Microsatellite instability and methylation of the DNA mismatch repair genes in head and neck cancer

S. Demokan1, Y. Suoglu2, D. Demir2, M. Gozeler2 & N. Dalay1*

1Oncology Institute, Department of Basic Oncology, 2Istanbul Medical Faculty, Department of Otorhinolaryngology, Istanbul University, Istanbul, Turkey

Received 8 February 2006; accepted 9 February 2006

Background: Methylation in the promoter region of the DNA mismatch repair genes hMLH1 and hMSH2 and microsatellite instability at three loci were analyzed in the tumor tissue from patients with head and neck cancer.

Methods: Microsatellite instability and promoter methylation were investigated by PCR, denaturing-polyacrylamide gel electrophoresis and digestion with methylation-specific restriction enzymes.

Results: Microsatellite instability was observed in 41% of the patients. hMLH1 and hMSH2 genes were methylated in 47% and 30% of the patients, respectively. BAT25 and BAT26 instability were associated with age and histopathology, respectively. Methylation frequency of the hMLH1 gene promoter was significantly higher in patients displaying a high level of microsatellite instability. Instability at the BAT 26 and D2S123 loci were associated with the MSI-high status.

Conclusions: Our results indicate that microsatellite instability and modifications in the hMLH1 and hMSH2 genes are implicated in a significant proportion of the patients with head and neck cancer.

Key words: methylation, hMLH1, hMSH2, microsatellite instability, head and neck cancer

Introduction

Among human malignancies, head and neck cancer is the sixth most common cancer type in the world [1]. Both environmental and genetic factors play an important role in the etiology but the causal relationship between these and tumor development is not yet clear [2]. Alternative modes of inactivation of genes during the development of cancer include an epigenetic process marked by promoter region hypermethylation associated with transcriptional loss [3]. Promoter hypermethylation is an important implication of gene silencing [4].

In recent years it has been shown that loss of activity of some key genes may occur through epigenetic means [5]. Loss of gene function by transcriptional silencing of selected genes may play a crucial role in the development and progression of sporadic human tumors [4]. Epigenetic changes occurring in the DNA repair genes impair the stability of DNA making it more vulnerable to damage. They increase the mutation rate allowing accumulation of new, permanent mutations that are no longer repaired which ultimately lead to cancer [6].

It has been suggested that impairment of the mismatch repair activity is an important step in carcinogenesis common to several types of cancer [7]. A significant proportion of carcinomas develop through DNA mismatch repair deficiency and exhibit frequent microsatellite instability [8, 9]. Recent studies indicate that a substantial fraction of sporadic tumors display MI although most tumors do not harbor mutations in the repair genes [10].

Microsatellite instability has also been described in squamous carcinomas of the head and neck (HNSCC) [11, 12]. The most prevalent abnormality in HNSCC is the relative high frequency of allelic losses at chromosome 3p which harbors the DNA mismatch repair gene hMLH1. hMLH1 and hMSH2 genes are responsible for DNA mismatch repair and inactivation of these genes is associated with increased mutations in simple repeats in genomic DNA and microsatellite instability [13].

Data on the mismatch gene alterations in head and neck cancer is rare. There are only four studies in the literature investigating inactivation of the hMLH1 gene and the results are inconsistent. Epigenetic silencing of the hMSH2 gene and its association with microsatellite instability has not been reported yet.

In this study, we aimed to investigate the role of microsatellite instability and promoter methylation of the DNA repair genes in head and neck carcinogenesis, and the correlation of these with the clinico-pathological parameters.

Materials and Methods

Fresh tumor and normal tissue samples of 116 patients (102 male, 14 female, mean age: 59.6 ± 11.8) with head and neck cancer admitted to the
Istanbul Medical Faculty, Department of Otorhinolaryngology were investigated. The patients had no prior treatment. The study design was approved by the local ethical committee. Characteristics of the patients are presented in Table 1. Most patients (74%) had laryngeal tumors. The remaining tumor types were parotid (n = 13), oral cavity (n = 10), nasal/paranasal sinus and nasopharynx (n = 6) and glomus (n = 1). Eighty-five percent of the patients (n = 99) had squamous cell carcinoma; the others were adenoma (n = 5), mixed tumors (n = 5), adenoid cystic carcinoma (n = 3), Warthin tumors (n = 2) and sarcoma (n = 2). Metastasis was not present in 80% of the patients. Tumor and normal tissue samples were collected during surgery and DNA was isolated by phenol/chloroform extraction after overnight incubation with proteinase K at 37°C.

Methylation in the promoter regions of hMLH1 and hMSH2 genes was investigated by methylation-specific restriction enzyme digestion. 100 ng of genomic DNA was digested by overnight incubation at 37°C using methylation-sensitive and methylation-resistant enzymes recognizing the same region. The methylation-sensitive enzyme HpaII cannot cut the specific region if methylation is present whereas MspI can cleave the region regardless of the methylation status. Undigested genomic DNA samples were used as controls. Following digestion the cleaved products were collected by precipitation with 0.3 M sodium acetate/ethanol (1:20) and the promoter regions were amplified by PCR using specific primers. Primer sequences (Integrated DNA Technologies, Iowa, USA) were: hMLH1 (forward 5’-GGCTGTAGTTGCGC-3’, reverse 5’-CAGCCAAATAGGAGCAGAG-3’), hMSH2 (forward 5’-GTCTGCCATGTGGTATTACCC-3’, reverse 5’-CTCTGGTGGGTGTGATGC-3’).

PCR reactions were performed in 50 µl 1 × PCR buffer containing 1.5 mM MgCl₂, 0.3 µM of each primer, 200 µM of each dNTP (MBI, Fermentas, Lithuania), 100 ng DNA and 2 U Taq polymerase (MBI, Fermentas, Lithuania). Amplification was performed by an initial denaturation at 95°C for 5 min, followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. The reaction was terminated by extension at 72°C for 7 min. The PCR products were separated electrophoretically on 2.2% agarose gels containing 0.1% ethidium bromide at 150 V for 45–60 min and analyzed using a video gel documentation system (Vilber Lourmat, Marine-La Vallée, France).

A microsatellite panel as recommended by the Bethesda Consensus and National Cancer Institute of USA [14] was used. DNA samples obtained from normal and tumor tissues were amplified using three different oligonucleotide pairs specific for the recommended microsatellite loci BAT25, BAT26 and D2S123. Primer sequences (Integrated DNA Technologies, Iowa, USA) were: BAT25 (forward 5’-TGCGCTTCAGAATGTAA GT-3’ and reverse 5’-CTCGCATTTAATCTGGCTC-3’), BAT26 (forward 5’-TGACTTTGTTGACTCCAGC-3’ and reverse 5’-ACCATTCAACATTATAACC-3’) and D2S123 (forward 5’-AAACAGGATGCTGGCCTTTA-3’ and reverse 5’-GGACTTTCCACCTATGGGAC-3’). PCR reactions were performed in 50 µl 1 × PCR buffer containing 2.5 mM (BAT25, BAT26) and 1.5 mM (D2S123) MgCl₂, 200 µM of each dNTP (MBI, Fermentas, Lithuania), 0.3 µM of each primer, 100 ng DNA and 2 U of Taq polymerase (Promega). Amplification was performed by an initial denaturation at 95°C for 5 min, followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. The reaction was terminated by extension at 72°C for 7 min. PCR products were run on 8% denaturing polyacrylamide gels at 180 V for 18 h, and visualized by silver staining.

### statistical analysis

Associations between clinical parameters and presence of microsatellite instability and methylation status were analyzed by the Yates’ χ² and Fisher’s exact tests using the Epistat package statistics programme. A probability value of <0.05 was considered significant. Kaplan-Meier and Log rank tests were used for survival analysis. Calculations were performed using the SPSS 7.5 statistical software (SPSS Inc., Illinois, USA).

### results

#### methylation in the promoter region of hMLH1 and hMSH2 genes

The genomic DNA samples from the patients were digested by methylation-sensitive (HpaII) and methylation-resistant (MspI) enzymes recognizing the same region. Since HpaII cannot digest a methylated site, PCR amplification of undigested DNA results in a 354 bp long fragment for hMLH1 (Figure 1A) and a 107 bp gene product for hMSH2 genes, respectively (Figure 1B). On the other hand, MspI can cut the site even if it is methylated and consequently no PCR product is observed on the gel after amplification.

Promoter methylation was observed in 69 (59%) patients. Methylation in the promoter region of the hMLH1 and hMSH2 genes was present in 47% (n = 55) and 30% (n = 35) of the

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Patients (n = 116)</th>
<th>MI (+) n (%)</th>
<th>MI (−) n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco use: (+)</td>
<td>97 (84)</td>
<td>41 (85)</td>
<td>56 (82)</td>
<td>0.853</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>19 (16)</td>
<td>12 (18)</td>
<td></td>
</tr>
<tr>
<td>Alcohol use: (+)</td>
<td>34 (29)</td>
<td>10 (21)</td>
<td>24 (35)</td>
<td>0.139</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>82 (71)</td>
<td>44 (65)</td>
<td></td>
</tr>
<tr>
<td>Lymph node involvement: (+)</td>
<td>31 (27)</td>
<td>12 (25)</td>
<td>19 (28)</td>
<td>0.893</td>
</tr>
<tr>
<td></td>
<td>(−)</td>
<td>85 (73)</td>
<td>49 (72)</td>
<td></td>
</tr>
<tr>
<td>Clinical stage: I + II</td>
<td>51 (44)</td>
<td>24 (50)</td>
<td>27 (40)</td>
<td>0.362</td>
</tr>
<tr>
<td></td>
<td>III + IV</td>
<td>65 (56)</td>
<td>41 (60)</td>
<td></td>
</tr>
<tr>
<td>Tumor size: ≤ 2 cm</td>
<td>51 (44)</td>
<td>20 (42)</td>
<td>31 (46)</td>
<td>0.817</td>
</tr>
<tr>
<td></td>
<td>&gt; 2 cm</td>
<td>65 (56)</td>
<td>58 (73)</td>
<td></td>
</tr>
<tr>
<td>Anatomical region:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>85 (74)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supraglottic</td>
<td>41 (48)</td>
<td>14 (29)</td>
<td>27 (40)</td>
<td>0.440</td>
</tr>
<tr>
<td>Glottic</td>
<td>40 (47)</td>
<td>18 (71)</td>
<td>22 (60)</td>
<td></td>
</tr>
<tr>
<td>Subglottic</td>
<td>4 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parotis</td>
<td>13 (11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral cavity</td>
<td>10 (9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal/paranasal sinus/</td>
<td>6 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nasopharynx</td>
<td>Glomus</td>
<td>1 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender: Female</td>
<td>14 (12)</td>
<td>5 (10)</td>
<td>9 (13)</td>
<td>0.865</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>102 (88)</td>
<td>43 (90)</td>
<td>59 (87)</td>
</tr>
<tr>
<td>Age: ≤ 45</td>
<td>10 (9)</td>
<td>5 (10)</td>
<td>5 (7)</td>
<td>0.398</td>
</tr>
<tr>
<td></td>
<td>&gt; 45</td>
<td>106 (91)</td>
<td>43 (90)</td>
<td>63 (93)</td>
</tr>
</tbody>
</table>

MI, microsatellite instability; n, number of patients.
patients, respectively. In 21 (18%) patients both genes were methylated. Among the individuals displaying methylation, the methylation rate was significantly higher for the hMLH1 gene (80% versus 51%) ($P = 0.0007$, $\chi^2 = 11.53$, df = 1). However, no association was found between hMLH1 methylation and the clinical parameters. Methylation of the hMSH2 promoter was associated with absence of metastasis (Fisher’s $P = 0.035$) and was more frequent in female patients ($P = 0.042$, $\chi^2 = 4.14$, df = 1). No other association was observed between methylation of the promoter region and clinicopathological parameters.

In patients with larynx cancer ($n = 85$), methylation rates in the hMLH1 and hMSH2 genes were similar to the total group of patients (46% and 28%, respectively). However, methylation of the hMLH1 promoter was still significantly more frequent ($P = 0.0037$, $\chi^2 = 8.4$, df = 1) whereas hMSH2 methylation was mainly observed in women patients ($P = 0.02$).

discussion

Differences in the ability to repair DNA damage may contribute to the cancer risk in the general population. Several links have been identified between alterations in the genes regulating DNA repair processes and development of tumors, indicating a key role of DNA protection.

Defects in the mismatch repair system may result in accelerated accumulation of mutations in critical genes and lead to progression to malignancy. Recent studies indicate that a variety of sporadic tumors exhibit frequent microsatellite instability associated with defects in the DNA mismatch repair pathways. This process is particularly affected by abnormal function of the hMLH1 and hMSH2 genes [15]. Genetic and epigenetic changes in these genes are likely to diminish mismatch repair, leading to a higher mutation rate and facilitating carcinogenesis. Very few studies on head and neck cancer have included ML in the analysis or evaluated the association between ML and hypermethylation in the promoter region of the mismatch repair genes. Methylation of the hMSH2 gene and its association with microsatellite instability has not been reported in head and neck cancer.

There are very little data on the association between the promoter methylation of the hMLH1 gene and presence of microsatellite instability in the head and neck carcinogenesis. In this study promoter hypermethylation was present in 59% of the tumors tested for both genes, confirming that this is an important and frequent epigenetic mechanism by which key genes are inactivated.

Figure 1. Methylation status of the (a) hMLH1 and (b) hMSH2 genes. The samples were separated on 2.2% agarose gels. No methylation is observed in samples 45, 11 and 17. Promoter region of the hMLH1 gene is methylated in samples 30, 43 and 57. Methylation in the hMSH2 gene is observed in samples 34 and 35. (H: digestion by HpaII, M: digestion by MspI, U: undigested product, PuC: Molecular weight marker).

Figure 2. Alterations in the band patterns for the (a) BAT25 (b) BAT26 (c) D2S123 microsatellite marker loci (N: Normal, T: Tumor).

Methylation of the hMLH1 promoter region was observed in 75% of MSI-H tumors compared to 33% MSI-L tumors (OR=6.0; CI 1.16–29.96; $P = 0.029$). Thus, the risk of developing cancer was six-fold higher in patients with methylated hMLH1 genes displaying a high level of instability. However, no meaningful difference was found for the hMSH2 gene methylation between the MSI-H (42%) and MSI-L (25%) tumors ($P = 0.22$) (Table 2).

Instability at the BAT26 and D2S123 markers was significantly associated with the MSI-H status (Fisher’s $P = 0.021$, Fisher’s $P = 0.000$ respectively) (Table 3).

There was no significant difference between the MSI-H and MSI-L/MSS tumors with regard to stage, differentiation, sex or any other clinico-pathological parameter. A meaningful association was observed between microsatellite instability and early disease only for patients with larynx cancer ($n = 85$) ($P = 0.037$, $\chi^2 = 4.33$, df = 1).

Survival analysis did not reveal a correlation with presence of genomic instability or methylation of the genes.

Microsatellite status in the tumor samples was investigated for three different microsatellite marker loci (BAT25, BAT26, D2S123) as recommended by NCI guidelines. BAT25 and BAT26 are mononucleotide-repeat markers, whereas D2S123 is a dinucleotide-repeat. Tumors not displaying instability at any of the markers were classified microsatellite-stable (MSS).

Microsatellite instability was observed in 41% of the patients. Instability rates for the BAT25, BAT26 and D2S123 loci were 15%, 19% and 19%, respectively (Figure 2).

The frequency of BAT25 instability was significantly higher in younger (≤45 years) patients ($P = 0.006$). Although the significance was borderline, the frequency of BAT25 instability was also higher in parotis tumors ($P = 0.05$). BAT26 instability was more frequent in squamous cell carcinomas (Fisher’s $P = 0.02$). There was a significant association between methylation of the hMLH1 promoter and instability at the BAT26 locus.

The MI status was scored MSI-L (Low) when instability was observed in only one of the markers. When instability was present in two or more markers, then the MI status was classified MSI-H (High). 12 patients (10%) were MSI-H, 36 patients (31%) were MSI-L and 68 patients (59%) were MSS.

Differences in the ability to repair DNA damage may contribute to the cancer risk in the general population. Several links have been identified between alterations in the genes regulating DNA repair processes and development of tumors, indicating a key role of DNA protection.

Defects in the mismatch repair system may result in accelerated accumulation of mutations in critical genes and lead to progression to malignancy. Recent studies indicate that a variety of sporadic tumors exhibit frequent microsatellite instability associated with defects in the DNA mismatch repair pathways. This process is particularly affected by abnormal function of the hMLH1 and hMSH2 genes [15]. Genetic and epigenetic changes in these genes are likely to diminish mismatch repair, leading to a higher mutation rate and facilitating carcinogenesis. Very few studies on head and neck cancer have included MI in the analysis or evaluated the association between MI and hypermethylation in the promoter region of the mismatch repair genes. Methylation of the hMSH2 gene and its association with microsatellite instability has not been reported in head and neck cancer.

There are very little data on the association between the promoter methylation of the hMLH1 gene and presence of microsatellite instability in the head and neck carcinogenesis. In this study promoter hypermethylation was present in 59% of the tumors tested for both genes, confirming that this is an important and frequent epigenetic mechanism by which key genes are inactivated.
It has been shown that methylation of the hMLH1 and hMSH2 genes is a situation previously reported for the hMLH1 gene promoter is higher than reported for larynx [16], gastric [17, 18], breast [19] or esophageal [20] cancer but are in accordance with recent reports on esophageal [21] and head and neck tumors [12]. Methylation of the hMSH2 promoter region was more frequently observed in female patients, a situation previously reported for hMLH1, MGMT, GSTP1 and TIMP genes in gastric cancer [22].

Our study showed no correlation between hypermethylation of the hMLH1 and hMSH2 promoters, individually or in any combination with tumor site, nodal status or T stage. Promoter methylation was also not associated with age or history of tobacco use. These observations are in accordance with recent reports [17, 23]. However, correlation between methylation of the hMLH1 promoter and lymphatic metastases has also been reported [20, 24]. Differences in these results may be attributed to differences in patient selection, different types of cancers, different ethnic groups and different sample sizes studied.

It has been shown that methylation of the hMLH1 gene is the most common way leading to microsatellite instability in colon tumors [25]. Aberrant methylation of the hMLH1 promoter is potentially a very important mechanism in the inactivation of the MMR system in human cancers [20]. Consistent with these observations the hMLH1 gene was preferentially methylated in our study.

There are no data in the literature on the methylation frequency of the hMSH2 gene in head and neck cancer. Previous studies on endometrial and colorectal cancer have shown that hypermethylation of the hMSH2 gene promoter seems to occur only in familial [19, 26], but not in sporadic [27, 28] cases. Our results are consistent with these previous observations in that hypermethylation of the hMSH2 promoter is also less frequent in sporadic head and neck cancer. However, our data indicate that silencing of the hMSH2 gene in addition to hMLH1 by promoter methylation is observed in a significant proportion of the patients.

Microsatellite instability was observed in 41% of our patients. This rate is somewhat lower than previously reported for head and neck cancer [29] and higher than observed for gastric [18], larynx [16, 30] or Turkish breast cancer [31] patients, but are in accordance with reports on esophageal [9] and breast [19] cancer.

The BAT25 instability rate matches data reported for colon cancer [32], BAT26 rates are concordant with reports on colon [33, 34], gastric [35], ovarian and endometrial [34, 36] cancers. The rate of D2S123 instability is in agreement with pooled data on gastric cancer from all countries [36].

In the present study 75% and 33% of the MSI-H tumors were associated with hypermethylation in the promoter regions of hMLH1 and hMSH2, respectively. These data are in accordance with recent reports on gastric tumors [22, 37]. An association between the MSI-H status and instability of the BAT25 locus is also in concordance with results observed for colon cancer [33].

In our study methylation of the hMSH2 gene promoter was more frequent in women. Similar data has been reported for the hMLH1 gene in gastric cancer [22].

It should be noted that hMSH proteins are implicated not only in postreplicative mismatch correction but also in cell killing by DNA modifying agents. It has been reported that inactivation of hMLH1 by promoter hypermethylation plays also a significant role in the development of resistance to chemotherapeutic agents [38, 39]. However, it has also been suggested that the loss of DNA repair genes may make tumor cells less able to repair DNA damage induced by radiation or cytotoxic chemotherapy and thus more responsive to treatment [23].

Methylation of DNA constitutes a stable marker that provides a high sensitivity in cancer detection since it cannot be masked by presence of normal tissue. Thus, methylation of the promoter region can be used as a specific molecular marker to detect cancer cells. Our results indicate that modifications in both the hMLH1 and hMSH2 genes are implicated in a significant proportion of the patients with head and neck cancer. Association of the mismatch repair gene inactivation with the response to chemotherapeutic agents requires further investigations.

**Acknowledgements**

This work was supported by the Istanbul University Research Fund (Project Number: T-149/06032003). The authors thank Burak Erdamar MD, for his assistance and coordinated support during the initial phase of the study.

**References**


