Featured Article

Overexpression of Matrix Metalloproteinase-1 and -9 mRNA Is Associated with Progression of Oral Dysplasia to Cancer

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ABSTRACT

Purpose: **Although an important risk factor for oral cancer is the presence of epithelial dysplasia, many lesions will not progress to malignancy. Matrix metalloproteinases (MMPs) are zinc-dependent proteinases capable of digesting various structural components of the extracellular matrix. Because MMPs are frequently overexpressed in oral squamous cell carcinoma (SCC), we hypothesized that they are also overexpressed in oral dysplasias; we also hypothesized that those dysplasias that progress to oral cancer express higher levels of MMPs than those lesions that do not progress.**

Experimental Design: **In this retrospective study, we examined changes in MMP-1, -2, and -9 mRNA expression using quantitative TaqMan reverse transcription-polymerase chain reaction in 34 routinely processed oral dysplasias and 15 SCCs obtained from 34 patients. After several years of close follow-up, 19 dysplasias progressed to oral SCC and 15 did not.**

Results: **Overall, MMP-1 mRNA was overexpressed (>2-fold) in 24 of 34 (71%) dysplasias and 13 of 15 (87%) oral SCCs. MMP-2 overexpression was seen in 11 of 34 (32%) dysplasias and 7 of 15 (47%) cancers; for MMP-9, overexpression was identified in 29 of 34 (85%) dysplasias and 15 of 15 (100%) cancers. MMP-1 and -9 levels were significantly higher in the SCCs compared with all oral dysplasias ($P = 0.004$ and $P = 0.01$, respectively). MMP-1 and -9 mRNA levels were significantly higher in the oral dysplasias that progressed to oral cancer compared with those that did not ($P = 0.04$ and $P = 0.002$, respectively).**

Conclusions: **Levels of MMP-1 and -9 mRNA may be markers of malignant transformation of oral dysplasia to oral cancer.**

INTRODUCTION

Degradation of the basement membrane and invasion of the underlying connective tissue by neoplastic cells is recognized as a fundamental step in the development of many epithelial cancers (1). It is now widely accepted that breakdown of these barriers is catalyzed by proteolytic enzymes released by the tumor and modulated protease inhibitors. A number of different proteases are involved in tumor invasion including the cathepsins, urokinase plasminogen activator, and matrix metalloproteinases [MMPs (2)].

MMPs are zinc-dependent proteases capable of digesting the various components of the extracellular matrix (ECM). Normally, MMPs are involved in remodeling the ECM, in which this function is integral to normal tissue growth and differentiation. Like many other proteins, the production of MMPs is regulated at the gene transcription level, and this expression is differentially regulated by several growth factors (3).

In several malignancies, overexpression of MMPs has been shown to be associated with tumor invasion. For example MMP-1 has been shown to be overexpressed in a number of different human tumors, and overexpression often correlates with poor prognosis (4–9). Overexpression of MMP-1, -2, and -9 has been identified in a large proportion of squamous cell carcinomas (SCCs) arising in the head and neck (10–14). MMP-9 overexpression is associated with inducible nitric oxide synthase (iNOS) activity, p53 status, and angiogenesis in patients with head and neck SCC (15, 16).

Although expression of some MMPs has been studied in the preneoplastic stages of several carcinomas including cervical, colorectal, and gastric premalignancy (17, 18), few studies have examined changes in MMPs in oral dysplasias. A single study examined six oral epithelial dysplasias using *in situ* hybridization and found overexpression of MMP-1 and -2 mRNA in two cases (19). To date, no study has examined changes in MMPs in relation to progression of oral dysplasia to cancer. The objectives of the present study were as follows: (*a*) to explore the prevalence of mRNA overexpression of MMP-1, -2, and –9 in oral epithelial dysplasias; and (*b*) to compare the level of expression of these proteases in dysplasias that subsequently progressed to SCC and in dysplasias that did not.

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MATERIALS AND METHODS

Case Selection. This study used 49 biopsy specimens collected from 34 patients with clinical diagnoses of leukoplakia or erythroplakia, who were followed at the University of California San Francisco (UCSF) Stomatology Clinical Center. We randomly selected 19 patients with specimens banked in the UCSF Oral Cancer Research Center whose initial biopsy revealed dysplastic changes and whose lesions eventually progressed to SCC ("progressors"). We also randomly selected 15 patients with an initial histopathologic diagnosis of epithelial dysplasia whose oral lesion had not progressed to SCC and who had been followed in the Stomatology Clinical Center for at least 4 years ("nonprogressors"). All patients but one are currently being followed; this one individual was followed for 8 years and had not progressed to oral cancer but was lost to follow-up. Four SCC tissue blocks were not available due to block depletion. Therefore, from 19 patients with dysplasias that progressed to cancer, there were 15 cases of SCC available for analysis.

All biopsies were excisional and came from the same intraoral site showing progressive development of epithelial dysplasia, carcinoma *in situ* (CIS), or SCC. All cases were reviewed to confirm the histologic diagnoses based on established histologic criteria (20). The 49 biopsies comprised 8 mild dysplasias, 16 moderate dysplasias, 10 severe dysplasias (for analysis, the 5 CIS cases were included in this group), and 15 SCCs. For the dysplasia cases, sites of involvement included tongue (20), floor of mouth (5), gingiva (4), labial mucosa (3), and buccal mucosa (2). For the SCCs, sites of involvement included tongue (6), gingiva (4), floor of mouth (3), labial mucosa (1), and buccal mucosa (1).

All biopsies had been routinely fixed in formalin for 24 hours before processing to paraffin. All cases were reviewed to confirm the histologic diagnoses based on established histologic criteria (20). Diagnoses for the preneoplastic specimens ranged from mild dysplasia to CIS.

For controls to normalize relative polymerase chain reaction (PCR) analyses, biopsies of 16 sections of normal mucosa were obtained from the UCSF Oral Cancer Research Center Tissue Bank. Tissues had been fixed and processed in a manner identical to that used for the sequential biopsies under study.

RNA Isolation from Formalin-Fixed Tissues. Total RNA was isolated from 5 × 10-μm paraffin sections using modifications to the Paraffin Block RNA Isolation kit (Ambion Corp., Austin, TX), as we have described previously (21). Briefly, sections were microtome cut from paraffin-embedded tissue samples onto clean, glass microscope slides. The microtome was cleaned after each use, and the tissue sections floated in a water bath containing diethyl pyrocarbonate-treated water before mounting on glass slides. Sections were deparaffinized in xylene, followed by an ethanol wash, and placed in 105 μL of RNA lysis buffer containing 950 μg/mL proteinase K and incubated for 18 hours at 56°C. RNA was obtained by extraction with an equal volume of 70% phenol (pH 4.3):30% chloroform at room temperature, and the aqueous phase was transferred to new microcentrifuge tubes. RNA was precipitated by the addition of an equal volume of isopropanol and 1 μg of linear acrylamide for 2 hours at −20°C. After centrifugation, the pellets were washed in 70% ethanol, air dried, and then resuspended in 50 μL of RNase-free water. Ten microliters of extracted RNA were then treated with 1 μL of DNase I (2 units/μL) in 2 μL of 10× DNase I reaction buffer and 7 μL of nuclease-free water and incubated for 15 minutes at 37°C. RNA was then extracted with acid phenol:chloroform; precipitated by adding 1 μL of linear acrylamide (5 mg/mL), 10 μL of 3 mol/L sodium acetate (pH 4.5), and 1 volume of isopropanol; incubated at -−20°C for 30 minutes; and resuspended in 50 μL of RNase-free water as the final volume.

Reverse Transcription. Reverse transcription was performed using the reverse transcriptase kit (Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol. Reactions were carried out in 20-μL volumes consisting of 1× buffer, 200 units of Moloney murine leukemia virus reverse transcriptase, 40 units/μL RNase inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), 5 μmol/L random hexamers (Life Technologies, Inc.), and 2 μL of total RNA. Reactions were incubated at 25°C for 10 minutes, 37°C for 50 minutes, and 70°C for 15 minutes at 4°C in a PCR thermocycler. After reverse transcription, samples were diluted by adding 60 μL of purified water (Sigma, St. Louis, MO). Negative controls were "no mRNA" where 2 μL of RNase-free were substituted for mRNA template in the reverse transcription reaction and "no cDNA" where water was substituted for cDNA template in the quantitative reverse transcription-PCR mixture.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction. The relative abundance of MMP mRNA was assessed using the 5′ fluorogenic nuclease assay to perform real-time quantitative PCR. The 5′ terminus of each probe was labeled with 6-carboxy-fluorescein (FAM), and the 3′ terminus was labeled with the quenching dye 6-carboxytetramethylrhodamine (TAMRA) . Intron-spanning primer and probe sequences were either obtained from previously published sequences (22, 23) or designed by us to meet specific criteria by using Primer Express design software (Table 1; PE Biosystems, Foster City, CA). Primers and probe were synthesized by Integrated DNA Technologies (Coralville, IA).

Samples were run on an ABI 7700 Prism (PE Biosystems) and monitored continuously during PCR amplification. Each tube was reexamined every 8.5 seconds. Computer software plotted amplification fluorescence *versus* time, represented by cycle number, to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot was examined at a point during the log phase of product accumulation by assigning a fluorescence threshold above background and determining the point at which each amplification plot crosses the threshold [defined as the threshold cycle number (Ct)]. Differences in Ct were used to quantify the relative amount of PCR target contained within each tube. Assuming that each reaction functions at 100% PCR efficiency, a difference of 1 Ct represents a 2-fold difference in the amount of starting template.

Real-time PCR was performed in triplicate 50-μL reaction volumes consisting of 1× PCR buffer (PE Biosystems), 5.5 mmol/L $MgCl_2$, 0.5 μmol/L forward and reverse primers, 0.2 μmol/L probe, 0.025 unit/μL AmpliTaq Gold (PE Biosystems), and 10 μL of cDNA from the reverse transcription reaction. Two-step PCR cycling was carried out as follows: (*a*) 95°C for

Table 1 Sequences of PCR primers and fluorogenic probes used in quantitative PCR

Gene	Primer/probe	Sequence
β-*Gus* (22)	β-Gus-F	CTCATTTGGAATTTTGCCGATT
	β-Gus-R	CCGAGTGAAGATCCCCTTTTTA
	β-Gus probe	FAM-TGAACAGTCACCGACGAGAGTGCTGG-TAMRA
MMP-1 (21)	MMP-1-F	GAGGGTCAAGCAGACATCATGA
	MMP-1-R	CAAGATTTCCTCCAGGTCCATC
	MMP-1 probe	FAM-TGTCAGGGGAGATCATCGGGACAA-TAMRA
MMP-2 (23)	MMP-2-F	TTCCTGGGCAACAAATATGAGA
	MMP-2-R	TGGTCGCACACCACATCTTT
	MMP-2-probe	FAM-AGCGCCGGCCGCAGTGA-TAMRA
MMP-9 (23)	MMP-9-F	CCCTGGAGACCTGAGAACCA
	MMP-9-R	AACCATAGCGGTACAGGTATTCCT
	MMP-9-probe	FAM-TCTCACCGACAGGCAGCTGGCA-TAMRA

NOTE. All probes were labeled with 3′ TAMRA quencher dye.

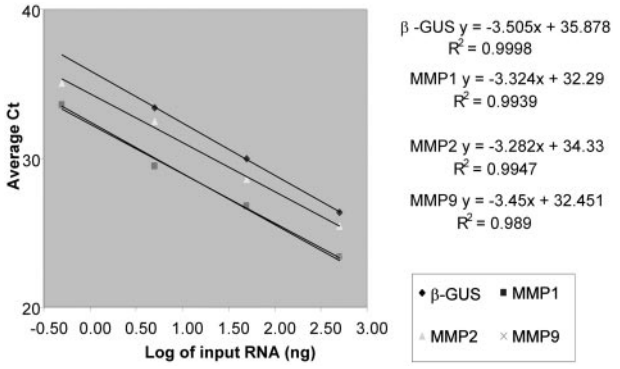

Fig. 1 The logarithm of the input RNA amount from the same sample plotted *versus* the Ct monitored during TaqMan quantitative reverse transcription-PCR. Amplification efficiency of β-*GUS* and *MMP* genes is shown as *regression lines*. All *points* represent the mean of triplicate PCR amplifications. Error bars are too small to be shown.

12 minutes (1 cycle); and (*b*) 95°C for 15 seconds and 60°C for 1 minute (40 cycles). At the end of the PCR, baseline and threshold values were established using ABI 7700 Prism software, and the Ct values exported to Microsoft Excel (Microsoft Corp., Redmond, WA) for analysis.

Quantitative Analysis. Relative expression of MMP-1, -2 and -9 mRNA was calculated using the comparative Ct method described previously (24). This method of analysis was selected because the slopes of the dilution standard curves for MMP and the reference gene β-*N-acetyl-glucuronidase* (β-*GUS*) were comparably similar across a range of input RNA, thus differences in relative abundance for low-quantity RNA species would not distort the analysis. Moreover, absolute Ct values for each sample were found to lie within the range of RNA quantities used for standard curve generation.

Analysis was carried out using the sequence detection software supplied with the ABI 7700 (PE Biosystems). This software calculates the Ct for each reaction, and this is used to quantify the amount of starting template in the reaction. All data were controlled for quantity of input RNA by performing measurements on an endogenous reference gene β-*GUS* (22, 25). The Ct values for each set of three reactions were averaged for all subsequent calculations. A difference in Ct values (ΔCt) was calculated for each marker by taking the mean Ct of triplicate tubes and subtracting the mean Ct of triplicate tubes for β-*GUS* [ΔCt = Ct (*MMP*) − Ct (β-*GUS*)].

Using a series of five normal RNAs, a mean ΔCt was calculated in the same manner as described above and used as a "calibrator." The ΔCt for a test sample was then subtracted from the ΔCt for the calibrator to generate a ΔΔCt [ΔΔCt = ΔCt (test RNA) − ΔCt (calibrator RNA)].

From this value, a relative RNA expression can be calculated using the following formula: relative RNA = $(1 + E)^{-\Delta\Delta Ct}$, where E is the PCR efficiency.

PCR efficiencies were measured as described previously (21) and found to be >96% (Fig. 1). For simplicity, PCR efficiencies were assumed to be 100%; therefore, this assumption introduced minimal error into the calculation. Thus, using this method, a relative RNA value of 1 represents no overexpression compared with the controls, a relative RNA value of <1 represents reduced expression compared with the controls, and a relative RNA value of >1 represents overexpression compared with the controls. To minimize bias of cases showing only minimal overexpression, we therefore defined overexpression when the relative RNA expression was >2 as representing a 2-fold increase (24).

Statistical Analysis. We initially summarized overexpression of MMP-1, -2, and -9 mRNA levels by computing proportions of specimens with a relative expression of >2.0. We used a contingency table approach and the χ^2 test to identify any association between overexpression (as a dichotomized vari-

Table 2 Proportion of cases showing MMP mRNA overexpression

	Dysplasias					
	Nonprogressors (N = 15)	Progressors (N = 19)	P	SCC (N = 15)	P*	P†
MMP-1	9 (60%)	15 (79%)	0.3	13 (87%)	0.2	0.7
MMP-2	3 (20%)	8 (42%)	0.3	7 (47%)	0.2	1
MMP-9	10 (67%)	19 (100%)	**0.01**	15 (100%)	**0.04**	–

NOTE. Overexpression is defined as relative mRNA expression of >2. Significant P values are shown in bold.

* P for χ^2 test comparing dysplasias from nonprogressors with SCCs.

† P for χ^2 test comparing dysplasias from progressors with SCCs.

Table 3 Summary of proportion of dysplasia cases showing overexpression and mean MMP mRNA levels by histological grade

Dysplasia grade	MMP-1	MMP-2	MMP-9
Mild			
Overexpressed	6/8 (75%)	3/8 (38%)	7/8 (88%)
Mean level (±95% CI)	14.8 ± 12.1	1.7 ± 0.7	54.0 ± 38.0
Moderate			
Overexpressed	10/16 (63%)	6/16 (38%)	12/16 (75%)
Mean level (±95% CI)	22.1 ± 18.2	2.0 ± 0.9	64.5 ± 46.6
Severe			
Overexpressed	8/10 (80%)	3/10 (30%)	10/10 (100%)
Mean level (±95% CI)	105.7 ± 56.9	1.8 ± 1.1	112.0 ± 68.9

NOTE. Overexpression is defined as a relative mRNA expression of >2.

Abbreviation: CI, confidence interval.

able) and progressor status. We then computed the mean and median of the MMP-1, -2, and -9 mRNA levels in the dysplasias from both groups of patients (progressors and nonprogressors) and in the SCCs. Because of the non-normality of the distribution of MMP-1, -2, and -9 mRNA levels, we used nonparametric statistical tests to compare these levels in the various groups: we compared mRNA levels in dysplasia specimens obtained from progressors *versus* those from nonprogressors using the Mann-Whitney test. The Wilcoxon signed-rank test was used to compare the dysplasias from the progressor group with the subsequent SCCs in that group.

Immunohistochemistry. To localize the source of the MMPs, five representative cases of each grade of dysplasia and five SCCs all showing overexpression (>2-fold) of MMP-1, -2, and -9 at the mRNA level were selected and stained by immunohistochemistry for protein expression as described previously (26). For MMP-1 staining, the monoclonal antibody Ab-1 (clone 41-1E5) was used at a dilution of 1:100 in PBS. For MMP-2 staining, the monoclonal antibody Ab-4 (clone 75-7F7) was used at a dilution of 1:50, and for MMP-9 staining, the monoclonal antibody Ab-7 (clone GE-213) was used at a dilution of 1:500. All antibodies were obtained from Oncogene Research Products (San Diego, CA). Microwave antigen retrieval in 0.1 mol/L sodium citrate buffer was used for MMP-1 and -9 but was not needed for MMP-2. Normal mouse serum containing mixed immunoglobulins at a concentration approximating the primary antibody was used as a negative control.

RESULTS

Among the 49 biopsies used in this study, there were 29 epithelial dysplasias, 5 CIS samples, and 15 SCCs obtained from 19 men and 15 women. Of the 34 patients, there were 19 patients who progressed to oral cancer over a median period of 2 years (range, 1–7 years) and 15 patients who did not do so after a median follow-up time of 7.13 years (range, 4–20 years).

RNA was extracted, converted to cDNA, and amplified by quantitative reverse transcription-PCR from all 49 formalin-fixed, paraffin-embedded tissue sections of oral dysplasia and SCC and from all 16 normal controls.

Overall, MMP-1 mRNA was overexpressed (>2-fold) in >70% of dysplasias and in >85% of oral SCCs. MMP-2 overexpression was seen in less than half of the dysplasias or cancers; for MMP-9, overexpression was identified in 29 of 34 (85%) dysplasias and 15 of 15 (100%) cancers (Table 2). Overexpression was independent of dysplasia grade (Table 3). MMP-1 and -9 levels were significantly higher in the SCCs compared with all oral dysplasias ($P = 0.004$ and $P = 0.01$, respectively). MMP-1 and -9 mRNA levels were significantly higher in the oral dysplasias that progressed to oral cancer compared with those that did not ($P = 0.04$ and $P = 0.002$, respectively; Table 4). By immunohistochemistry MMP-1, -2, and -9 protein expression was localized to the cytoplasm of tumor cells and dysplastic epithelium (Fig. 2), although a few stromal inflammatory cells also showed expression of these proteins.

DISCUSSION

MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) initiate basement membrane degradation and further degrade collagenous and noncollagenous parts of the ECM. Neoplastic keratinocytes express MMP-2 and –9, and in culture, oral SCCs with high levels of MMP-2 and -9 show greater invasion capacities compared with those with low levels of expression (27). Increasingly high levels of MMP-2 and -9 have been associated with poor prognostic factors for patients with

Table 4 Summary of levels of MMP mRNA in progressing and nonprogressing oral dysplasia and cancers

	Dysplasias ($N = 34$)		
	Nonprogressors ($N = 15$)	Progressors ($N = 19$)	SCCs ($N = 15$)
MMP-1			
Mean (±95% CI)	14.65 ± 18.27	34.97 ± 19.41	100.00 ± 63.99
Median (range)	2.14 (0.24–129.18)*†	18.89 (0.33–150.80)*†	74.02 (0.86–407.28)*†
MMP-2			
Mean (±95% CI)	1.84 ± 0.98	2.00 ± 0.77	2.75 ± 1.72
Median	1.06 (0.70–7.19)	1.62 (0.32–6.64)	1.73 (0.15–12.18)
MMP-9			
Mean (±95% CI)	37.78 ± 23.54	108.36 ± 51.72	119.41 ± 40.44
Median	12.28 (0.99–137.03)*†	78.52 (6.92–400.32)*†	124.64 (2.57–256.89)*†

* MMP-1 and -9 mRNA levels were significantly higher in the SCCs compared with dysplasias that progressed to oral cancer and those dysplasias that did not progress to oral cancer ($P = 0.004$ and $P = 0.01$, respectively).

† MMP-1 and -9 mRNA levels were significantly higher in the oral dysplasias that progressed to oral cancer than in those dysplasias that did not progress to oral cancer ($P = 0.04$ and $P = 0.002$, respectively).

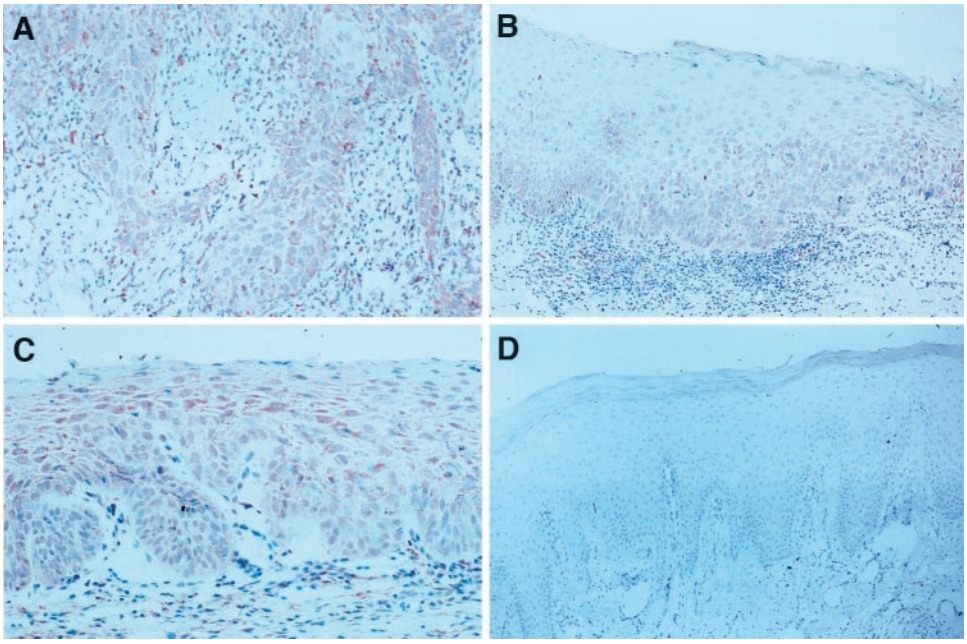

Fig. 2 Localization of MMP protein expression in sections of dysplasia and SCC. *A*, moderately differentiated SCC showing diffuse cytoplasmic expression of MMP-1 (×200). *B*, mild epithelial dysplasia showing cytoplasmic expression of MMP-2 (×100). *C*, severe epithelial dysplasias showing diffuse cytoplasmic expression of MMP-9 (×200). *D*, negative control (×100).

oral cancer including the development of lymph node metastases and poor survival (13, 28). We found that MMP-9 is frequently overexpressed in oral SCC. We found that almost all oral SCCs showed overexpression of MMP-9, and these results are consistent with other reports of high levels of MMP-9 mRNA in oral SCC or tumor cell lines (10, 16, 19).

MMP-1 is a potent interstitial collagenase that has been shown to be frequently activated in invasive oral SCC (19, 21). MMP-1 mRNA expression has been shown in epithelial cells within tumor islands but also with the fibrous connective tissue adjacent to the tumor (29). Using quantitative PCR methods, we found that MMP-1 was overexpressed in 71% of oral dysplasias and 87% of oral SCCs. These results are similar to those of other studies examining the expression of MMP-1 in head and neck SCC and showing frequent overexpression or enhanced activation of this molecule (16). Only one other study has used semiquantitative PCR methods to show that MMP-1 is overexpressed in fresh biopsies of laryngeal and pharyngeal carcinomas (30). We also found that mRNA levels of MMP-1 were significantly higher in the oral SCCs compared with all oral dysplasias, in keeping with other reports showing that overexpression is associated with stromal invasion by tumor cells (11, 19).

Many oral SCCs are preceded by white or red lesions showing varying degrees of epithelial dysplasia from mild to severe (20, 31–33) Although an important risk factor for oral cancer is the presence of epithelial dysplasia, many dysplasias will not progress to malignancy (34). We found that higher levels of MMP-9 mRNA are significantly associated with oral dysplasias that progress to oral cancer compared with those that did not progress. This finding is interesting in view of the relatively small sample size ($n = 34$ patients) from which the material is drawn. Although other molecular markers have been identified that may identify oral premalignancy at greater risk of malignant transformation (32, 35), assay of MMP-1 or -9 may prove useful because it is a single molecular test and, technically, it is a relatively simple test to perform. The assay described here does not rely on microdissection of sections for lesional tissue. Moreover, these findings also raise the possibility of using immunohistochemistry to assess MMP-1 and -9 protein levels because MMP mRNA and protein levels often correlate strongly (21, 36, 37). It is important to stress that not all progressing dysplasias overexpressed MMP-1 and that this fact may result in a small false-negative rate. Larger, prospective trials assessing MMP-1 and -9 expression for the prediction of oral cancer development may be important to further assess this finding and to determine clinical applicability. Moreover, whether a single or a panel of markers will be necessary for cancer prediction is also an important issue to address.

We found overexpression of both MMP-1 and -9 in almost all oral SCCs. Formalin fixation of the study material did not permit assessment of MMP activity. Activation of MMPs is tightly regulated at several biological levels, including at mRNA expression, by growth factors, hormones, other chemicals, and, importantly, the tissue inhibitors of metalloproteinases (2). However, in cervical cancer, overexpression of some MMPs correlates well with increased activation. Therefore it is likely that a large proportion of oral dysplasias and cancers in our study showing overexpressed MMPs at the mRNA level would also show activated proteinases (36, 37).

In conclusion, this study has used quantitative reverse transcription-PCR to show that MMP-1, -2, and -9 are frequently overexpressed in both oral dysplasias and oral SCCs. We have also shown that elevated levels of MMP-1 and -9 mRNA are significantly associated with oral dysplasias that progressed to oral cancer compared with those dysplasias that did not progress; levels of MMP-1 and -9 mRNA may be markers of malignant transformation at this site.

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