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DNA Promoter Hypermethylation in Saliva for the Early Diagnosis of Oral Cancer

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ABSTRACT Oral health care professionals could drastically improve the quality of life for patients with potentially malignant oral lesions by using a noninvasive test that could be used to detect cancer using saliva. Promoter DNA hypermethylation is a critical step in oral carcinogenesis and has a number of significant advantages over genetic and protein diagnostic markers. Methylight is a recently developed assay that rapidly quantifies promoter hypermethylation and could potentially be applied into a clinical setting.

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DISCLOSURE

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Oral cancer continues to be a significant health problem that affects 40,000 people in the U.S. each year.¹⁻² In the U.S. more people die from oral cancer than melanoma, cervical, and ovarian cancer combined.³ Early diagnosis significantly improves tumor control and survival. The five-year survival for patients with stage I or II oral cancer is 70 percent to 80 percent. In contrast, patients with stage III or IV oral cancer have a survival rate of only 40 percent to 50 percent. Dental health care professionals are commonly required to evaluate patients with potentially malignant oral lesions. There is currently no method to differentiate between patients with oral cancer and oral dysplasia without performing a biopsy. The development of a noninvasive diagnostic

method, such as salivary analysis, could be used for oral cancer screening leading to an early diagnosis and an improvement in a patient's quality of life.

For a diagnostic test to be implemented clinically, the test must detect an event in oral cancer patients that is not otherwise present in normal individuals. One such event is methylation of cancer-associated genes, which leads to a loss of function for the gene. Methylation is an epigenetic alteration that involves the addition of methyl groups to cytosine residues in a CpG dinucleotide. It occurs in the promoter region of a gene, where there is a high density of CpG dinucleotides. In fact, promoter hypermethylation is a more frequent mechanism in gene silencing than genetic mutation.⁴ It is one of the earliest events in oral carcinogenesis, preceding changes in

protein expression level. These advantages make promoter hypermethylation a very attractive diagnostic marker for the early detection of oral cancer.

The hypothesis of this project is that the presence of oral cancer leads to changes in promoter hypermethylation that could be detected in the saliva of patients. Moreover, salivary analysis based on hypermethylation could be used to noninvasively screen patients at risk for oral cancer.

The first objective of this project, therefore, is to quantitatively analyze promoter hypermethylation of five genes (*APC*, *E-cadherin*, *MGMT*, *p15*(*INK4B*), and *p16*(*INK4A*)) in the saliva of three groups of patients: normal, dysplasia, and cancer. Current studies with tissue DNA reveal these genes are most often inactivated by promoter hypermethylation, allowing for the progression of oral cancer.⁵⁻⁷ **TABLE 1** lists the five genes and their role in carcinogenesis.

Even though quantifying promoter hypermethylation from saliva DNA could be a robust diagnostic tool due to the noninvasive nature of the test and the possibility of detection at the earliest stages, to date, the studies analyzing promoter hypermethylation in saliva DNA have only been qualitative.⁸⁻¹⁰ This project is unique because it will be the first to quantitatively measure promoter hypermethylation in saliva DNA, which would allow for higher sensitivity.

Tissue DNA has successfully been used to quantify promoter hypermethylation, but high-quality saliva DNA is much more difficult to isolate than tissue DNA.¹¹⁻¹³ Since this is the first time anyone is quantifying promoter hypermethylation in saliva DNA, it is necessary to establish the validity of using saliva DNA in a quantitative assay. Therefore, the second objective of this project is to determine the correspondence between

TABLE 1**Role of Genes in Carcinogenesis**

Gene	Role in Carcinogenesis
<i>APC</i>	Tumor suppressor
<i>E-cadherin</i>	Synthesizes calcium-dependent adhesion protein involved in metastasis
<i>MGMT</i>	O6-methyl-guanine repair gene
<i>p15</i>	Tumor suppressor; inhibits activity of cyclin-dependent protein kinases, which controls normal progression through G1 of cell cycle
<i>p16</i>	Tumor suppressor; inhibits activity of cyclin-dependent protein kinases, which controls normal progression through G1 of cell cycle

saliva and tissue promoter hypermethylation. A high positive agreement value between the methylation status of saliva DNA and tissue DNA would indicate saliva is a valid diagnostic medium.

MATERIALS AND METHODS**Promoter Methylation Quantification in Saliva****SALIVA COLLECTION**

A faculty mentor routinely sees patients with oral dysplasia and oral cancer. Fourteen patients with either biopsy-proven oral SCC or oral dysplasia at the sites indicated in **FIGURE 1** who have not been previously treated for oral SCC or oral dysplasia, were recruited into the study from the clinical practice of the faculty mentor at the University of California, San Francisco. Saliva was collected before surgical resection. Five normal subjects with no history of oral lesions were also recruited. Whole saliva, 7.5 ml, was collected from oral cancer patients, dysplasia patients, and normal subjects between 6:30 a.m. and 8 a.m. Following collection, saliva samples were stored in a -80-degree Celsius freezer.

DNA Extraction and Modification

Genomic DNA was extracted from 1000 µl saliva with a commercially available DNA extraction kit (QIAamp Blood Kit; Qiagen Hilden, Germany). The DNA

was chemically modified with sodium bisulfite to convert all unmethylated cytosines to uracils while leaving methylated cytosines unconverted (EpiTect Bisulfite Kit, Qiagen Hilden) and eluted in 20µl elution buffer. Such modification allowed for differentiation between methylated and unmethylated DNA using Methylight.

Methylight

Methylight, a fluorescence-based real-time PCR assay, was employed to detect methylation in the promoter region of the genes of interest. Three oligonucleotides, a forward primer, reverse primer, and probe were designed for each of the five genes, *APC*, *E-cadherin*, *MGMT*, *p15*, and *p16*. These oligonucleotides annealed within the promoter region of each gene to quantify its methylation status. They were specific to the methylated version of DNA that was unconverted by the sodium bisulfite treatment. The primers, therefore, only amplified methylated DNA and left unmethylated DNA unamplified. The probe was linked to a 5' FAM, 6-carboxyfluorescein, reporter and a 3' Black Hole Quencher (BHQ) dye. During PCR amplification, the reporter dye was separated by the 5'→3' exonuclease activity of DNA polymerase and its fluorescence was measured.

The fluorescence was plotted on an amplification curve; the Ct value obtained from the amplification curve was used to quantify the amount of DNA



FIGURE 1. The location of oral lesions is indicated with blue representing cancer and red representing dysplasia. Sites include: tongue, floor of mouth, maxillary and mandibular gingiva, and hard and soft palate.

that was amplified. In addition to the five genes of interest, oligonucleotides were also designed for *COL2A1*, an internal reference gene that would be amplified regardless of the methylation status of the DNA. *COL2A1* was used to normalize for differences in genomic template amounts in each reaction.¹⁴

For each amplification reaction, 2 μ l of bisulfite converted DNA was used. PCR was performed in a 30 μ l reaction consisting of 0.3 μ M of each primer, 0.1 μ M of probe, 200 μ M each of dATP, dCTP, and dGTP, 400 μ M of dUTP, 6.7 mM MgCl₂, 1x TaqMan Buffer A, 1x stabilizer, and 2 U of AmpliTaq Gold polymerase at the following conditions: 95-degrees Celsius for 10 min, followed by 50 cycles at 95-degrees Celsius for 15 s and 60-degrees Celsius for 1 min. Male peripheral blood leukocyte DNA (PBL-DNA; Promega) was modi-

fied with SssI-CpG methylase enzyme to generate fully methylated human genomic DNA, and was used as a positive control.

Statistical Methods

PCR was performed for each saliva DNA sample with primers and probes from 1) the gene of interest (*APC*, *E-cadherin*, *MGMT*, *p15*, and *p16*) and 2) the reference gene (*COL2A1*). The Ct value of each sample was used to calculate the quantity of DNA that had been amplified during the run. A ratio of the quantity of DNA amplified from the gene of interest (*GENE*) and the quantity amplified from *COL2A1* was obtained. The percentage of methylation value (PMR) was calculated for each sample by dividing the *GENE/COL2A1* ratio of a sample by the *GENE/COL2A1* ratio of the positive control DNA and multiplying by 100.¹⁵

A PMR value was calculated for all normal, dysplasia, and cancer saliva DNA samples. A PMR cutoff value was calculated, above which samples were considered to be positive for methylation, by taking the median PMR value of normal samples (if > 0) plus one percentage point. If the median PMR value of the normal samples was 0, the PMR cutoff was 1.¹⁶

COMPARISON OF PROMOTER HYPERMETHYLATION IN TISSUE AND SALIVA DNA

Paraffinized tissue blocks were obtained from patients whose saliva was collected. Ten 10 micron sections were cut from the blocks and genomic DNA was harvested following a protocol for paraffinized tissue (QIAamp Blood Kit). Bisulfite treatment and Methylight were performed at identical conditions to saliva DNA.

Statistical Methods

PMR values were calculated for each tissue DNA sample. A PMR cutoff was determined from the normal tissue DNA using the same calculations as described previously. Cancer or dysplasia samples with a PMR value above the threshold were considered positive for methylation. A positive agreement value between tissue and saliva DNA was calculated for each of the five genes by comparing the methylation status of the tissue and saliva DNA for each sample.

RESULTS

Promoter Hypermethylation Quantification in Saliva

Results from the calculations showed the proportion of samples in the cancer/dysplasia group that were positive for methylation. From **FIGURE 2**, 35 percent of the samples were methylated at *p16*; 29 percent at *MGMT* and *p15*; 14 percent at *APC*; and 7 percent at *E-cadherin*.

Furthermore, the results showed that 71 percent of the oral cancer/dysplasia samples were methylated for one or more genes; 29 percent for two or more genes; 7 percent for three or more genes; and 7 percent for four or more genes (**FIGURE 3**).

COMPARISON OF PROMOTER HYPERMETHYLATION IN TISSUE AND SALIVA DNA

After performing Methylight on saliva and tissue DNA from corresponding patients, the positive agreement value between tissue and saliva DNA at each of the five genes was determined. From **TABLE 2**, the positive agreement of tissue and saliva DNA of *p16*, *E-cadherin*, *p15*, *MGMT* and *APC* were 87.5 percent; 87.5 percent; 62.5 percent; and 62.5 percent; and 12.5 percent, respectively, with a mean value of 62.5 percent.

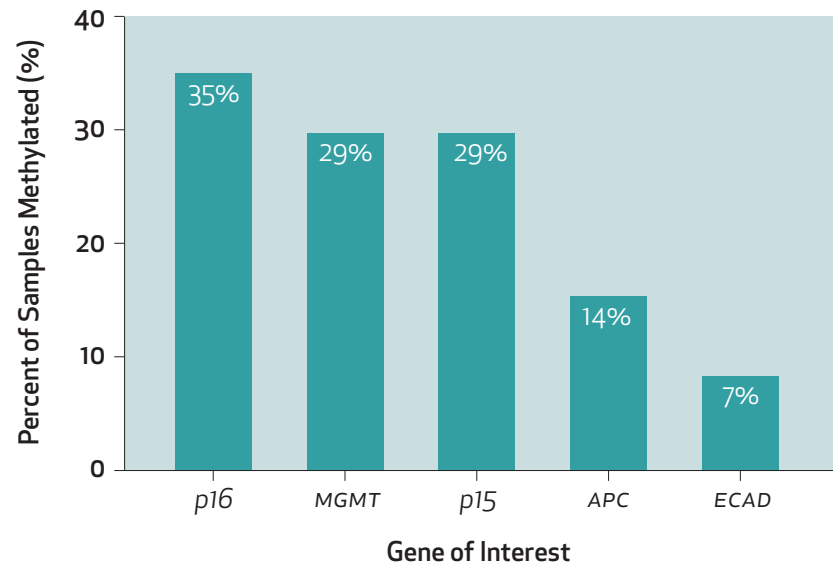


FIGURE 2. The percentage of cancer/dysplasia samples methylated at the corresponding genes is shown.

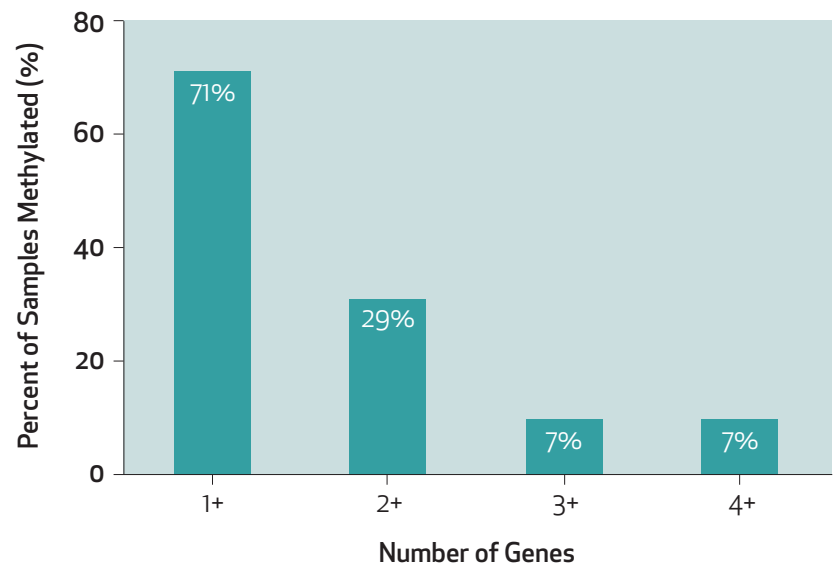


FIGURE 3. Proportion of dysplasia/cancer samples with promoter hypermethylation. The degree of methylation (indicated as PMR value) was measured in dysplasia/cancer (n=14) and normal samples (n=5). PMR values of the normal sample were used to set a normal threshold. Of the dysplasia/cancer samples, 71 percent crossed the threshold and were considered methylated at one or more genes.

DISCUSSION

The assay developed in this project quantified the promoter hypermethylation status of five selected genes in saliva DNA. The methylation frequency ranged from 7 percent to 35 percent for the five genes. Most importantly, using all five genes as a composite biomarker allowed for the detection of 71 percent of the samples.

Furthermore, there was a 62.5 percent agreement between the matched tissue and saliva samples. Saliva has a heterogeneous cell population that makes DNA isolation from cancer cells in saliva more difficult than in tissue. However, the high positive agreement obtained from

the assay proved that saliva is a valid diagnostic medium in quantitative methylation-specific PCR techniques.

These results indicate the assay composed of the five selected genes is a promising early marker for cancer detection. Moreover, saliva serves as a potentially ideal medium for future noninvasive diagnostic tests.

The major advantage of using saliva DNA with the MethyLight assay is the convenience and noninvasiveness of the assay. MethyLight allows for the concurrent analysis of multiple genes in multiple patients in less than two hours. In addition, the assay quantitatively measures the methylation of DNA in cancer

ABLE 2

Correlation of Promoter Hypermethylation between Tissue DNA and Saliva DNA

An average correlation of 62.5% was obtained, indicating that saliva DNA is reliable in quantitative methylation-specific PCR techniques.

Gene	Correlation
<i>p16</i>	87.5%
<i>E-cadherin</i>	87.5%
<i>p15</i>	62.5%
<i>MGMT</i>	62.5%
<i>APC</i>	12.5%

and dysplasia samples, providing higher sensitivity than previous qualitative studies that have had to use a gel-electrophoresis-based method to visualize the

amplified DNA and estimate the amount of methylation from the intensity of the DNA bands. Higher sensitivity enables detection of methylation in samples that would have otherwise been overlooked in such qualitative assays.

The convenience, sensitivity, and noninvasive nature of the assay make it a practical method in monitoring disease progression. The assay is currently being evaluated for its capability of predicting dysplasia progression. To date, there is no clinical or molecular method to determine which oral dysplasias progress into cancer.

Furthermore, the assay will be utilized to monitor oral cancer patients after surgical resection. Patients treated for oral SCC have a 26 percent to 47 percent of developing a recurrence within two years of surgical resection and an annual 5 percent chance of developing a second oral primary SCC.¹⁷ Once a second oral cancer develops, the five-year survival drops to 25 percent.¹⁷

Patients treated for oral cancer are extremely difficult to evaluate for oral cancer recurrence because of their distorted oral and pharyngeal anatomy secondary to scarring following surgery and/or radiation. This salivary assay would improve tumor surveillance in this patient population by identifying a threshold or rate of change in promoter hypermethylation. Early recognition of oral cancer through noninvasive salivary analysis would significantly improve the lives of patients at risk for this disease. ■■■■

REFERENCES

1. Boring CC, Squires TS, Tong T, Cancer statistics. *Calif Cancer J Clin* 41(1):19-36, 1991.
2. Silverman SJ, Epidemiology. D.C. Decker Inc., 1998.
3. Vokes EE, Weichselbaum RR, et al, Head and neck cancer. *N Engl J Med* 328(3):184-94, 1993.
4. Stebbing J, Bower M, et al, Epigenetics: an emerging technology in the diagnosis and treatment of cancer. *Pharmacogenomics* 7(5):747-57, July 2006.
5. Ishida E, Nakamura M, et al, Promoter hypermethylation of p14ARF is a key alteration for progression of oral squamous cell carcinoma. *Oral Oncol* 41(6):614-22, 2005.
6. Uesugi H, Uzawa K, et al, Status of reduced expression and hypermethylation of the APC tumor suppressor gene in human oral squamous cell carcinoma. *Int J Mol Med* 15(4):597-602, 2005.
7. Viswanathan M, Tsuchida N, Shanmugam G, Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. *Int J Cancer* 105(1):41-6, 2003.
8. Lopez M, Aguirre JM, et al, Gene promoter hypermethylation in oral rinses of leukoplakia patients — a diagnostic and/or prognostic tool? *Eur J Cancer* 39(16):2306-9, 2003.
9. Nakahara Y, Shintani S, Detection of p16 promoter methylation in the serum of oral cancer patients. *Int J Oral Maxillofac Surg* 35(4):362-5, 2006.
10. Rosas SL, Koch W, et al, Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. *Cancer Res* 61(3):939-42, 2001.
11. Eads CA, Danenberg KD, et al, MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* 28(8):E32, 2000.
12. Eads CA, Lord RV, et al, Epigenetic patterns in the progression of esophageal adenocarcinoma. *Cancer Res* 61(8):3410-8, 2001.
13. Goldenberg D, Harden S, et al, Intraoperative molecular margin analysis in head and neck cancer. *Arch Otolaryngol Head Neck Surg* 130(1):39-44, 2004.
14. Ogino S, Kawasaki T, et al, Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. *J Mol Diagn* 8(2):209-17, 2006.
15. Trinh BN, Long TI, Laird PW, DNA methylation analysis by MethyLight technology. *Methods* 25(4):456-62, 2001.
16. Sova P, Feng Q, et al, Discovery of novel methylation biomarkers in cervical carcinoma by global demethylation and microarray analysis. *Cancer Epidemiol Biomarkers Prev* 15(1):114-23, 2006.
17. Loree TR, Strong EW, Significance of positive margins in oral cavity squamous carcinoma. *Am J Surg* 160(4):410-4, 1990.

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