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Original article

Study on expression of p16 and human papillomavirus 16 and 18 (E6) in OLP and its malignant transformation

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ABSTRACT

Objective: The aim of this study is to investigate the expressions of p16 and HPV16/18(E6) in oral lichen planus (OLP) and malignant transformed OLP (MT-OLP).
Study design: The expression of p16 and HPV16/18(E6) in 40 cases of OLP and 6 MT-OLP was assessed by immunohistochemical staining. Twenty four cases of normal oral mucosa were used as controls.
Results: Compared to normal oral mucosa, the expression of p16 and HPV16/18(E6) protein increased in OLP and MT-OLP. And there was a correlation between p16 expression and HPV infection in OLP and OLP malignant lesions ($p < 0.0001$).
Conclusions: The expression of p16 protein might predict HPV16/18 infection in OLP. And HPV16/18(E6) infection might contribute to OLP malignant transformation.

1. Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease that affects oral mucous membranes and seems to be related with cell-mediated immune process [1]. OLP can cause general erosions of oral mucosa, which lead to pain, discomfort, inquietude and low quality of life [2]. A small section of OLP present for a long time might undergo malignant transformation, resulting in the development of oral squamous cell carcinoma(OSCC) [3]. Most researches have shown that patients with OLP develop into OSCC at an increased ratio than does the normal population. Although there are many differences in experimental designs and regions, it is remarkable that the majority of papers report a rate of malignant transformation of OLP to 0.5–2% in a five year period [4].

Epithelial carcinogenesis is a multi-step process accompanied gene mutation and protein expression changes of the corresponding gene, resulting in uncontrolled cellular growth. To be the end result of the multi-step, oral carcinoma always occurs earlier than morphological alterations, and morphological alterations are not always predictive of a possible carcinoma progression [5–7]. Therefore, molecular markers of diagnose can be useful for recognizing the malignant transformation in clinical application.

The *p16(CDKN2A)* gene maps on 9p21, binds to and inhibits cyclin-dependent kinases (CDK) 4 and 6, which can negatively regulate the cell cycle progression through the G1 to S phase [8]. Mutation and deletion of *p16* gene lead to abnormal cell growth. *p16* has been reported to be a target of deletions of the chromosome 9 in various human cancers [9]. Recently, many studies shown that *p16* has displayed to be methylated in several human cancers, resulting in decreased p16 expression [10–12]. Shintani et al. revealed that p16 expression was obvious in normal epithelium assessed by immunohistochemical staining, but its expression was decreased in dysplasia and almost absent in OSCC [13]. Sopee Poomsawat et al. showed that expression of p16 was detected in 65.2% of OLP [14]. And some studies revealed that the very high frequency of p16 overexpression in OSCC was thought to be related with HPV infection [15,16]. Recent data showed a higher frequency of HPV16/18 in OLP compared with normal mucosa [17–20].

Human papillomavirus (HPV) infection may cause the development of head and neck mucosal lesions. Some types of HPV, such as HPV16, 18, 31, 33 and 35, have been revealed to related with specific types of precancerous and malignant lesions [21]. Eespecially, HPV 16 is a pathogenic factor in 20–25% of OSCC [22,23]. Recently, some studies reported that the HPV infection may be involved in the carcinogenesis of OSCC [15,24,25]. However, some epidemiologic studies on the

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initiation and progression of oral cancer involvement of HPV have generated contradictory results. The discrepancy observed maybe due to the differences of sensitivity of the experimental methods and the characteristics of the patient to be examined. Considering the sensitivity of different detection methods, the infection rate of HPV16/18 is 0–87% in of OLP, 40–67% in leukoplakias and 2.5–76% in OSCC [26]. Some researchers have found a strong association between HPV16/18 infection and oral premalignant lesions, particularly in OLP [17,18]. HPV-positive malignant lesions are characterized by the over-expression of E6 and E7 viral oncoproteins that result in inactivation of retinoblastoma protein and p53 and inhibition of apoptosis [27]. Several studies have suggested that detection of p16 overexpression can be a surrogate marker to identify active HPV infections in different cancers [28–30].

Up to now limited information can be obtained about p16 expression and HPV infection in the malignant transformed OLP. The aim of this study was to analyze the p16 expression in OLP and OLP malignant lesions and compared the expression with normal mucosa to evaluate whether it can be used as a marker for detection of carcinogenesis. The relationship between p16 expression and HPV16/18 infections was further investigated.

2. Materials and methods

2.1. Tissue specimens

The studies involving human materials were approved by the Research Ethics Committee, Dalian Medical University, China. And our research was in full accordance with the Helsinki declaration of the World Medical Association and obtained written consent from all participants. The clinical information of 40 cases of OLP was shown in Table 1. The patients were diagnosed using established clinical and histopathological WHO criteria, as followings: (1) Clinical criteria: presence of white papule, reticular, annular, plaque-type lesions, gray-white lines radiating from the papules; presence of lacelike network of slightly raised gray-white lines (reticular pattern); presence of atrophic lesions with or without erosion. (2) Histopathological criteria: presence of thickened ortho- or parakeratinized layer in sites that are normally keratinized, and, if site is normally nonkeratinized, this layermay be thin; presence of civatte bodies in epithelium, basal layer, and superficial part of connective tissue; presence of a well-defined band-like zone of cellular infiltration that is confined to the superficial part of the connective tissue, consistingmainly of lymphocytes; signs of liquefaction degeneration in the basal cell layer. Tissue specimens of 6 patients with malignant transformed OLP (MT-OLP) were studied, according to the criteria as described in previous studies [31]. The tissue specimens had been fixed in neutral-buffered formalin and embedded in paraffin. Diagnosis was made according to the hematoxylin and eosin (HE) stained sections by two clinical pathologists. Twenty-four normal oral mucosal tissue specimens without necrosis and inflammation were used as control group. All the patients and healthy controls were from the First Affiliated Hospital of Dalian Medical University.

Table 1
Characteristics of the OLP cases.

Characteristics	Cases
Gender	
Male	16
Female	24
Age	
< 54years	19
≥54years	21
Type	
AE	17
non-AE	23
Site	
Buccal mucosa	25
Tongue	10
Lip	5

OLP, Oral lesion planus.

2.2. Immunohistochemical staining

Immunohistochemical staining was performed on 4-μm-thick paraffin serial sections using the streptavidin-biotin complex technique. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol, and nonspecific binding was blocked with 10% normal goat serum. The sections were incubated with p16 (DCS50; 1:200; Santa Cruz Biotechnology Inc.) or HPV16/18 (E6) (C1P5; 1:100; Santa Cruz Biotechnology Inc.) antibodies overnight at 4 °C. After washing with phosphate-buffered saline (PBS), they were incubated with horseradish peroxidase-conjugated secondary antibodies (Invitrogen). The antigen bound peroxidase activity was visualized by staining the sections with diaminobenzidine chromogen. The sections were observed after nuclear staining with hematoxylin. Negative control experiments were carried out by replacing the primary antibodies with PBS.

2.3. Evaluation of immunohistochemical staining

The expression of p16 was considered positive when the nucleus or cytoplasm was stained brown. The HPV16/18(E6) protein expression was mainly in the nuclei and considered as positive when at least a nucleus was stained clearly. The sections were observed and evaluated by three observers independently. Images were obtained on a microscope of Olympus BX43. Integrated optical density (IOD) and the area of target distribution were measured by Image-Pro Plus 6.0 software. We calculated the mean density of each field and took the average of the mean density of ten fields as the mean density of each case.

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 16.0 for Windows software. The difference of expression of target protein between OLP or MT-OLP and the normal controls was analyzed by the Mann-Whitney U test. Correlations were evaluated by the Spearman correlation coefficient test. The significance was considered statistically when $p < 0.05$.

3. Results

3.1. Characteristics of OLP and MT-OLP

The clinical information of 40 cases of OLP was shown in Table 1. The patients included 16 male patients (40%) and 24 female patients (60%). The average age of OLP at diagnosis was 54 years old, and the most common site was buccal mucosa. As shown in Table 2, six cases of MT-OLP included 2 males and 4 females. The average age of these patients at OLP diagnosis was 60 years old. After 3–20 years' follow-up,

Table 2
General information about MT-OLP patients included in the study.

Case	Gender	OLP age	MT-OLP age	Site	Type of tissue	Died of disease
1	F	76	81	Tongue	OSCC II	Yes
2	M	65	71	Tongue	CIS	No
3	F	35	55	Tongue	OSCC I	No
4	F	65	73	Lower lip	CIS	No
5	M	56	61	Lower lip	CIS	No
6	F	65	68	Buccal mucosa and gingiva	OSCC I	No