Reduced Expression of hMLH1 and hGTBP/hMSH6: A Risk Factor for Head and Neck Cancer

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Abstract

Head and neck cancer, like lung cancer, is considered a paradigm of an environmentally induced disease. Genetically determined variation in DNA repair capacity is thought to contribute to susceptibility to tobacco-related cancers. In this molecular epidemiology study, we investigated the association between DNA mismatch-repair (MMR) gene expression and the risk of head and neck cancer. Using our newly developed multiplex reverse transcription-PCR assay, we simultaneously evaluated the relative expression levels of five MMR genes (hMSH2, hMLH1, hPMS1, hPMS2, and hGTBP/hMSH6) in the peripheral blood lymphocytes of 78 patients (mean age 59.6 ± 12.4 years) with newly diagnosed head and neck cancer and 86 healthy controls (mean age 58.2 ± 12.9 years). The relative MMR gene expression was not correlated with disease stage or tumor site in the cases or with smoking and alcohol use in the controls. The expression levels increased with age in both cases and controls, but the mean expression of hGTBP/hMSH6 increased with age in both cases and controls and was significantly lower in the cases than in the controls (P < 0.05). Using the median expression level in controls as the cutoff value, significantly increased odds ratios (ORs) were associated only with low expression of hMLH1 (OR = 4.4; 95% confidence interval = 2.1–9.1) and hGTBP/hMSH6 (OR = 2.1; 95% confidence interval = 1.1–4.1) after adjustment for age, sex, ethnicity, smoking status, and alcohol use. The results suggest that low hMLH1 and hGTBP/hMSH6 expression is associated with an increased risk of head and neck cancer. Additional studies with a larger number of subjects are warranted to confirm these findings.

Introduction

Head and neck cancers include squamous cell carcinomas of the oral cavity, pharynx, and larynx (1). In 1997, it is estimated that there were 30,750 new cases and 8,440 deaths from this disease in the United States (2). Whereas tobacco use is the dominant etiological factor for head and neck cancer in the United States (3), alcohol consumption is also a major risk factor (4). It has been shown that an interaction between tobacco use and alcohol consumption is probably multiplicative for laryngeal cancers but is more variable for oral and pharyngeal cancers (5). Head and neck cancer, like lung cancer, can therefore be considered the paradigm of an environmentally induced disease. Its occurrence in young adults and in nonusers of tobacco and alcohol, however, suggests a possible genetic predisposition as well (6).

One possible susceptibility factor is DNA repair capacity (7, 8), which may influence the rate of fixation of mutations (9, 10) and thus cause the genetic or chromosomal instability associated with enhanced cancer risk (11–14). MIN1 and LOH have been frequently observed in head and neck tumors (15), indicating the possible involvement of defective DNA repair in the former and subsequent genomic instability in the latter. Although genomic instability may be important in tumor progression (12), evaluation of genetic susceptibility and inherited DNA repair capacity, in particular, should facilitate the identification of individuals at increased cancer risk. In this molecular epidemiological study, using a newly developed multiplex RT-PCR assay with the β-actin gene as an internal control (16, 17), we simultaneously evaluated the relative expression of five human MMR gene homologues (hMSH2, hMLH1, hPMS1, hPMS2, and hGTBP/hMSH6) in human peripheral lymphocytes and tested the hypothesis that genetically determined alterations in DNA repair gene expression are associated with increased risk of head and neck cancer.

Materials and Methods

Study Subjects. A case-control study design was used to investigate the association between reduced MMR gene expression in peripheral blood lymphocytes and risk of head and neck cancers. The cases studied were patients from The University of Texas M. D. Anderson Cancer Center with newly diagnosed squamous cell carcinomas of the oral cavity, pharynx, and larynx who had not received prior chemotherapy or radiotherapy. Patients with a prior cancer history or recurrent head and neck cancer were excluded.

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3 The abbreviations used are: MIN, microsatellite instability; LOH, loss of heterozygosity; RT, reverse transcription; MMR, mismatch repair; OR, odds ratio; CI, confidence interval; HNSCC, head and neck squamous cell carcinoma.
Molecular Analysis by Multiplex RT-PCR. A newly developed multiplex RT-PCR assay using the β-actin gene as an internal control was used to simultaneously evaluate the expression of five human MMR gene homologues (hMSH2, hMLH1, hPMS1, hPMS2, and hGTBP/hMSH6) in each blood sample. These genes were chosen primarily because we were able to obtain their cDNA clones for verification of the PCR products at the time this project was initiated (16, 17). One of the MMR genes, hMSH3, was not included because we did not have the cDNA clone. The internal control allows for evaluation of possible contamination of genomic DNA and normalization of variation in the amount of PCR product loaded (16, 17).

To amplify the five MMR genes, we used the previously described multiplex RT-PCR protocol (17) and previously described primers (16) synthesized by Life Technologies, Inc. (Gaithersburg, MD). Briefly, by using Tri-Reagent, a RNA/DNA/protein isolation reagent (Molecular Research Center, Inc., Cincinnati, OH; Ref. 20), we isolated total RNA. Then, cDNA was synthesized by RT with 0.5 μg of random primers (Promega Biotech, Madison, WI). Approximately 10 ml of blood were drawn from each subject who completed a self-administered risk factor questionnaire. From this pool, we selected control subjects matched to the lung cancer cases on age, sex, ethnicity, and smoking status. To evaluate the expression of possible contamination of genomic DNA and normalization of variation in the amount of PCR product loaded.

Statistical Analysis. The relative expression of each MMR gene was first analyzed as a continuous variable. The box-and-whisker plot technique (21) was used to explore the differences in the distributions between cases and controls. Student’s t test was used to compare the differences between cases and controls. Correlation analyses were performed on the expression levels and selected host factors. For calculation of crude ORs and CIs, the median expression level of the repair genes in the controls was used as the cutoff value for statistical comparison. Values greater than this median were considered high expression, and values below the median were considered low expression. Those who smoked more than 100 cigarettes in their lifetimes were considered ever smokers, and those who had at least 1 drink/week were considered alcohol users. To evaluate the dose-response relationship, the subjects were categorized into four groups by quartile of expression level in the controls (Fig. 2) and assigned a value of 1–4, which was fit as a continuous variable in an unconditional logistical regression model (22). All of the statistical analyses were performed with Statistical Analysis System software (Version 6; Statistical Analysis System Institute, Inc., Cary, NC).

Results

This report is based on 78 cases and 86 controls for whom we had sufficient RNA for the experiments. Table 1 shows the distribution of cases and controls by age, race, smoking status, and alcohol use. The mean age was 59.6 ± 12.4 years for the cases and 58.2 ± 12.9 years for the controls. Non-Hispanic whites accounted for 91 and 88% of the cases and controls,
Approximately three-quarters of both cases and controls were ever smokers and alcohol users. There were more men among the cases (57%) than the controls (42%).

Among the 86 controls, the greatest variation in expression was in hMSH2 (2.9–92.4%; approximately 32-fold), and the least variation was in hMLH1 (14.2–59.5%; approximately 4.2-fold; Fig. 2). Although previous experiments suggested that intrasample variation assessed by repeated measurements of a sample (cell line) was less than 2-fold (17), we did not test for variability within or among samples in this study because of the limited amount of blood available. On the basis of our previous experience in performing RT-PCR, we believe that much of the observed variation resulted from interindividual variation rather than intrasample variation. Correlation analysis indicated that the expression level increased with age (which ranged from 29–83 years) for hMLH1 (r = 0.28; P < 0.01) and hPMS1 (r = 0.26; P < 0.05). Although smoking status and alcohol use were correlated (r = 0.22; P < 0.05), there was no correlation between the gene expression levels of hMLH1 or hPMS1 and smoking status or alcohol use. However, decreased expression of hGTBP/hMSH6 was correlated with alcohol use (r = 0.22; P < 0.05). The expression levels of all five MMR genes were highly correlated, with the strongest correlation between hMLH1 and hPMS1 (r = 0.76; P < 0.0001), and the weakest correlation between hGTBP/hMSH6 and hPMS1 (r = 0.25; P < 0.05).

In the 78 cases, the proportions of patients with stage I, II, III, and IV tumors were 19, 29, 21, and 31%, respectively, and no differences in the expression levels of the five genes were found among these stages. Stratified analysis by tumor site (i.e., oral cavity (50%), larynx (27%), pharynx (19%), and others (unknown primary, 4%)) revealed no significant differences in the expression levels of the five genes, although patients with tumors of the pharynx tended to have slightly higher expression of each gene (data not shown). The expression levels of the five genes were also highly correlated, as observed in the controls.

Although the distribution of the expression levels of the five genes varied greatly in both cases and controls, particularly
Percent difference
From two-sided $t$ test analysis.

Table 2 Differences in MMR gene expression between cases and controls

<table>
<thead>
<tr>
<th>Gene</th>
<th>Case (n = 78) Mean ± SD</th>
<th>Control (n = 86) Mean ± SD</th>
<th>Percent differencea</th>
<th>p*b</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSH2</td>
<td>59.6 ± 12.4</td>
<td>58.2 ± 12.9</td>
<td>−13.7</td>
<td>0.485</td>
</tr>
<tr>
<td>hPMS2</td>
<td>32.7 ± 22.7</td>
<td>37.9 ± 19.3</td>
<td>−11.8</td>
<td>0.118</td>
</tr>
<tr>
<td>hGTBP</td>
<td>56.0 ± 13.9</td>
<td>59.5 ± 15.3</td>
<td>−3.5</td>
<td>0.282</td>
</tr>
<tr>
<td>hMLH1</td>
<td>43.0 ± 12.6</td>
<td>47.3 ± 13.2</td>
<td>−9.1</td>
<td>0.035</td>
</tr>
<tr>
<td>hPMS1</td>
<td>29.1 ± 8.12</td>
<td>35.1 ± 9.80</td>
<td>−17.1</td>
<td>0.000</td>
</tr>
<tr>
<td>hMLH1</td>
<td>33.1 ± 11.3</td>
<td>36.9 ± 12.1</td>
<td>−10.3</td>
<td>0.044</td>
</tr>
</tbody>
</table>

a Percent difference = [(meancase − meancontrol)/meancontrol] × 100.
b From two-sided $t$ test analysis.

Table 3 Crude and adjusted ORs and CIs for MMR gene expression level

<table>
<thead>
<tr>
<th>Gene expression level</th>
<th>No.</th>
<th>Crude OR (CI)</th>
<th>Adjusted OR (CI)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>hMSH2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>29</td>
<td>43</td>
<td>1.00</td>
</tr>
<tr>
<td>Low</td>
<td>49</td>
<td>43</td>
<td>1.69</td>
</tr>
<tr>
<td>hPMS2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>37</td>
<td>44</td>
<td>1.00</td>
</tr>
<tr>
<td>Low</td>
<td>41</td>
<td>42</td>
<td>1.16</td>
</tr>
<tr>
<td>hGTBP/hMSH6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>28</td>
<td>44</td>
<td>1.00</td>
</tr>
<tr>
<td>Low</td>
<td>50</td>
<td>42</td>
<td>1.87</td>
</tr>
<tr>
<td>hMLH1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>43</td>
<td>1.00</td>
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<tr>
<td>Low</td>
<td>61</td>
<td>43</td>
<td>3.59</td>
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<td>hPMS1</td>
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<td></td>
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<tr>
<td>High</td>
<td>23</td>
<td>43</td>
<td>1.00</td>
</tr>
<tr>
<td>Low</td>
<td>55</td>
<td>43</td>
<td>2.39</td>
</tr>
</tbody>
</table>

a Adjusted for age (in years), sex, ethnicity, smoking status, and alcohol use in a logistic regression model.

for $hMSH2$, the mean expression levels of all five MMR genes tended to be lower in the cases than in the controls (Fig. 2); the most significant difference in the mean expression level between cases and control was observed for $hMLH1$: the mean in the cases (29.1 ± 8.12) was 17% lower than that of the controls (35.1 ± 9.80; $P < 0.0001$; Table 2). There were no statistically significant differences in the mean levels of gene expression between gender and ethnic groups, although the number of nonwhites was small (7 cases and 10 controls).

To adjust for potential confounders, we fit the data into logistic regression models incorporating age, sex, ethnicity, smoking status, alcohol use, and the expression level of each gene (Table 3). Only the reduced expression levels of $hGTBP$/hMSH6 and $hMLH1$ remained significant risk factors (OR = 2.10 and 95% CI = 1.07-4.12 and OR = 4.35 and 95% CI = 2.09-9.06, respectively). A dose-response relationship was found for both these gene expression levels (trend test, $P < 0.0001$ for $hMLH1$ and $P < 0.05$ for $hGTBP$/hMSH6) with subjects in the lowest quartile of the expression levels having the greatest risk for head and neck cancer.

Discussion

In this study, we found an association between reduced expression of $hMLH1$ and $hGTBP$/hMSH6 and increased risk of head and neck cancer that was independent of age, sex, ethnicity, smoking, and alcohol use. The variation in the MMR gene expression levels, particularly the $hMSH2$ level, suggests genetic heterogeneity of gene expression levels and possibly an unmeasured environmental influence on epigenetic regulation of gene expression (23) in addition to the assay variation. The common splicing variants of MMR genes observed in normal lymphocytes probably also contribute to the wide range in overall expression levels (24, 25). The correlation between the relative expressions of the MMR genes may reflect the overall MMR capacity, in which each gene has an exclusive and necessary role (13, 14). The positive correlation between age and expression of MMR genes, however, may reflect the need to repair damage due to cumulative environmental carcinogen exposure (23), the increasing level of endogenous DNA damage with aging (26), and the age-related decrease in DNA repair capacity (7, 27). Alternatively, this increase in MMR gene expression may be a compensation for inefficient MMR challenged by an increasing mutation rate and genomic instability as a result of aging (26, 28). Nevertheless, the age-related increase in expression was independent of the reduced gene expression level associated with increased risk of head and neck cancer in this study population, indicating that such reduced gene expression may be genetically determined. However, the exact mechanisms for such age-related increases in the expression remain unclear. Further study on the correlation between reduced transcription or transcript stability and related protein function should shed light on the underlying molecular mechanisms.

Although the validity of peripheral blood lymphocytes as a surrogate for head and neck tissue has not been tested, collecting head and neck tissue for such an epidemiological study is not practical. However, the use of unstimulated peripheral blood lymphocytes, which is the easiest tissue to col-
fect for a molecular epidemiological study, provides information on the genetically determined expression of the genes tested, although the level of expression may be modulated by an exposure or risk factor not measured in this study. The observed differences in the gene expression levels between cases and controls were relatively small and could be the results of chance. However, consistently lower mean expression levels of the five MMR genes in the cases would not be expected by chance, because the cases tended to be older than the controls and hence might have been expected to have higher mean gene expression levels, given an observed age-related increase in gene expression in both cases and controls in this study.

The finding of low hMLH1 and hGTBP/hMSH6 expression associated with an increased risk of head and neck cancer is consistent with previous reports. The MMR genes are thought to be housekeeping genes, because they are expressed (although at various levels) in all tissues tested (29, 30). Defective MMR is believed to be responsible for MIN, which is a hallmark of hereditary nonpolyposis colon cancer (31) and a common genetic alteration observed in many malignancies (32), including HNSCC (15). A number of reports have suggested that MIN and LOH are frequent in HNSCC, occurring in 15–72% of the tumors examined (33–35). One recent microsatellite marker study showed that 51% (17 of 34) of tumors with oral leukoplakia, a precancerous lesion, had LOH on chromosome 9p21, and 37% of those patients (but only 6% of patients without LOH) developed HNSCC (35). Furthermore, a recent report suggested that G-T p53 transversions, a mutation considered to be characteristic of exogenous DNA-damaging agents, including tobacco smoke carcinogens, occur in 14% of HNSCCs (36), indicating the involvement of inefficient DNA repair activities. It is therefore likely that inherited low gene expression or inadequate gene expression in response to environmental carcinogen exposure may be correlated with low DNA repair capacity, as seen in lung cancer (8).

In summary, our findings suggest that reduced expression of MMR genes in blood lymphocytes may vary from individual to individual, and that this interindividual variability may be genetically determined or epigenetically influenced by cumulative environmental carcinogen exposure. Low expression of the MMR genes hMLH1 and hGTBP/hMSH6 was associated with an increased risk of head and neck cancer. However, we were not able to evaluate the effect of tobacco and alcohol use on gene expression because of the frequency-matching design. This issue should be addressed in future studies, which are needed to validate the utility of this assay and the significance of the findings.

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Q Wei, S A Eicher, Y Guan, et al.


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