MicroRNA-137 promoter methylation in oral lichen planus and oral squamous cell carcinoma

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Oral lichen planus (OLP) is a common oral mucosal disease, which is generally considered a potentially malignant lesion. To identify efficiently prognostic biomarker, we investigated the *microRNA-137* (*miR-137*) promoter methylation in OLP and compared with the samples from healthy volunteers and patients with oral squamous cell carcinoma (OSCC). A total of 20 OLP and 12 patients with OSCC as well as 10 healthy subjects were subjected to *miR-137* promoter methylation analysis using methylation-specific PCR (MSP). To address the malignancy prediction potential from *miR-137* promoter methylation status, methylation of the *p16* gene, a well-known tumor suppressor, was investigated in the same samples. The *p16* methylation and *miR-137* promoter methylation were found to be 25% and 35% in patients with OLP, 50% and 58.3% in patients with OSCC, and 0% and 0% in healthy subjects, respectively. The differences between *miR-137* and *p16* methylation levels were statistically significant between healthy controls and patients. Methylation levels of the two promoters were also influenced by age, gender, and lesion duration. Interestingly, aberrant promoter methylation of the *p16* and *miR-137* genes was only found in the epithelium but not in the connective tissue from patients with OLP. This raises the possibility to use *miR-137* methylation as a biomarker for malignant prediction in patients with OLP.

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Introduction

Oral lichen planus (OLP) is one of the most common chronic oral mucosal diseases, and females have more liability to it (1, 2). Oral lichen planus is classified as a potentially malignant disorder by WHO (1). However, a controversy still exists in literature regarding the malignant potential of OLP (3–5). In recent years, some retrospective studies revealed a frequency of malignant transformation from 1.5 to 6.4%, with a follow-up period of at least 6 months (6–9). These facts suggest that patients with OLP have a risk of malignancy, but the mechanisms of the development of malignancy have not been clearly described yet, especially there is a lack of efficient biomarkers to predict the malignant outcome.

DNA promoter methylation is an epigenetic change that happens frequently to regulate the expressions of genes (10). Recently, multiple studies have showed that DNA promoter methylation usually is an early event in carcinogenesis, including oral cancer (11–14). The *p16* gene, a well-known tumor suppressor, plays an important role in regulating the cell cycle, and it has been reported that *p16* promoter methylation is mainly responsible for *p16* inactivation during the malignancy in many types of human cancers (13, 15). *p16* methylation was also detected in oral squamous cell carcinoma (OSCC) and oral pre-cancerous lesion (16, 17). In a prospective cohort study including 78 cases of dysplasia with a 45.8-month follow-up, the patients with *p16* methylation were found undergoing malignant transformation from epithelial dysplasia more frequently than the ones with unmethylated *p16* promoter (18), and it is suggested as a biomarker for the early malignant alteration in oral potentially malignant lesions, such as oral leukoplakia. However, *p16* methylation in patients with OLP has not been reported.

MicroRNAs (miRNAs) are a 21–23 nucleotide-long non-coding RNA molecules that functions to regulate the expression levels of one or more target genes. For instance, in the early stage of carcinogenesis, the altered miRNA expression has already been demonstrated, and this may

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play a key role in the transformation of the cell (19, 20). Thus, changes in miRNA expression patterns provide a great diagnostic tool and prognostic prediction power for human cancers (21, 22). Candidate miRNAs with potential roles in oral cancer etiology and progression have been proposed including loss of *miR-34b*, *miR-100*, *miR-125b*, *miR-137*, *miR-193a*, and *miR-203* (19, 21–24). Usually, upregulation of a miRNA has more prediction power than negative biomarkers. Indeed, miRNA deregulation and complete silencing have been linked to a number of cancer types, accompanying with aberrant CpG methylation within the promoter region. *miR-137* gene has a wide-spread CpG island, and methylation of these segments has been found in OSCC (19), gastric cancer cell lines (25), and colon cancer (26). Furthermore, it has been reported that *miR-137* promoter methylation could be detected in mouth rinse and saliva in patients with oral carcinomas (27, 28). miRNA alterations have also been found in patients with OLP where *miR-125* and *27b* are downregulated (29), but there are no reports studying miRNA methylation in OLP. Here, we investigate the potential for *miR-137* promoter methylation to serve as positive biomarkers in OLP.

During the malignant progress of oral mucosal lesions, the interaction between epithelium and underlying connective tissue has been a subject of interest. As a completely different gene methylation patterns between epithelium and connective tissue in healthy volunteers have been demonstrated previously (14, 28), we here designed the experiment to detect promoter methylation in epithelium and connective tissue separately. This approach enabled us to investigate the differences of methylation profiles between both kinds of tissue and help us to understand their role in the possible malignant transformation progression for OLP.

We discovered that methylation of *miR-137* and *p16* occurred in patients with OLP, but the frequency was lower than in patients with OSCC. Interestingly, the methylation was only found in epithelium but not in connective tissues in OLP, indicating the early malignant alteration of OLP lesion may originate from the epithelium. Our finding adds novel information to the possibility of the development OSCC from OLP and provides a novel biomarker for the malignant potential.

Materials and methods

Tissue samples

All the oral mucosa tissues were obtained from School of Stomatology, Fourth Military Medical University (FMMU), Xi'an, China, between October 2010 and August 2012. The samples were collected from 20 patients with OLP (the duration of disease from 0.5 to 12 years, average 5.6 years), 12 patients with OSCC, and 10 healthy volunteers (Table 1). Tissues were separated into two parts, one was stored in -20°C for DNA extraction, and another one was used for histopathological diagnosis. Final diagnoses were made based on the clinical and pathological characterizations according to the criteria made by WHO (30). There is no one with epithelial dysplasia in this group of patients with OLP and no LP lesion from other area of their body. All the patients were untreated before sample collection. Clinical data from the patients were collected at the time of

Table 1 Description of the study population

	OSCC, <i>n</i> = 12	OLP, <i>n</i> = 20	HM ^a , <i>n</i> = 10
Age, mean years	60.4	49.6	27.8
Gender			
Male	8	8	5
Female	4	12	5
Family history			
Yes	0	0	0
No	12	20	10
Years smoking			
Never	4	13	7
< 10 years	0	2	2
> 10 years	4	2	1
> 20 years	4	3	0
Alcohol Use ^b			
Never	8	17	10
A little	4	3	0
Much	0	0	0
Combined tobacco/alcohol use	4	1	0
Wear dentures			
Yes	3	4	0
No	9	16	10
Lesion site (Biopsy)			
Buccal	5	16	—
Tongue	6	4	—
Palate	1	0	—
Lesion also in other sites in the mouth			
Yes		9	
No		11	
Duration period			
< 5 years	—	9	—
5–10 years	—	6	—
> 10 years	—	5	—
Stage of tumor			
I	7	—	—
II	5	—	—

^aHealthy Mucosa.

^bA little: one drink a week; Much: one drink every day. One drink is considered to be one beer (12 oz), one glass of wine (5 oz), or one shot of liquor (1.5 oz).

enrollment. The protocol has been approved by the Ethics Committee of the School of Stomatology, FMMU.

Tissue separation and DNA extraction

Mucosal tissues from patients with OLP and healthy volunteers were separated into epithelium and connective tissue in 2 mM EDTA buffer (pH = 7.2, containing EDTA, NaCl, KCl, Na_2HPO_4 , and KH_2PO_4) for 1-h incubation at 37°C (31). DNA was extracted by routine method using a TIANamp Genomic DNA kit (Tiangen, Beijing, China).

Methylation analysis

Genomic DNA was treated with sodium bisulfite as described elsewhere (32). Methylation-specific polymerase chain reaction (MSP) was used for the analysis of *p16* and *miR-137* promoter methylation, and then, the PCR products were resolved on 2% agarose gel. The primer sequences used to assess *p16* promoter methylation were as follows: methylated alleles—5'-TTATTAGAGGGTGGGGCGGA TCGC-3' and 5'-GACCCCGAACC GCGACCGTAA -3' with an annealing temperature of 57°C and expected amplicon of 243 bp; and unmethylated alleles—5'-TTAT TAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCCC

AAACCACAACCATAA-3' with an annealing temperature of 57°C and expected amplicon of 243 bp (11). The primer sequences used to assess *miR-137* promoter methylation were as follows: methylated alleles—5'-GCGGTAGTAGTAGCGGTAGC-3' and 5'-ACCCGTCACCGAAAAAAA-3' with an annealing temperature of 58°C and expected amplicon of 86 bp; and unmethylated alleles—5'-GGTGGTAGTAGTGGTAGT-3' and 5'-TACCCATCACCAAAAAAA-3' with an annealing temperature of 51°C and expected amplicon of 86 bp (27).

Positive control of methylated PCR was included using fully methylated DNA obtained from DNA treated with SssI methyltransferase (New England Biolabs Inc., Ipswich, MA, USA).

Statistical analysis

A Student's *t*-test was used to compare the age differences between groups, and Fisher's exact test was used for comparison among categorical variables.

Results

p16 gene promoter methylation status

The *p16* promoter methylation was detected in 5 of 20 patients with OLP, 6 of 12 patients with OSCC, and 0 of 10 healthy subjects. All methylation events in OLP samples were found in epithelium but not in connective tissue (Table 2). There was statistically significant difference of *p16* gene promoter methylation frequency between patients with OLP or OSCC and controls [$P < 0.05$ or < 0.005 , respectively]. However, no statistically significant difference was found between OLP group and OSCC group [$P > 0.05$]. Representative examples of MSP for the *p16* gene promoter methylation were showed in Fig. 1.

Table 2 *p16* and *miR-137* gene promoter methylation rates in each group

	<i>p16</i>		<i>miR-137</i>	
	M(%)	U(%)	M(%)	U(%)
OLP				
Ep	5 (25)	15 (75)	7 (35)	13 (65)
Cn	0 (0)	20 (100)	0 (0)	20 (100)
OSCC	6 (50)	6 (50)	7 (58.3)	5 (41.7)
HM				
Ep	0 (0)	10 (100)	0 (0)	10 (100)
Cn	0 (0)	10 (100)	0 (0)	10 (100)

M, methylated; U, unmethylated; Cn, connective tissue; Ep, epithelial tissue; HM, healthy mucosa.

Figure 1 Examples of methylation of *p16* and *miR-137* promoter. A: *p16* promoter methylation; B: *miR-137* promoter methylation. SssI, positive controls for methylated allele of two genes. M, methylated; U, unmethylated; Cn, connective tissue; Ep, epithelium; Tu, tumor tissue.

miR-137 gene promoter methylation status

miR-137 promoter methylation was detected in 7 of 20 patients with OLP, 7 of 12 patients with OSCC, and 0 of 10 of normal subjects. Similarly to *p16*, methylation of *miR-137* in OLP samples was not found in connective tissue but only in epithelium (Table 2). There were statistically significant differences in *miR-137* gene methylation levels between healthy individuals and OLP group as well as OSCC group [$P < 0.025$ and < 0.005 , respectively]. No statistically significant difference was found between the OLP and OSCC groups [$P > 0.05$]. Representative examples of MSP for the *miR-137* gene promoter methylation were also showed in Fig. 1.

Correlation analysis between methylation- and disease-associated risk factors

The significance of the associations between promoter methylation and various risk factors were analyzed using Student's *t*-test for the correlation with age and Fisher's exact test for correlations with other factors. Even with relative limited objects in the present study, we found a significant correlation of methylation of both *p16* and *miR-137* with age ($P < 0.05$), and higher methylation frequency was in male than in female patients with OLP. Duration of disease was also associated with methylation status of the *miR-137* promoter methylation, which patients with OLP with a longer duration of disease (> 5 years) demonstrated a higher methylation rate of *miR-137* ($P < 0.05$). In addition, the *miR-137* methylation status was also correlated with alcohol use in patients with OSCC ($P < 0.05$). More details for correlation of *miR-137* methylation with risk factors were summarized in Table 3.

Discussion

The malignant transformation of OLP to OSCC has been recognized since 1910 (33). However, there are also some controversial reports that question its malignant potential (3–5). In this study, we investigated the alterations of two molecular biomarkers in OLP and compared with the levels in OSCC. We observed higher frequency of *miR-137* and *p16* promoter methylation in the mucosal tissues of patients with OLP than in the mucosal tissues from healthy individuals, but the frequency was significantly lower than in patients with OSCC. Interestingly, methylation occurred exclusively in epithelium in our present study; no methylation was found for either *p16* gene or *miR-137* gene in connective tissue in patients with OLP. This observation indicates tissue-specific pattern of methylation, which is in agreement with previous reports studying methylation patterns in the *PAI-1* and *miR-137* promoter region in oral malignant and normal epithelium (14, 19, 28). It also indicates that the malignant alterations of OLP lesion probably initiate from epithelium and imply that studying the specific molecular alteration in epithelium may enable early detection of malignant neoplasms of epithelial origin.

In general malignant progression is accompanying the abnormal histopathological alteration, that is, epithelial dysplasia. The OLP samples in the present study did not reveal any epithelial dysplasia, indicating that the methylation may occur prior to the histological alteration during

Table 3 Association of exposure variables with *miR-137* methylation status

<i>miR-137</i>	<i>OLP</i>		P-value	<i>OSCC</i>		P-value
	M, n = 7	U, n = 13		M, n = 7	U, n = 5	
Age, mean years	55.9	46.2	0.05 ^{a*}	62.4	57.6	0.2 ^a
Gender						
Male	5	3	0.05 ^{b*}	7	1	0.005 ^{b*}
Female	2	10		0	4	
Years smoking						
Never	3	10	0.25 ^b	1	3	0.1 ^b
< 10 years	1	1		0	0	
> 10 years	0	2		2	2	
> 20 years	3	0		4	0	
Alcohol use ^b						
Never	5	12	0.25 ^b	3	5	0.05 ^b
A little	2	1		4	0	
Much	0	0		0	0	
Combined tobacco/ alcohol use	1	0	0.5 ^b	4	0	0.1 ^b
Wear dentures	2	2	>0.99 ^b	1	2	0.5 ^b
Lesion site						
Buccal	5	11	>0.99 ^b	2	3	0.9 ^b
Tongue	2	2		4	2	
Palate	0	0		1	0	
Duration period						
< 5 years	1	8	0.05 ^b	—	—	
5–10 years	2	4		—	—	
> 10 years	4	1		—	—	
Stage of tumor						
I	—	—		3	4	0.1 ^{b*}
II	—	—		4	1	

^aStudent's *t*-test.

^bFisher's exact test.

*Significant correlation was also found between factors indicated and *p16* methylation.

M, methylated; U, unmethylated.

progression of the disease. The similar observation has previously been reported in histologically normal epithelium adjacent to the tumor site in patients with oral carcinoma, for example, aberrant expression and mutation of *p53* (34) as well as aberrant methylation of the *ABO* (35) and *DBCCR1* (36) genes. In patients with OLP, the *p53* and *p21* aberrant expression was found in higher ratio than in patients with oral non-specific mucositis, suggesting that more attention should be paid to OLP for its potential role in malignant transformation (37). All those findings suggest that our promoter methylation biomarkers may be even more useful for early predication of malignancy.

In 1994, the *p16* gene was demonstrated by Kamb (38) to be a tumor suppressor gene and also called *multiple tumor suppressor 1 (MTS-1)*, and its important role in regulation of the cell cycle has been subject of numerous studies. It has clearly been demonstrated that a decline in *p16* expression increases the risk of developing a variety of cancers, and aberrant promoter methylation is considered to be a common way in the inactivation of *p16* (15, 39–41). Inactivation of *p16* and associated promoter methylation has been considered a robust biomarkers for oral cancer (15, 42, 43) and other types of human cancers (44–46). In the present study, *p16* promoter methylation was detected in 5 of 20 (25%) patients with OLP, and 6 of 12 (50%) patients

with OSCC in contrast to none of normal subjects. The results indicate that the aberrant *p16* gene promoter methylation in patients with OLP may be used as an indicator for its malignant potential. This finding agrees with previous studies by others (16, 47) that have drawn similar conclusions in patients with oral leukoplakia accompanying with or without epithelial dysplasia.

Compared to *p16*, a classical biomarker, microRNAs have attracted more attention since 2006 and been reported to be hypermethylated in many types of cancer (19, 25, 26). miRNA alterations in oral cancer have been reported, that is, *miR-375* was repressed, and *miR-127* activated in OSCC tumors (19, 28). Altered expressions of *miR-21*, *miR-424*, and *miR-137* were also detected in OSCC (27, 48–50), and miRNA alterations have also been found in leukoplakia (51) and OLP (52). For example, both *miRNA-146a* and *miRNA-155* were increasingly expressed in OLP lesions (52). In a comparison between patients with OLP and healthy controls, 46 miRNAs were found to be differentially expressed with more than 2-fold change, including 8 upregulated and 38 downregulated miRNAs (53). OSCC-correlated upregulation of *miR-21*, *miR-223*, and *miR-143* was also found in OLP (54). *miR-137* is located on human chromosome 1p22 and shown to have tumor suppressor potential in several types of solid tumors, and *miR-137* methylation is commonly observed in human cancers (27, 55), including OSCC (19, 28). In this study, *miR-137* promoter methylation was found positively in 7 of 20 (35%) patients with OLP and 7 of 12 (58.3%) patients with OSCC. Our data indicate that methylation of the *miR-137* promoter may have a potential to be served as a biomarker for malignant predication in patients with OLP during the follow-up. However, the difference was not statistically significant between OLP group and OSCC group in *miR-137* promoter methylation rates due to the relative less case number.

In the analysis of gene promoter methylation-associated risk factors, we found relatively higher frequency of gene promoter methylation in aged, male patients and/or with longer duration of disease. Aging-related hypermethylation has been demonstrated in many different genes like *IGF2* gene (56) and *p21* gene (57). Dysregulated expression of microRNAs in aging-dependent diseases has been also described (58), and it was further addressed that miR-17-92 cluster might be important regulators on the crossroads between aging and cancer (59). To exclude the impact of age on methylation in malignancy, age-matched control group may be necessary to be included, but this is practically a difficult task. For gender-related DNA methylation, there are also several reports demonstrating female predominant gene methylation, such as *MTAP* (60) in gastric cancer, *p14^{arf}* (61) in colorectal cancer, and *CDH1* (62) in lung cancer. Male-correlated methylation has also been reported, that is, *RASSF1A* in lung cancer (62–64). This could be influenced by sex hormones or by physiological differences between the target organs. Promoter methylation of *miR-137* obtained from oral rinse in patients with OSCC has previously been shown (27, 65) where women were found to have a five times higher risk of *miR-137* methylation compared to men (27). The disagreement to our results may need to be clarified in future using an enlarged number of samples. Also, the incidence of OLP is

generally higher in female than male. It is possible that the higher methylation frequency in males in our study may rely on other factors, for example smoking or other environmental factors. Although smoking is not associated with methylation in this study, some studies have reported an association between methylation and smoking in lung (62, 63), prostate (66), and bladder (67) cancer tissue. One recent report indicated that the methylation of multiple microRNA loci was not correlated with smoking habit for most of miRNAs analyzed in non-small-cell lung cancer patients, except for miR-124-2 and miR-124-3 (68). Epigenetic regulation of miR-34a was associated with ethanol-induced apoptosis in human hepatocytes, indicating the roles of epigenetic alteration of miRNA in alcohol-related diseases (69). It is widely accepted that alcohol/smoking can cause cancers, but whether it can influence the gene methylation rates in OLP and furthermore promote malignant transformation of OLP still needs to be uncovered. Multiple miRNA alteration in OLP was found in a small group of patients with relative shorter duration of disease – 6.2 years compared with 10.7 years for whole group of patients (54). Our contradictory finding that longer duration of disease yielded higher methylation frequency may be due the short follow-up period (5.6 years) in general for patients involved in present study.

Overall, our findings suggest that promoter methylation of *p16* and *miR-137* is relatively common event in OSCC and, to a somewhat lower extent, in OLP. In addition, the exclusivity of methylation in the epithelium from patients with OLP indicates that the abnormal methylation of *p16* and *miR-137* may represent an early alteration during the malignancy and may preserve a potential to serve as biomarker for early malignant prediction for patients with OLP. However, further follow-up studies may be needed to evaluate its prediction power in clinic. Furthermore, we have found several risk factors including aging that are associated with aberrant methylation of *p16* and *miR-137*. In this way, our current finding added in novel information for the malignant potential in OLP.

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