Oral Diseases (2009) 15, 206–213. doi:10.1111/j.1601-0825.2008.01510.x

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ORIGINAL ARTICLE

Promoter hypermethylation of mismatch repair genes, *hMLH1* and *hMSH2* in oral squamous cell carcinoma

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OBJECTIVES: Major risk factors of oral squamous cell carcinoma (OSCC) are environmental and can lead to DNA mutagenesis. Mismatch repair (MMR) system functions to repair small DNA lesions, which can be targeted for promoter hypermethylation. We therefore wanted to test whether hypermethylation of MMR genes (*hMLH1, hMSH2*) could contribute to oral carcinogenesis by correlating the information to patient clinical data.
METHODS: Genomic DNA was extracted from 28 OSCC and six normal oral epithelium samples. The methylation status of the two MMR genes was assessed using Methylation Specific PCR after DNA modification with sodium bisulfite. Serial sections of the same tissues were immunostained with antibodies against hMLH1 and hMSH2 protein.
RESULTS: Promoter hypermethylation was observed in 14/28 OSCC cases. Remarkably, 100% of patients with multiple oral malignancies showed hypermethylation in *hMLH1* or *hMSH2* compared with 31.5% of single tumor patients. In 10 cancer cases, expression of the *hMLH1* and *hMSH2* genes by immunostaining showed reduced or absence of expression of one of the genes, although some did not reflect the methylation status.
CONCLUSIONS: Hypermethylation of *hMLH1* and *hMSH2* might play a role in oral carcinogenesis and may be correlated with a tendency to develop multiple oral malignancies.
Oral Diseases (2009) **15**, 206–213

Keywords: oral cancer; hypermethylation; hMLH1; hMSH2; multiple malignancies

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Received 4 October 2008; revised 22 November 2008; accepted 27 November 2008

Introduction

Squamous cell carcinoma (SCC) accounts for more than 90% of oral cavity and oropharynx cancer cases worldwide (Johnson *et al*, 2005). The 5-year survival rate is about 50% (Greenlee *et al*, 2001) and depends, most significantly, on the disease stage upon diagnosis. Treatment of oral squamous cell carcinoma (OSCC) results in significant morbidity. While risk groups include tobacco and alcohol users (Hasihibe *et al*, 2007), exposure to papilloma virus type (D'Souza *et al*, 2007) and UV light, patients with OSCC, nonetheless, run an increased risk (36%) for developing secondary tumors in the head and neck region (Licciardello *et al*, 1998). Thus, improved early detection of oral malignancy and focusing on high-risk patients might minimize morbidity and improve survival.

Although some environmental and life style factors influence the outcome of the disease, oral carcinogenesis, nonetheless, is a multistep process with increasing evidence of various genetic changes as described in several studies (Califano *et al*, 1996; Scully *et al*, 2000a,b). In this context, epigenetic changes are now recognized as an important mechanism in oral cancer development (Shaw, 2006). The correlation of specific epigenetic changes with tumor behavior may be of potential benefit for early detection of OSCC as well as new therapeutic strategies.

Several studies have now reported a direct association between inactivation of DNA repair genes and cancer. The DNA mismatch repair (MMR) pathway, functions to correct replicate mismatches that escape DNA polymerase proof-reading, and hence plays an important role in the maintenance of genetic stability (Modrich, 1994). Defects in this system can consequently lead to somatic variations in the microsatellite length, designated as a marker for microsatellite instability (MSI). The mutator phenotype indicated by MSI also causes accumulation of mutations in the oncogenes and/or tumor suppressor genes (Loeb, 1991). Notably, MSI has also been detected in head and neck squamous carcinomas

(HNSCC), (Piccinin *et al*, 1998; Nunn *et al*, 1999; Wang *et al*, 2001). Several genes are now known to be involved in MMR processes in mammalian cells (*hMSH2*, *hMSH3*, *hMSH6*, *hMLH1*, *hMLH3*, *hPMS1*, *hPMS2*, *hMSH4*, *hMSH5*) with *HMSH4* and *hMSH5* likely playing roles exclusively in meiosis and not in mechanisms of cancer prevention (Kolodner, 1995; Chao and Lipkin, 2006). Therefore, it follows that defects in these genes have been associated with a wide variety of malignancies including HNSCC. For instance, low expression levels of the MMR proteins: hMSH6 (Wei *et al*, 1998), hMLH1 (Wei *et al*, 1998; Lo Muzio *et al*, 1999), and hMSH2 (Lo Muzio *et al*, 1999) has been demonstrated in HNSCC.

An important epigenetic mechanism of gene inactivation during carcinogenesis is gene silencing caused by hypermethylation of the promoter region. Approximately half of all human genes have stretches of GC pairs on their promoters, known as 'CpG islands'. Methylation of the 5-cytosine of the CG dinucleotides prevents transcription and consequently expression of the subsequent gene. *De-novo* methylation of normally unmethylated CpG islands has been observed in different types of human tumors. Indeed, loss of protein expression in cancer cells because of promoter hypermethylation of genes is a consistent and an early marker of tumorigenesis (Baylin and Herman, 2000).

Hypermethylation of several genes (*p16INK4A*, *p15INK4B*, *p14ARF*, *DCC*, *DAP kinase*, *MINT1*, *MINT2*, *MINT27* and *MINT31*) have been reported in OSCC (Ogi *et al*, 2002; Ha and Califano, 2006), with fewer findings regarding *hMLH1* and *hMSH2*.

We therefore wanted to assess whether hypermethylation of the MMR genes, *hMLH1* and *hMSH2*, plays a role in oral carcinogenesis and if this could be correlated with clinical parameters and tumor behavior.

The aims of the present study were therefore to investigate the role of hypermethylation of the promoter region *of hMLH1* and *hMSH2* in oral carcinogenesis by analyzing DNA from normal and squamous cell carcinoma of human oral tissues, and to correlate these findings with histopathological grading as well as clinical outcome and gene expression.

Materials and methods

Patient samples

Following IRB approval, formalin-fixed paraffin-embedded (FFPE) OSCC tissues archived at the Oral Pathology Department at the Hebrew University-Hadassah School of Dental Medicine between 1990 and 2000 were analyzed for inclusion. For this, H&E stained tissues sections were histologically evaluated and only those cases where the majority of the tissue ($>80\%$) was malignant were selected for analysis. The histological grading of well, moderately and poorly differentiated tissues, was given according to World Health Organization (1969–1981). Genomic DNA was extracted from 50 different lesions from the oral cavity (excluding pharyngeal) and only samples giving high yields of genomic DNA (detailed below) and those able to amplify DNA fragment from beta globin gene by PCR were included in the study and the final group consisted of 28 samples from 28 patients with oral cavity cancer. Notably, the clinical information for this final group was kept blind until the analysis was concluded.

Additional four gingival and two buccal mucosa tissues from non-cancerous patients (three male and three female) served as control.

Immunohistochemistry

Ten of the 28 OSCC specimens and 10 normal oral epithelium tissues were examined for the expression of hMLH1 and hMSH2 proteins by immunohistochemistry. Five-micrometer serial sections from FFPE specimens were cut and mounted on precleaned Superfrost Plus glass slides. One slide was stained with H&E to confirm the presence of sufficient epithelial tissue.

After deparaffinization, the slides were processed for immunostaining by incubating in blocking solution (Histostain SP kit; Zymed Laboratories, CA, USA) for 10 min followed by the primary antibodies (mouse anti-MLH1 1:50 or mouse anti-MSH2 1:250; Zymed Laboratories diluted in blocking solution) at room temperature overnight and following the procedure as described (Molinolo *et al*, 2007). Mouse colon specimens and substitution of non-immune 1% BSA for primary antibodies in each specimen served as positive and negative controls, respectively.

All tumoral fields were evaluated for staining intensity and where available, the adjacent normal epithelium served as an internal control. As methylation is not a whole tissue change, count of positively stained cells was not performed. Intensity of staining was scored as 'positive' (++), weak (+) or absent (−) by two independent investigators (RC and Yoram Zilberman) who were blinded to the clinical information and the methylation status.

DNA extraction from paraffin embedded tissues

Five unstained tissue sections of 5 μm thickness from each sample were used for DNA extraction after assessing H&E stains to ensure the presence of the appropriate tissue. Paraffin sections were deparaffinized and DNA was extracted with a QIAmp tissue kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Briefly, lysed tissue sections were incubated overnight at 55°C with proteinase K, followed by an additional incubation for 10 min at 70°C in lysis buffer. Then, the DNA was passed through column, washed with AW buffer before eluting in PCR-grade water. The resulting solution containing the genomic DNA was stored at −20°C until used.

Bisulfite modification of DNA

The genomic DNA extracted from the tissue samples was modified with sodium bisulfite as described by Shteper *et al*, 2001).

The dNTPs concentration was calibrated for each PCR reaction to obtain gentle adjustment of the magnesium concentration. PCR products from the second PCR round were analyzed on 2% agarose gels and the results of both reactions were compared. Details

of the primer sequences for each locus as well as the concentration of dNTP and cycling parameters are shown in Table 1.

Sequence analysis

Sequence analysis was performed on samples demonstrating methylation. Sequencing of the first round PCR products was performed to exclude the possibility of false positive results. In the first round of PCR, only DNA molecules successfully modified by bisulfite undergo amplification and consequently two types of PCR products, representing the methylated and unmethylated sequences were obtained in the same test tube. The PCR product were excised and extracted from 2% agarose gels using the QIAquick Gel Extraction Kit (Qiagen) and directly sequenced using a Sequenase TM Version 2.0 DNA Sequencing Kit (USB, Cleveland, Ohio, USA). Direct sequencing of the second round PCR products was performed to check for the presence of methylation in the internal part of the sequence amplified with methylation specific primers. PCR products were resolved on 2% agarose gel. Bands were subsequently cut and extracted using a Qiagen extraction kit. Sequence analysis was carried out at the Sequencing Unit, Hebrew University, Givat Ram, Jerusalem.

Statistical analyses

The resulting data were tabulated and analyzed with SPPS (version 14 for Windows) with two-tailed alpha for significance set at <0.05. Pearson's (χ^2) was used to analyze correlations between methylation (dependent variable; dichotomous outcome of yes or no) *vs* gender, or the presence of multiple oral or extra-oral tumors (independent variables).

Results

Clinical information

To address this part, 28 relevant cases consisting of tongue tumors (8), lip (4), and palate (3) with the remaining (14) from the oral mucosa (buccal and vestibular), formed the basis of this study. The tissues were taken from equal ratios of male:female and the age ranged from 41 to 85 years, with a mean of 70.2 years. Of these, 23 of 28 (82%) OSCC samples studied were classified as moderately or poorly differentiated SCC, while the remaining five were well differentiated SCC. After the slides were selected and the analysis concluded, appropriate medical files were retrieved and reviewed for clinical correlation. Thirty-two per cent (9/28) of all the patients in the study group were found to have a history of multiple malignancies. In patients with multiple biopsies, same site lesions were considered as recurrent lesions, while different sites were considered as second primaries (i. e. tongue and palate). In case of adjacent tissues (i. e. mandibular ridge and oral mucosa) these were considered as second primaries if more than 3 years had passed between diagnosis. Five patients had a second primary oral carcinoma, one patient developed a second primary oral carcinoma during the course of the study and samples were taken from two patients with an extra-oral malignancy (neuroblastoma, breast and prostatic carcinoma), with one case having both intra- and extra-oral (prostate) malignancies. The patient data are summarized in Table 2.

Table 2 Summary of patients' history

Patients and clinical details	*Study group*	*Control group*
Gender		
Male	14	3
Female	14	3
Age		
< 50(range 41–50)	3	6
≥ 50(range 56–85)	24	0
Unavailable	1	
Biopsy site		
Tongue	8	0
Palate	2	0
Oral mucosa (Buccal, ridge, vestibular)	13	2
Lip	4	0
Gingiva	0	4
Unavailable	1	0
Histological grade		
Well-moderately differentiated	5	–
Moderately–poorly differentiated	23	–
Multiple malignancies		
Oral	6	–
Extra-oral	3	–

hMLH1 and hMSH2 expression

All sections of normal epithelium ($n = 10$) showed positive staining for both proteins, with a nuclear localization as shown for the mouse colon tissue serving as a positive control. The staining was prominent in the basal and parabasal layers, the most proliferating parts of the epithelium (Figure 1a,b). In all OSCC samples analyzed, the staining was predominately nuclear

Table 1 Methylation specific PCR: primers and reactions conditions (Shteper *et al*, 2001)

Locus/Amplimer	*I/II PCR*	*M/U*	*Forward primer*	*Reverse primer*	*T°C*	*dNTPs mM*
MSH2 island	I	–	ggtggtaatttatttttgtatatatttt	ccatatacttaatcacccccctaaa	60	1.0
MSH2	II	M	gtcgtggtcggacgtcgttc	caacgcgtcctcgccgtacg	55	1.6
	II	U	ggttgttgtggttggatgttgttt	ccaacaacacatcctcaccataca	55	1.6
MLH1 island	I	–	tttatgtattggtatataaagttt	atacctctactaaaataatctaa	53	0.8
MLH1	II	M	cgttcgtcgttcgttatatatcgttc	gacgaaactctaattttccgacccg	65	0.8
	II	U	tgtgtgtttgttgtttgttatatattgttt	caacaaaactctaattttccaaccca	65	0.8

U, unmethylated; M, methylated.

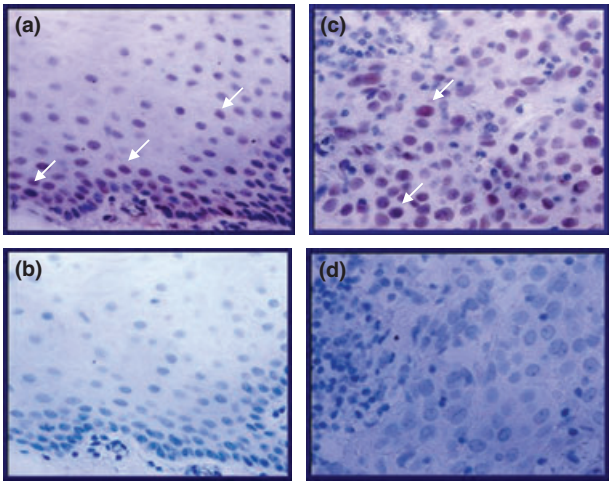

Figure 1 Immunohistochemistry results of hMLH1 and hMSH2 proteins in OSCC and normal oral epithelium samples. Left column – Anti-hMLH1 in normal oral epithelium: **(a)** Nuclear staining, basal and parabasal cells (arrows) (400×) **(b)** Unstained control (400×). Right column – Anti-hMLH1and Anti-hMSH2 in same OSCC tissue: **(c)** Anti-hMLH1staining Positive nuclear staining (arrows) (400×) **(d)** Anti hMSH2 – lack of immunostaining (400×)

(Figure 1c). Of the 10 OSCC tissue samples analyzed, low to no expression of *hMLH1* or *hMSH2* was found in six of the samples (Figure 1d). Of note, two of these cases showed reduced expression of both proteins.

Methylation status

Detection of DNA methylation is based on bisulfite deamination, which converts unmethylated cytosine into thymidine, leaving methylated cytosines unaltered. Subsequent PCR amplification enables assessment of the methylation status by using primers specific either for modified or for unchanged cytosines. Using this notion, none of the six normal epithelium samples showed any evidence of hypermethylation in the promoter region of the two genes. Promoter hypermethylation (*hMLH*1 or *hMSH2*) was detected in 50% (14⁄28) of the DNA from the cancer patients. Representative results of Methylation-specific PCR and direct sequence of the second round of PCR for hMSH2 are shown in Figure 2. All five SCC and normal samples were unmethylated while two SCC samples showed evidence of methylation (Figure 2a). Of note, sample number 6 failed to show any positive PCR results likely indicating insufficient input of DNA.

Direct sequencing of CpG island area in one of the SCC samples (Case #8) reflected the methylation (Figure 2b,c). Table 3 summarizes the clinical data and immunohistochemistry results of positive methylated sample group. A statistical significant correlation between hypermethylation and the presence of multiple oral malignancies was observed ($\chi^2 = 7.64$, $P = 0.006$ degree of freedom df = 1) (Table 4). All patients (100%) with multiple oral malignancies showed methylation in one of the two genes compared with 31.5% of patients who did not have multiple primary tumors. No significant correlation was observed between hypermethylation and the presence of multiple extra-oral malignancies ($\chi^2 = 1.17$, $P = 0.28$ df = 1). No significant correlation was observed between hypermethylation and gender ($\chi^2 = 2.84$, $P = 0.24$ df = 2). However, a higher percentage of positive methylation was found in females (9⁄14), in patients over 50 years of age but because of the small group numbers, no conclusions can be drawn from this finding. When comparing between the methylation status and the immunostaining results, no specific pattern was observed (Table 3). Unaffected expression of the mismatch repair genes, *hMLH1* and *hMSH2*, without any evidence of promoter hypermethylation was found in all normal oral epithelia. In OSCC tissues, four cases (#1, 2, 6, and 9) showed promoter hypermethylation and absence of or reduced expression of *hMLH1* and *hMSH2* genes. In the other five OSCC cases (# 3, 8, 10, and two other negative methylated samples) hypermethylation of the promoter region did not correlate with lower expression of the mismatch repair genes. Promoter hypermethylation of *hMSH2* was detected in 36% (10⁄28) of the patients, while *hMLH1* was found in 17% (5⁄28) of the patients. These numbers include one sample (#1), which showed promoter hypermethylation in both genes.

Discussion

This study investigated the methylation status of *hMLH1* and *hMSH2* in OSCC samples, compared with non-malignant oral epithelium. Expression of the gene product was examined by immunohistochemistry. We found promoter methylation of *hMLH1* and *hMSH2* genes in half of the OSCC samples, with a higher incidence of *hMSH2* methylation than the *hMLH1* counterpart and, partially reduced protein expression of both genes by immunohistochemistry. Our results also showed that hypermethylation of the main mismatch repair genes, *hMLH1* and *hMSH2*, in OSCC is correlated to multiple oral malignancy patients.

OSCC is highly connected to environmental changes, and life style is an important factor in tumor development (Neville and Day, 2002). Hypermethylation, as an epigenetic change, is suggested as one of the pathways leading to oral cavity carcinogenesis (Ha and Califano, 2006). In this context, a direct association between hypermethylation of mismatch repair genes (*hMLH1* and *hMSH2*) and cancer has been reported in colon cancer (Muller and Fishel, 2002) but reports on methylation status of the same genes in HNSCC are inconclusive and ranged from none to 88%. Previous studies on mismatch repair genes (summarized in Table 5) indicated that information correlating patient's medical history with the grade of tumors was not readily available for oral cavity cancers. Indeed, hypermethylation of *hMLH1* was found in 0–47% of HNSCC (Ogi *et al*, 2002; Liu *et al*, 2003; Viswanthani *et al*, 2003; Puri *et al*, 2005 and Sengupta *et al*, 2007), but the results pertaining to samples exclusively from oral tumors are representative of only one study (Viswanthani *et al*, 2003). Similarly, *hMSH2* was found to be methylated in

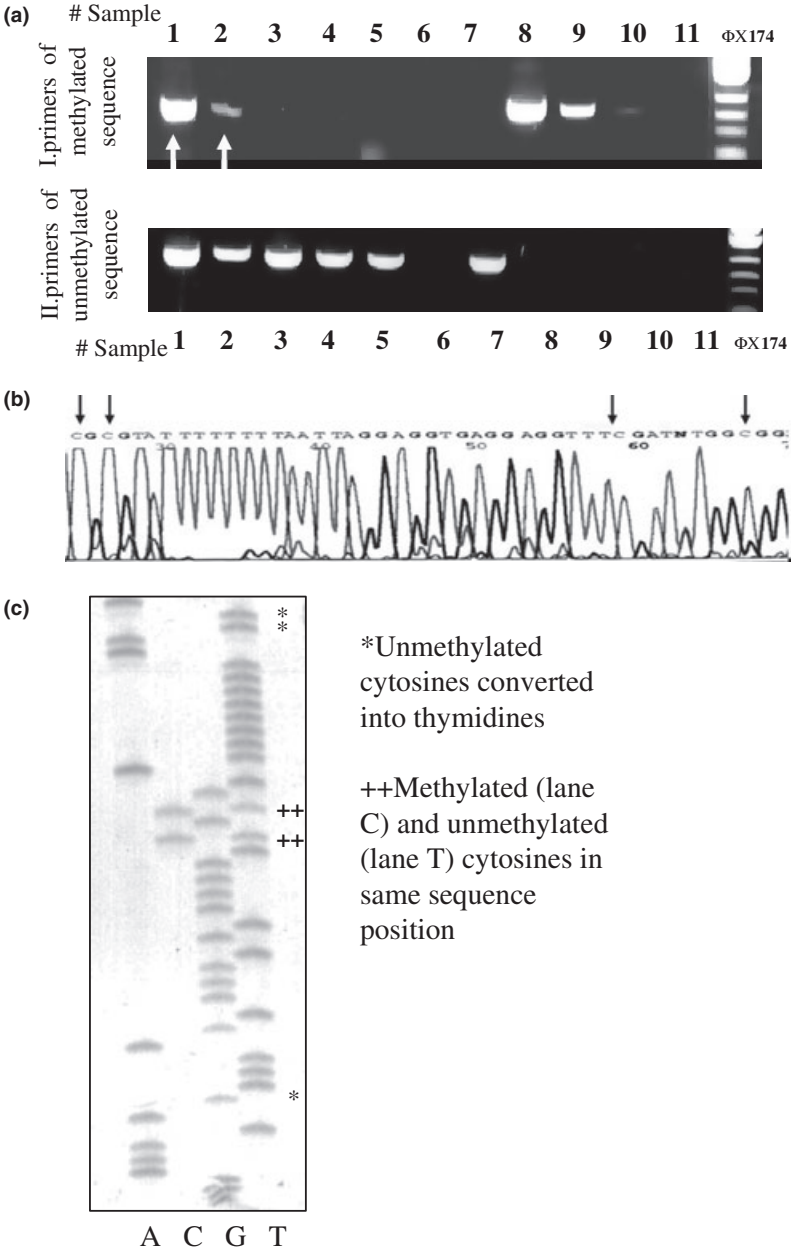

Figure 2 **(a)** Methylation specific PCR for *hMSH2* promoter region in OSCC and normal oral epithelium. I: primers specific for methylated sequence. II: primers specific for unmethylated sequence. Lanes 1–6: Oral squamous cell carcinoma from different patients: sample#6 negative in both methylated and unmethylated due to lack of DNA for analysis. Lane 7: Normal oral epithelium sample. Lanes 8–10: Positive (*in vitro* methylated) control with reduced DNA amount: undiluted, diluted 1:10, diluted 1:100. Lane 11: Blank control with H_2O. ΦX: ΦX174 DNA/HAE III. **(b)** Results of direct sequencing of the second round PCR fragment of the *hMSH2* promoter region – Case #8 (Table 3) presented in land 1. Methylated Cytosines (in the CpG islands of the *hMSH2* promoter region) remain cytosines after bisulfite modification (arrows), while other cytosines were converted into thymidines. *hMSH2* gene complete cds (Accession number gi46488017 base pairs 1591-1636). **(c)** Sequence presentation of methylated and unmethylated CpG molecules in the same DNA Case #8 (Table 3). *hMSH2* gene complete cds (Accession number gi46488017, base pairs 1568–1619) Note all cytosines were converted except in those in the frame

0–30% of HNSC (Sengupta *et al*, 2007), but in these studies, oral mucosal tissues were not included.

Two studies did not find methylation in either of the two genes (Wang *et al*, 2001) from 57 samples or in *hMLH1* gene (Ogi *et al*, 2002) from 96 samples. Other studies, however, did find 8–37% *hMLH1* methylation (Viswanthani *et al*, 2003; Puri *et al*, 2005; Sengupta *et al*, 2007). Demokan *et al*, 2006 reported 47% of methylation of *hMLH1* in HNSCC, similar percentage to the current study, although only 10 samples were taken from the oral cavity. High percentage of *hMLH1* methylation (88%) was reported by Liu *et al*, 2003; but only in samples previously found to have loss of expression of the gene product (7/8). The widely divergent findings relating to the methylation status of the mismatch repair genes might be attributable to the different sensitivities of the techniques used as well as the availability of fresh tissue compared with FFPE tissues. Differences of the studied population may also be a factor in these diverse results. Higher frequency of methylation in the current study could be because of the unique characteristics of the study group, for example, over 80% of the samples were graded as moderately to poorly differentiated SCC. Other groups studied tumors of various histological grading (for example, Ogi *et al*, 2002) or failed to mention this information (Wang *et al*, 2001; Liu *et al*, 2003; Viswanthani *et al*, 2003; Puri *et al*, 2005). The importance of histological grading for *hMLH1* expression was asserted by Wright and Stewart, 2003, who reported that a high sample number lacked expression of *hMLH1* (70%) and *hMSH2* (22%) in poorly differentiated colon carcinoma.

Table 3 Summary of clinical data and immunohistochemistry results of positive methylated sample group

Biopsy number	*Sex/age (years)*	*Tumor site (Other malignancies sites)*	*Tumor grade*[a]	*Meth. hMSH2*[b]	*Meth. hMLH1*[b]	*hMSH2*[c] *protein immunostain*	*hMLH1*[c] *protein immunostain*
1	F/85	Palate	P	+	+	+	–
2	M/50	Tongue	P	–	+	+	+
3	F/82	Lip	P	–	+	+	*
4	F/56	Oral mucosa	U	+	–	*	*
5	M/72	Oral mucosa	MP	+	–	*	*
6	M/59	Palate	P	+	–	+	*
7	M/70	Tongue (neuroblastoma)	W	+	–	*	*
8	F/80	Mandibular ridge (buccal mucosa)	P	+	–	++	–
9	F/77	Mandibular vestibulum (palate)	P	–	+	–	+
10	M/80	Mandibular mucosa (buccal mucosa prostate)	M	–	+	*	*
11	F/75	Lip (hard palate, upper lip soft palate)	M	+	–	*	*
12	F/66	Tongue (breast)	M	+	–	*	*
13	F/69	Tongue (floor of mouth soft palate oral mucosa)	W	+	–	++	–
14	F/71	Mandibular ridge (buccal mucosa)	W	+	–	*	*

[a]Degree of differentiation :W, well; M, moderately; P, poorly; MP, moderatly–poorly differentiated; U, unavailable.
[b]+ Positive methylation, – Negative methylation.
[c]++ normal staining, + reduced staining. – Negative staining; *not performed/failure.

Table 4 Correlation of methylation status with multiple oral malignancies

Multiple oral malignancies (Total n*)*	*Methylation of hMLH1/ hMSH2* n *(%)*	*No methylation* n *(%)*
Yes (6)	6 (100)	0 (0)
No (22)	8 (36)	14 (64)
Total (28)	14 (50)	14 (50)
	$P = 0.006$	

hMSH2 promoter methylation was found in some solid tumors in a range of 8–34% (Wang *et al*, 2003; Hsu *et al*, 2005; Kawaguchi *et al*, 2006). In this context, methylation of *hMSH2* in oral SCC samples has been less frequently studied. Of those reported, Wang *et al* (2001) reported no methylation of hMSH2, while Demokan *et al*, 2006 and Sengupta *et al* (2007) indicated 27% and 30% of *hMSH2* methylation in HNSCC and OSCC, respectively. Our finding (36% methylation) was aligned with those reported, and the current study is the first to report positive methylation of the *hMSH2* gene in oral SCC from FFPE tissues.

Our results of higher frequency of methylation in female patients are similar to Demokan *et al* (2006) study and this gender profile has also been reported for *hMLH1, MGMT, GSTP1,* and *TIMP* genes in gastric cancers by Hong *et al*, 2005.

Notably, we found a significant correlation between the methylation of mismatch repair genes and multiple oral malignancies. All patients with multiple oral neoplastic tumors (from different oral sites) showed methylation in one of these genes. Although a small number of patients were included in the present study, our results indicate that patients with hypermethylation of the *hMLH1* or *hMSH2* genes have a tendency to develop multiple malignancies. A high risk of developing secondary carcinoma in the gastrointestinal tract was found in patients with defective protein expression of *hMLH1* and/or *hMSH2* (Yamamoto *et al*, 2006). Verma *et al* (2003) claimed that alterations in DNA methylation have emerged as one of the most consistent molecular changes in multiple neoplasms. Thus, our finding of methylation of one of the MMR can be linked to those reported findings. To the best of our knowledge, this is the first study of hypermethylation of oral epithelial cells in patients with multiple malignancies.

Immunohistochemical analysis showed a difference in the intensity of the nuclear staining of hMLH1 and hMSH2 proteins in the OSCC samples, similar to the results of Nunn *et al* (2003). Three of four OSCC samples examined had reduced expression of *hMLH1*, 6/9 samples had reduced expression of *hMSH2*, and one of four samples had reduced expression of both genes. Lack of expression of both *hMLH1* and *hMSH2* in OSCC was also reported by Lo Muzio *et al* (1999), and might be a hallmark of the mutator phenotype. In contrast, Wang *et al* (2001) found neither an abnormal expression level of those genes in all 12 moderately differentiated OSCC cases nor methylation of the *hMLH1* and *hMSH2* genes. In our study, matching was not found between the expression of the genes and their methylation status.

Liu *et al* (2003) reported similar mismatching in a patient with HNSCC who was moderately positive for the hMLH1 protein and also showed promoter hypermethylation. Mismatching was also found by Saito *et al* (2003) in alveolar soft part sarcoma.

A lack of complete correlation between the methylation status and immunohistostaining may be attributable to the fact that silencing of one allele by methylation does not affect the protein expression of the other, unmethylated allele. Another possibility is allelic misbalance (Nunn *et al*, 2003) resulting in reduced protein expression without evidence of hypermethylation. Other genetic

Table 5 Literature review of methylation status of *hMLH1* and *hMSH2* genes in OSCC

References	*Origin of samples and type of analysis*	*HNSCC samples* (N)	*Oral samples* (N)	*hMLH1 methylated %*	*hMSH2 methylated %*	*Tumor data*
Wang *et al*, 2001	Fresh and Paraffin PCR analysis of – genomic DNA after digestion with methylation-specific restriction enzymes; IHC	57	36	0	0	Site, stage
Ogi *et al*, 2002	Paraffin PCR analysis of genomic DNA after bisulfite modification	96	96	0	NS	UICC stage T-category invasion of the bone
Viswanthani *et al*, 2003	Restriction multiplex PCR	99	99	8 of oral	NS	NS
Liu *et al*, 2003	Paraffin methylation specific PCR; IHC	16	No data	88 of MSI + 0 of MSI–	NS	NS
Puri *et al*, 2005	Paraffin methylation specific PCR	51	23	23 of HNSCC	NS	Stage
Demokan *et al*, 2006	Fresh PCR with restriction analysis	116	10	47 of HNSCC	30 of HNSCC	Site, stage
Sengupta *et al*, 2007	Fresh PCR with restriction analysis	143	81	37 of HNSCC	27 of HNSCC	Site, stage

events in the context of mutation also can lead to loss of function of MMR genes. The discrepancy between the results of different studies may reflect the heterogeneous study groups and illustrate the multifactorial nature of OSCC (Califano *et al*, 1996).

A combination of several different mechanisms may result in SCC, promoter methylation being only one of them. This may explain the widely diverging findings on the methylation status in OSCC. According to our findings, we suggest that hypermethylation of the main mismatch repair genes is a hallmark of the potential mutator phenotype in OSCC. Hypermethylation of *hMLH1* and *hMSH2* may play a role in oral carcinogenesis. Although a limited number of patients are included in the present study, our results indicate a correlation to develop multiple malignancies in subjects with hypermethylation of *hMLH1* and *hMSH2*. Evidence of hypermethylation of mismatch repair genes in oral cancer might serve as a predictor for the development of malignancy. As methylation cannot be masked by the presence of normal tissue, it can serve a sensitive marker of malignancy.

Acknowledgements

We thank Dr Yoram Zilberman PhD, DMD, Senior Scientist, Skeletal Biotechnology Laboratory, Hebrew University-Hadassah Medical Center, for help with the immunohistochemistry evaluation.

The research was partially supported by the Dr Izador I. Cabakoff Research Endowment Fund.

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