infected with HPV.

In this study, we found that there are still 2 cases of E6 mRNA and 5 cases of E7 mRNA overexpression in the cells of benign lung lesions. We think that it may be related to the transient infection of HPV16. The specific pathogenesis needs further study in the future.

In conclusion, we detected by qRT-PCR the expression levels of E6 mRNA and E7 mRNA in the bronchial brushing and TBNA of 310 patients with lung disease, especially for small cell lung cancer. The results showed that the expression levels of E6 mRNA and E7 mRNA in small cell lung cancer group are significantly higher than those in benign cell group and slight higher than those in squamous cell carcinoma group. Therefore, we speculate that the occurrence of some small cell carcinoma is the same as that of some squamous cell carcinoma, which is closely related to HPV16 infection. In addition,
we also found that the expression levels of E6 mRNA and E7 mRNA in the central type of SCLC were significantly higher than those in the peripheral type of SCLC. The overexpression of E6 mRNA and E7 mRNA was in some benign lesion cells, which may be related to HPV transient infection.

Authors’ Note
Ethical approval was obtained for the experimental procedures by the Ethics Committee of the First Hospital of China Medical University, Shenyang, China. All procedures in this study were conducted in accordance with the First Hospital of China Medical University’s (APPROVAL NUMBER/2016-125) approved protocols. This article does not contain any studies with animals. Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

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The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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