p16 Promoter Methylation Is a Potential Predictor of Malignant Transformation in Oral Epithelial Dysplasia

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Abstract

Management of the patient with oral epithelial dysplasia depends on the ability to predict malignant transformation. Histologic grading of this condition fails in this regard and is also subject to interpathologist and intrapathologist variability. This study uses longitudinal clinical samples to explore the prognostic value of a previously validated panel of methylation biomarkers in a cohort of patients with histologically proven oral dysplasia. Methylation enrichment pyrosequencing assays were used to provide the sensitivity of traditional methylation-specific PCR with the additional specificity advantages of a subsequent confirmatory sequencing reaction. In 57% (8 of 14) patients with a lesion that transformed to oral squamous cell carcinoma, 26% (26 of 100) of longitudinal samples collected over ≥3 years showed *p16* methylation. Only 1% (2 of 184) of samples from 8% of patients (2 of 24) not undergoing malignant transformation within 3 years had *p16* methylation. Both of these samples with *p16* promoter methylation were the most recently collected and the patients remain under continuing clinical review. Promoter methylation of *MGMT*, *CYGB*, and *CCNA1* did not correlate with malignant progression. We thus conclude that methylation of the *p16* gene promoter shows promise as a predictor for malignant transformation (Fisher's exact, $P = 0.002$) in a subset of patients. (Cancer Epidemiol Biomarkers Prev 2008;17(8):2174–9)

Introduction

Leukoplakia and erythroplakia (1) are the most common precursor lesions of oral squamous cell carcinoma (OSCC). Dysplasia is reported to be present in up to 25% of biopsies of leukoplakia, whereas the majority of erythroplakia are expected to harbor dysplastic features, which frequently amount to severe dysplasia or carcinoma *in situ* (2). Optimal management of these patients depends on the ability to predict malignant transformation, thus allowing for closer monitoring, risk factor reduction, surgical excision, or pharmacologic intervention.

The conventional management of oral epithelial dysplasia (OED) is based on histologic grading into mild, moderate, and severe dysplasia and carcinoma *in situ*. Sites such as floor of mouth and ventral tongue, and nonhomogenous and red lesions are cited as high risk (3, 4). However, only two significant prognosticators were identified in one recent study, namely clinical appearance of lesion and size (3). The grading of dysplasia did not correlate with the course of the lesions, a finding reported by others (5). Additionally, interpathologist and intrapathologist variability in grading of dysplasia is well-described and may act as a confounding factor (6). A further limitation in the search for predictive markers is the relatively low frequency (<1-18%) with which such premalignant lesions actually acquire invasive potential, although this may be as high as 50% when severe dysplasia or carcinoma *in situ* is present (2).

Allelic imbalance and ploidy analysis have been reported to be useful in prediction of progression, although neither has translated into the clinical setting. Loss of heterozygosity at 3p and 9p has been suggested to increase the risk of malignant progression by a factor of 3.8, whereas additional loss on chromosome arms 4q, 8p, 11q, or 17p leads to a 33-fold increase in risk (7). However, a significant number of dysplastic lesions (47%) investigated by Tabor et al. (8) showed no detectable loss of heterozygosity, and 4 nondysplastic lesions showed extensive losses. The significance of ploidy analysis remains controversial with the recent retraction of several prominent studies by Sudbo (9, 10). Some evidence supports ploidy abnormalities in leukoplakia, although the previously reported prognostic importance has not been substantiated (11).

Few studies have investigated promoter methylation in the setting of OED. Unfortunately, none of the previously published reports have longitudinal clinical follow up and, therefore, cannot correlate molecular changes with malignant progression. Some of these studies investigated OED in clinically normal mucosa at the periphery of invasive squamous cell carcinoma (12, 13), although this strategy clearly introduces bias.

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Variable rates of *p16* methylation ranging from 28% to 75% have been reported (11-13), whereas the *MGMT* gene has been shown to be methylated in 56% of leukoplakia (14) and in 41% of normal-appearing mucosa adjacent to tumor (12).

Investigation of promoter methylation of tumor suppressor genes in the setting of OED seems appropriate, given the relatively high frequency of this epigenetic change in OSCC itself (15) and the numerous reports of methylation in premalignant lesions at other sites (16) including breast (17), pancreas (18), cervix, esophagus (19), and stomach (20). Early epigenetic changes could predispose cells to further genetic abnormalities that allow progression of the neoplastic process. For example, silencing of *p16* may allow epithelial cells to escape senescence, leading to genetic instability and permit accumulation of genetic mutations (17), and *p16* methylation has been suggested as a valuable biomarker in the prediction of malignant change in gastric dysplasia (20). Furthermore, the potential for demethylation and re-expression of *p16* and retinoic acid receptor-β by 5-aza-2-deoxycytidine has been shown in OED cell lines (21).

Methods of evaluating and quantifying methylation in clinical samples continue to evolve. All previous studies of OED have used methylation-specific PCR. Although highly sensitive, this method lacks internal control for adequacy of bisulfite treatment and is prone to false positives, which can occur relatively frequently, particularly when a high number of PCR cycles are used (22). Pyrosequencing offers the advantage of relatively high throughput with confirmation of adequacy of bisulfite treatment and is semiquantitative. Methylation enrichment pyrosequencing (MEP) is a relatively recently described modification of these two techniques that uses methylation-specific primers but is followed by a confirmatory pyrosequencing step that allows elimination of false positives (22). This is particularly appropriate in the analysis of samples where target DNA concentrations may be low, such as mucosal scrapes and saliva. These surrogate samples have been shown to be useful in the investigation of OSCC, with studies demonstrating good agreement of loss of heterozygosity patterns between OSCC and DNA extracted from saliva and lesion brushings (23). Surrogate samples are noninvasive and seem more acceptable to the patient as a means of OED surveillance than continual biopsy.

To exploit the preponderance of molecular data generated in cancer research to develop clinically valuable strategies in OED, two challenges are apparent. First, to identify biomarkers that predict those lesions at high risk for progression with high sensitivity and specificity and, second, to determine if the underlying molecular aberrations are amenable to pharmacologic intervention.

The aim of this study is to explore the prognostic value of a panel of methylation biomarkers previously validated in OSCC (15) in a cohort of patients with OED. This study uses longitudinal tissue collection from a patient cohort with prolonged clinical follow-up. We describe the utility of noninvasive oral scrapes as a surrogate for repeated invasive biopsies, and our assays make use of MEP to improve the specificity and sensitivity of the methylation assays.

Materials and Methods

Patients. Patients with biopsy-proven OED were prospectively enrolled in the study subject to appropriate ethics and consent. Scrapes of visible leukoplakia and erythroplakia were obtained, as well as clinically normal, usually contralateral, oral mucosa. When multiple lesions were present, each was sampled separately. Patients whose lesions were perceived to be at high risk based on existing clinical criteria underwent surgical excision. These patients were retained within the observed cohort and the surgical site or any residual lesion subsequently sampled after healing. Scrapes were obtained using fecal sampling spoons, snap frozen in liquid nitrogen, and stored at −80°C. Rebiopsy was done as clinically indicated and, if sufficient tissue was available, a portion was separated and snap frozen in liquid nitrogen for research use. Clinical variables, site, and past medical and habit history were recorded.

Analysis of clinical data from all enrolled patients diagnosed between 2000 and 2003 was undertaken in 2006 to establish two cohorts. First, a subset of 24 patients was identified with stable or nontransforming dysplasia (''NT'' group). The criteria for inclusion were a histologic diagnosis of dysplasia in a patient who had been followed for a period of at least 36 mo with no evidence of clinical or histologic progression, or perhaps even regression. The second sample set (transformers, ''T'') consisted of 14 patients who, after a biopsy diagnosis of dysplasia, developed invasive carcinoma at the same site at a time interval of at least 6 mo after the index biopsy. This time criteria allowed exclusion of an inadequate or nonrepresentative biopsy. For those patients with multiple premalignant lesions, development of carcinoma at any one of the sites triggered inclusion to the transforming group. In total, 284 scrape samples were collected from 38 patients with OED over 3 y (NT group mean, 7.6; median, 8; range, 2-15 samples per patient; T group mean, 7.1; median, 8; range, 2-25 samples per patient).

DNA Extraction. For both scrapes and tissue samples, DNA was extracted using a phenol-chloroform–based extraction procedure that gives superior yield from small samples (24). Bisulfite treatment of each sample was undertaken using the EZ DNA methylation kit (Zymo Research). A quality check of converted DNA was done using pyrosequencing methylation assay primers (15) specific for bisulfite-treated DNA, which confirmed the DNA content before analysis and assessed the efficiency of bisulfite conversion using an internal control (a C residue not followed by a G residue in the sequence being examined, which will not be subject to methylation and therefore should be completely converted to Uracil). Any samples showing <100% conversion were rejected as these are likely to produce false positives in any methylation-dependent assay.

Methylation Analysis. MEP was used as previously described (22). Hot-start PCR was carried out with HotStar Taq Master Mix kit (Qiagen Ltd.) using 120 ng bisulfite-treated DNA. MEP primers and annealing temperatures are shown in Table 1. PCR conditions were as follows: 1 cycle at 94°C for 15 min; 40 cycles of (94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s); 1 cycle of 72°C for 5 min. The presence or absence of PCR products and freedom from PCR contamination was

Table 1. Gene PCR and pyrosequencing primers and PCR annealing temperature

Gene	Forward primer	Reverse primer	Pyrosequencing primer	Annealing temperature
CCNA1	Biotin-TTTGCGTAGTTTCGAGGATTTC	CCGTTCTCCCAACAACCG	CTAACAACCCCCTCTA	60°C
CYGB	Biotin-TCGATCGTTAGTTCGTTC	ACTAACTCGAAAACGCG	ACCCAACTAAATCCAC	56°C
P16	CGGAGGGGGTTTTTTCGTTAGTATC	Biotin-TCCCCCTCTCCGCAACCG	GGTTGGTTATTAGAGGGT	62°C
MGMT	CGGTTTCGTTTTCGCGTTTC	Biotin-ACCGCGAAAACCTACGAACG	GGATATGTTGGGATAGT	62°C

NOTE: Biotin: the indicated primers were 5′ biotin labeled to enable preparation of the appropriate ssDNA for subsequent pyrosequencing reactions.
Abbreviations: *CCNA1, cyclin A1*; *CYGB, cytoglobin*; *MGMT, 6-O-methylguanine DNA methyltransferase*.

established on 2% agarose gels with ethidium bromide staining. Gel-positive PCR products were subject to confirmatory pyrosequencing to ensure that methylation was >95% at all CpG dinucleotides interrogated. This constituted our criterion for designation of a sample as ''methylation positive.'' These specific CpG targets in four genes have previously been shown to be methylated in a tumor-specific pattern in OSCC (15). Pyrosequencing was carried out using the PSQ96MA System (Biotage) according to manufacturer's protocol, as previously described (15).

Statistical Analysis. Differences in methylation frequency between NT and T groups were tested using Fisher's Exact test. Tests involving samples were not undertaken because interdependence of samples from the same patient was assumed. Differences in the number of methylated genes between NT and T patient groups were tested using the Mann-Whitney test.

Results

Clinical. Of the 24 patients in the NT group, 13 (54%) had mild, 5 (21%) moderate, and 6 (25%) had severe OED on the initial biopsy. Of the 14 patients in the T group, 6 (43%) had mild, 3 (21%) moderate, and 5 (36%) severe OED, although several patients underwent subsequent biopsies that showed a progression to a higher grade. The differences were not significant. There was also no significant difference in the distribution of affected subsites between NT and T groups (data not shown). Smoking history was obtained in 22 of the 24 NT patients. Only 2 had never smoked, most having sustained the habit over a prolonged (>20-year) period. Four of the 14 T patients denied ever smoking, 6 were current long-term smokers, and there were 3 ex-smokers.

Gene Promoter Methylation Analysis. In total, 293 gel positives were obtained from all investigated genes, of which 65% were eliminated either as unmethylated or as nontarget PCR products (i.e., the pyrosequencing reaction failed).

***p16*.** Methylation of the *p16* promoter predicted for malignant transformation, being present in 26% (26 of 100) of samples from T patients compared with 1% (2 of 184) of samples from the NT group (Table 2). Overall, 57% of patients from the T group were positive in at least one scrape compared with only 8% of patients from the NT group (Fisher's Exact, $P = 0.002$). Furthermore, this *p16* promoter methylation was retained in subsequent samples in four of five patients where the first sample testing positive was not the most recently collected. Almost twice as many of the positive samples in the T group were from lesions than from normal tissue (Table 3). Notably, only two samples showed p16 promoter methylation among the NT group, and these samples were the most recently collected from those patients. Transforming patients who showed methylation of the *p16* promoter ($n = 8$; Table 2) displayed variable time intervals between their first presentation of *p16* promoter methylation and the development of OSCC (range, 0-70 months; median, 18 months), which did not seem to correlate with pathologic staging.

***Cyclin A1*.** Methylation at this gene promoter was similar between T (50%) and NT (58%) patient cohorts (Table 2); however, a tendency for methylation in the T samples was observed (12% versus 22%). Within both groups, the majority of positive samples were derived from clinically apparent lesions (Table 3).

***Cytoglobin*.** Methylation at *CYGB* was frequent in both cohorts, seen in 46% and 57% of patients in the NT and T groups, respectively (Table 2). Methylation was seen in 10% of NT samples compared with 25% of samples from the T group. Methylation was observed more consistently in longitudinal samples from the T group, whereas a more sporadic distribution of positive results were seen in the NT group, leading to the frequency of positive samples from normal and lesional tissue shown in Table 3.

***MGMT*.** *MGMT* showed the lowest level of methylation with only 3% and 4% of NT and T samples respectively being positive, corresponding to 21% and 29%

Table 2. Frequency of gene promoter methylation in the NT and T cohorts

	CCNA1		*CYGB*		*p16*		*MGMT*	
	NT	T	NT	T	NT	T	NT	T
Samples	22/184 (12%)	22/100 (22%)	18/184 (10%)	25/100 (25%)	2/184 (1%)	26/100 (26%)	6/184 (3%)	4/100 (4%)
Patients	14/24 (58%)	7/14 (50%)	11/24 (46%)	8/14 (57%)	2/24 (8%)	8/14 (57%)	5/24 (21%)	4/14 (29%)
Fishers Exact test (P)*	0.74		0.74		0.002		0.70	

NOTE: Results are summarized for total samples and for individual patients in separate rows.
*Fishers Exact test relates to patient data (see Materials and Methods).

Table 3. Distribution of gene promoter methylation in normal and dysplastic lesions in the NT and T cohorts

	CCNA1		*CYGB*		*p16*		*MGMT*	
	N	D	N	D	N	D	N	D
NT	5/22 (23%)	17/22 (77%)	9/18 (50%)	9/18 (50%)	1/2 (50%)	1/2 (50%)	3/6 (50%)	3/6 (50%)
T	5/22 (23%)	17/22 (77%)	5/25 (20%)	20/25 (80%)	9/26 (35%)	17/26 (65%)	2/4 (50%)	2/4 (50%)
NT + T	10/44 (23%)	24/44 (55%)	14/43 (33%)	29/43 (67%)	10/28 (36%)	18/28 (64%)	5/10 (50%)	5/10 (50%)

Abbreviations: N, normal; D, dysplastic.

of patients. The positive samples were usually solitary findings among multiple negative samples, thus explaining the discrepancy between the percentages of positive samples and patients.

Methylation at Multiple Gene Promoters. The number of genes showing promoter methylation in each patient was compared between the two groups (Table 4). Methylation of one gene seemed to be a relatively frequent event among stable (NT) patients. A higher proportion of patients with 3 or 4 methylated gene promoters was observed in the T group (6 of 14 versus 1 of 24, T versus NT), but this was not significant. Neither single gene methylation, nor an overall pattern of increasing methylation, correlated with the histologic grade of malignancy (data not shown).

Methylation in Oral Scrapes versus Corresponding Biopsy Tissue. Where both incisional biopsy and corresponding scrape material was available from the same lesion, a comparison was made of methylation status between the two sources of DNA. Concordance of methylation status between scrape and tissue was seen in 80% of cases (61 concordant results, 15 nonconcordant). Interestingly, in the discrepant cases, the scrape was more likely to show positivity than the biopsy, perhaps reflecting a masking of methylation in dysplastic cells by contaminating unmethylated DNA from stroma and inflammatory cells in the latter samples.

Discussion

This study shows that methylation of *p16* in OED is potentially a specific predictive marker of malignant transformation. However, it has limited sensitivity as only 57% of the transforming patients showed this change. In our cohort, histologic grading of dysplasia neither correlated with development of invasive carcinoma, in keeping with previous reports (3), nor with *p16* promoter methylation. Our study is the first to analyze methylation patterns in multiple longitudinal samples from OED patients with no previous history of OSCC, and who were later divisible on the basis of remaining stable for >36 months or showing progression to carcinoma. However, no clear conclusions could be drawn about the timing of *p16* promoter methylation and disease progression in this small sample of patients, although this is clearly an important consideration when determining the suitability of *p16* promoter methylation as a potential biomarker.

The OED/OSCC continuum provides a model in which to study molecular aberrations triggering malignant change in precancerous states. The study of disease progression at any site is often limited by the difficulty in obtaining samples from the same lesion over time and correlation with clinical and histologic change. The opportunities offered by the oral cavity are its relative accessibility and the ability to obtain surrogate tissue samples such as mucosal scrapes and saliva. Few previous studies have investigated methylation in the setting of OED and most have used methylation-specific PCR, which is prone to false-positive results (22). This was highlighted in our study by the proportion of samples in which ''gel-positive'' products were subsequently rejected after pyrosequencing. These failures include priming from unmethylated alleles, thus generating unmethylated products and mispriming, which is a problem with low concentrations of target DNA and high numbers of PCR cycles. MEP offers several advantages in clinical samples of this type. A relatively large sample set can be processed at moderate cost because only gel-positive samples require the confirmatory pyrosequencing step. Validation by pyrosequencing allows the exclusion of all false-positive results and also checks for adequacy of bisulfite conversion. Furthermore, the quantity of DNA obtained from a mucosal scrape may be small and will almost certainly include contaminating oral tissue and salivary DNA. In these situations, MEP is an ideal technique due to its high sensitivity and ability to detect methylation in clinical samples with low concentrations of methylated DNA.

p16 promoter methylation offers promise as a predictive biomarker differentiating between stable and transforming OED. It is noteworthy that in the two NT patients demonstrating *p16* methylation, the change was detected only in the most recent sample. Attempts to compare our data with other published studies are difficult due to differences in methods and lack of clinical follow-up in the literature. Kresty et al. (25) reported methylation of *p16* in 58% patients with severe dysplasia, whereas Lopez (14) noted relatively high rates of methylation at *p16* (44%) and *MGMT* (56%) in DNA

Table 4. Frequency of multiple gene promoter methylation in the and cohorts

	Methylation negative	1 gene positive	2 genes positive	3 genes positive	4 genes positive
NT (n = 24)	3 (13%)	12 (50%)	8 (33%)	1 (4%)	0 (0%)
T (n = 12)	4 (29%)	3 (21%)	1 (7%)	2 (14%)	4 (29%)

extracted from mouthrinse (14), and for *p16*, this was slightly increased in patients with persistent leukoplakia and a history of OSCC. *p16* was shown to be methylated in 75% of OED patients enrolled in a chemopreventive trial (13), with 38% demonstrating reversal during the course of the study. However, correlation of methylation with outcome was not specifically stated and an apparent association between methylation and increasing severity of dysplasia was noted, in contrast to our findings. More recently, Kato and colleagues (12) determined methylation status of *p16* and *MGMT* in OSCC, tumor adjacent mucosa, and mucosa from healthy volunteers. No methylation was found in the latter group, but it was present at high rates in OSCC (51% and 56% for *p16* and *MGMT*, respectively). Clinically normal oral mucosa adjacent to tumor showed an intermediate frequency of methylation (27% and 40%). In our current study, 26% of all patients showed *p16* methylation, clearly at the lower end of the previously reported rates (27-75%). One explanation may be false-positive methylation-specific PCR results in previous studies. The prevalence of *p16* methylation in our previous (unrelated) cohort of OSCC patients (27%; ref. 15) was lower than that of our transforming cohort of OED (57%). This might suggest that p16 methylation may be more common in OED-derived OSCC, or perhaps represents a transient stage in malignant transformation. However, we are cautious in this conclusion because in our previous study, the criteria for *p16* methylation in tumor tissue were significantly more stringent than in this study (>5% methylation using a quantitative assay [pyrosequencing methylation assay] compared with a qualitative [yes/no] assay [MEP]).

To our knowledge, this is the first study that investigates *CCNA1* and *CYGB* in OED. Although methylation at the promoters of these genes was prevalent in OED, this did not predict malignant transformation. Our data for *MGMT* conflicts with that previously published in OED with very few samples showing methylation (3% and 4%). In a cohort of OSCC in which the same CpG islands were analyzed (15), 31% of patients were methylated at this gene. One possible explanation is that silencing of this gene occurs at a later stage and that methylation does not contribute to acquisition of dysplasia or early invasion. Alternatively, it is well-recognized that a subset of OSCC may arise *de novo* without any clinically or histologically detectable lesion, and abnormalities of *MGMT* may represent a subset of tumors with no clinically recognizable premalignant state.

In this study, the finding of methylation rates from 23% to 50% in clinically normal mucosa from OED patients (Table 3) is in keeping with concept of field change (26) and implies that molecular aberrations extend far beyond the limits of histologic and clinical detection. As previously reported, the histologic grade of dysplasia did not predict malignant transformation (3), but notably, there were a higher proportion of non-smokers among the T group. This is consistent with the findings that although smoking is a risk factor for development of OED, the habit seems not to be associated with additional cancer risk (27). This is also corroborated by our previous work, which showed methylation at the *p16* gene in only 4% of clinically/histologically normal mucosa from 70 patients, 57% of whom were heavy smokers (15).

Limitations of the present study include the small number of genes selected for study and the relatively small size of the cohort. The 4 genes chosen for analysis were selected from 10 reported in the literature as most frequently methylated in OED and OSCC (28), and were subjected to validation as tumor specific by pyrosequencing (15). To address the lack of sensitivity of a single methylation marker such as *p16* in predicting transformation, global methylation analysis may prove helpful in the selection of additional markers, and such methods are rapidly evolving.

Confirmatory studies are now required in other cohorts to validate that *p16* methylation is seen specifically in transforming patients and additional methylation markers are required to form a clinically useful predictive panel. This study also suggests that there is some merit in investigating demethylating agents in OED, perhaps delivered topically. Methylation assay of mucosal scrapes would provide a surrogate end point, at least to show pharmacologic effect. Our findings may also have generalized relevance in other upper aerodigestive tract malignancies such as laryngeal, esophageal, and bronchial squamous cell carcinoma, which have common aetiological factors and similar histologic appearances in both the premalignant and invasive stages.

In summary, this is the first study to apply sensitive methylation assays to serial OED scrape samples with corresponding clinical outcome data. Promoter methylation of *p16* is a promising biomarker for malignant transformation in OED.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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