hMLH1 and hMSH2 Expression Correlates with Allelic Imbalance on Chromosome 3p in Non-Small Cell Lung Carcinomas

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ABSTRACT

DNA mismatch repair genes have been implicated in the pathogenesis and predisposition of certain malignancies through a mutator phenotype. In this study, we investigated, in 150 non-small cell lung carcinomas, the expression levels of hMLH1 and hMSH2 proteins in relation to loss of heterozygosity on chromosomes 3p and 2p, the mutational status of these genes' promoters and the hot spot exons. We have demonstrated that 88 of 150 (58.6%) tumor specimens had reduced expression levels of the hMLH1 protein, whereas 85 of 147 (57.8%) specimens had reduced expression levels of the hMSH2 protein. Reduced expression levels of both proteins were observed in 51 of 150 (34%) specimens. In adenocarcinomas, the reduction of hMSH2 expression was more frequently observed than that of hMLH1 (P<0.003), whereas in squamous cell carcinoma of the lung hMLH1 expression was more frequently reduced than hMSH2 (P<0.006). Reduced expression of hMLH1 correlated with allelic imbalance on loci D3S1289 (P<0.0002) and D2S391 (P<0.05). It is of note that an inverse correlation was found between hMSH2 reduced expression and loss of heterozygosity at locus D3S1300 (P=0.016). In addition, hMLH1 reduced expression was more frequently associated with heavy smokers, assessed by daily tobacco uptake (P=0.018) and total smoking exposure (pack-years; P<0.05). In addition, a correlation between hMLH1 reduced expression and nodal metastasis in squamous cell carcinoma of the lung was observed (P=0.015). No mutations were identified in the promoters or exons examined in these two genes. These findings indicate that hMLH1 and hMSH2 gene inactivation is a common event in the development of non-small cell lung carcinoma and allelic loss seems to be a major genetic event involved in hMLH1 silencing. In addition, we propose that a putative negative regulator of hMSH2 gene may be located at the locus 3p14.

INTRODUCTION

hMLH1 and hMSH2 are two of the genes known to be implicated in the DNA MMR3 system. Inactivation of the MMR machinery has been closely associated with a mutator phenotype that is a hallmark of almost all human cancers (1). Molecular defects in one or both of the genes account for a significant proportion of HNPCCs and a small proportion of sporadic colorectal cancer cases (2, 3). Inactivation of DNA MMR genes occurs in two steps, following the same pattern as in tumor suppressor genes (4). Previous studies have demonstrated that LOH at the DNA MMR loci is a frequent genetic event in human cancers, including lung cancer (5–9). It has also been shown that methylation of the promoter region of the hMLH1 gene leads to lack of expression of the encoding protein (10–12). However, hMSH2 promoter methylation has not been demonstrated in tumors lacking expression of the relative protein (13–15). Recent reports have indicated that reduced expression levels of the DNA MMR genes may be implicated in the pathogenesis of certain human cancers and may predict disease-free survival after primary chemotherapy (16–22). A possible role of hMLH1 and hMSH2 overexpression in the induction of apoptosis has also been suggested (23).

Multiple molecular defects have been identified to play a role in the molecular pathogenesis of lung tumors, including alterations in oncogenes and tumor suppressor genes (24–25). Mutations in the p53 and K-ras genes as well as allelic losses and deletions at chromosomes 3p and 9p seem to be among the most commonly found genetic defects in carcinomas of the lung (25–29). Although the role of the hMLH1 and hMSH2 genes in the molecular pathogenesis of HNPCC and sporadic colorectal carcinoma has been well studied, little is known about the involvement of these genes in lung cancer. In this study, we investigated the expression levels of hMLH1 and hMSH2 proteins in relation to LOH at chromosomes 2p and 3p in NSCLC. We also examined the mutational status of the promoter regions and the most frequently mutated exons reported of the hMLH1 and hMSH2 genes.

MATERIALS AND METHODS

Patients. Lung tumor tissue samples were obtained from 150 patients, 59 males and 91 females, who were operated in the Cardiothoracic Center of Broadgreen (Liverpool, Merseyside, United Kingdom). The age of the patients ranged from 41–95 years (median, 65). The histology of the specimens included in this investigation was: 49 adenocarcinomas, 85 squamous cell carcinomas, 8 adenosquamous, 6 large cell carcinomas, and 2 unclassified NSCLCs. Smoking history (daily consumption, current status) was available for 111 individuals: fifty-four current smokers, 16 recently stopped smokers (1–4 years prior to presentation), 35 former smokers (>5 years prior to presentation), and 6 nonsmokers. However, complete data for calculating the total smoking exposure was available for only 65 smokers. Total smoking exposure is expressed in pack-years: pack-years = [(age at operation) − (age started) − (years stopped)] × (packs/day).

In this study, the patients’ pack-years ranged from 17–165 (median, 69).

Immunohistochemical Detection of hMLH1 and hMSH2 Protein Expression. Protein expression was demonstrated immunohistochemically by a modified avidin-biotin complex method. Formalin-fixed paraffin tissue sections were sectioned at 4-μm thickness, mounted on APES-coated slides, and dried at 37°C overnight. Sections were deparaffinized in xylene and rehydrated in a series of graded alcohols to tap water. Heat-mediated antigen retrieval was required to expose the epitopes and was performed by microwaving the sections on full power in 0.01 M citrate buffer (pH 6.0) for 15 min in a 800-W microwave oven. The sections were left to stand for 15 min to cool and then rinsed for 5 min in running tap water. Endogenous peroxidase activity was blocked by 1.5% hydrogen peroxide in methanol for 10 min. Sections were incubated in the primary antibody buffer (5% goat serum in PBS) for 20 min.

Monoclonal antibodies against hMLH1 and hMSH2 (Serotec Ltd., Oxford, United Kingdom) were diluted 1:10 and 1:20, respectively, in the primary antibody buffer and incubated for 1 h at room temperature. The primary antibodies were visualized with Dako LSAB 2 Peroxidase kit (DAKO, Cam-
bridgeshire, United Kingdom). The secondary and tertiary reagents were incubated for 30 min each and rinsed in-between each stage with 0.05 M Tris-buffered saline (pH 7.6). The signal was developed with diaminobenzidine (Merck, Dorset, United Kingdom) and hydrogen peroxide. The sections were counterstained with Gill’s Hematoxylin. Normal mouse IgG replaced the primary antibody as a negative control. The frequency of the nuclear staining was scored on a scale from (−) to (+++) [as absent (−), weak (+), moderate (++), and strong (+++)] without the knowledge of clinical, pathological, and 3p LOH status data. The staining was scored by two of the authors independently.

**DNA Extraction.** Paired tumor-normal frozen tissue specimens were available from 85 individuals. Five 10-μm sections of each sample were microdissected to ensure presence of >75% tumor cells. Sections were lysed in 400 mM Tris-HCl pH, 150 mM NaCl, 60 mM EDTA, 1% SDS, and 100 μg/ml proteinase K and incubated at 42°C for 16 h in an orbital shaker. Deproteinization included extraction with phenol/chloroform and chloroform. DNA was precipitated by the addition of an equal volume of isopropanol. DNA was spooled onto sterile microbiology loops, washed with 70% ethanol, and resuspended in 200 μl of 10 mM Tris (pH 8)-1 mM EDTA. Working stocks were prepared by 5-fold dilution in double distilled H2O.

**LOH Analysis.** Four markers located proximal and distal to hMLH1 gene (D3S1289, D3S1266, D3S1300, and D3S1304) and one marker proximal to hMSH2 gene (D2S391) were available for 85 individuals from a previous study of ours (30). An additional marker (D2S2259) also located on 2p16 was examined in this study and added in the existing database. All fluorescent microsatellite markers were selected from the Linkage Mapping Set V2.0 (PE Applied Biosystems, Warrington, United Kingdom), and analysis was performed on a 377 ABI-PRISM automatic sequencer. The reaction conditions, details, and analysis parameters have been previously described (30).

**Mutational Analysis.** Mutational analysis was performed in 120 samples. Screening of the hMLH1 promoter region and exons 9, 13, and 16 and the hMSH2 promoter region and exons 5, 7, and 8 was performed by PCR, followed by SSCP and HA. The primers used for hMLH1 (exons 9, 13, and 16) and hMSH2 (exons 5, 7, and 8) and PCR amplification parameters have been described previously (31). The primers used for the amplification of the promoter regions of hMLH1 and hMSH2 are:

- hMLH1 promoter: 5’ AGGCTCCACCACAAATAAC 3’ (sense), 5’ CGCTGTCGCCGCTTCTCATATT 3’ (antisense);
- hMSH2 promoter: 5’ CCTTGACATACCCCCACCCA 3’ (sense), 5’ GCGACCCCCACCCCCACTAA 3’ (antisense).
PCR reactions were performed in a 25-μl reaction volume and contained 100 ng of genomic DNA, 200 μM of each dNTP, 8 μM of each primer, 0.6 unit of BIOPRO polymerase (Bioline, London, United Kingdom), and 2.5 μl of 10× buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, and 1.5 mM MgCl2]. Samples were subjected to 37 cycles of amplification.

Results

hMLH1 and hMSH2 Expression in Lung Tumors. Fifteen normal lung tissues, adjacent to tumors examined in this study, were also investigated for the expression of hMLH1 and hMSH2. In all cases, normal bronchial epithelium demonstrated strong staining (+ + +) for both proteins (Fig. 1, A and F). Hence, tumors demonstrating strong (+ + +) immunoreactivity were classified as “normal expression”, whereas tumors demonstrating absent, weak, and moderate immunoreactivity were classified as “reduced expression.”

hMLH1 expression was examined in 150 NSCLC tissues. Sixty-two (41%) were found to have intense staining (+ + +; Fig. 1E), and 88 (59%) showed reduced (absent, weak, or moderate) staining (−, +, or + +; Fig. 1B–D). Of the specimens with reduced expression, 8 showed absence (−) of hMLH1 expression whereas 22 showed weak (+) and 58 showed moderate (+ +) expression (Table 1). On examining the NSCLC subtypes, 26 of 49 (53%) adenocarcinomas and 56 of 85 (66%) SqCCL showed reduced hMLH1 expression (Table 1).

It is of note that hMLH1 reduced expression was more frequently found in heavy smokers (>1 pack per day). Forty-six of 71 heavy smokers and 13 of 32 moderate smokers had reduced hMLH1 expression (Fisher’s exact test, $P = 0.018$; Table 2). In addition, hMLH1 reduced expression was more frequently found among patients with total smoking exposure higher than the median (69 pack-years; $P < 0.05$; Table 2). No association was found between the nonsmoker/former/current smoker status and hMLH1 expression levels. A correlation between hMLH1 reduced expression and nodal metastasis was found in SqCCL ($P = 0.015$). In particular, hMLH1 reduced expression was found in 29 of 51 (57%) SqCCL specimens with negative nodes and in 24 of 29 (83%) SqCCL specimens with positive nodes. No significant associations were found between hMLH1 expression and other clinicopathological parameters (age, gender, differentiation, and T stage).

hMSH2 expression was examined in 147 NSCLC tissues, because we ran short of tissue for three samples that had already been examined for hMLH1. Sixty-two (42%) were found to have strong expression (+ + +; Fig. 1J), and 85 (58%) showed reduced (absent, weak, or moderate) expression (−, +, or + +; Fig. 1, G–I). Nine of 49 adenocarcinomas (18%) demonstrated hMSH2 strong expression, whereas reduced expression was observed in 40 (82%). Forty-five of 82 (55%) SqCCL showed strong expression whereas 37 (45%) showed reduced expression (Table 1). No significant associations were identified between hMSH2 expression and T stage, nodal metastasis, differentiation, smoking status, age, or gender of the patient.

The comparative analysis of expression levels of hMSH2 and hMLH1 in different histological types (Table 1) demonstrated that, in adenocarcinomas, hMSH2 was more frequently reduced than hMLH1, 40 of 49 and 26 of 49, respectively ($P < 0.003$). In contrast, in SqCCL, hMLH1 expression was more frequently reduced than hMSH2, 56 of 85 and 37 of 82, respectively ($P < 0.006$; Fig. 2). Simultaneous reduced expression of both hMLH1 and hMSH2 was found in 51 of 150 (34%) samples examined (Fig. 3). Samples with reduced expression of both MMR proteins, comparatively with sam-

### Table 1

<table>
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<th>hMSH2 expression</th>
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<td>14</td>
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<tr>
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<tr>
<td>Total NSCLC</td>
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$^a$ AdenoCa, adenocarcinoma.

### Table 2

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<th>Former (1–4 yr)</th>
<th>Daily tobacco consumption</th>
<th>Total tobacco exposure$^a$</th>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>19</td>
<td>12</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
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<td>6</td>
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<td>31</td>
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<td></td>
<td>Reduced</td>
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<td>29</td>
<td>22</td>
<td>9</td>
<td>21</td>
<td>38</td>
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</tbody>
</table>

$^a$ The patients have been grouped according to their pack-year data based on the mean pack-year value found in this study (69 pack-years).
and D3S1289 the level of the hMSH2 protein and LOH at loci 3). In addition, a trend was observed between a higher expression D3S1300 an inverse correlation was found between LOH at locus D3S1289. No mutations or polymorphisms were detected in any of the examined regions. We have also performed automated sequencing analysis for both genes in 20 randomly selected samples to check possible SSCP false negatives, however, no mutations were found.

hMLH1 and hMSH2 Expression Correlates with Allelic Imbalance at Chromosomes 2p and 3p. Eighty-five of the 150 samples examined in the current study were previously investigated for allelic imbalance using fluorescent microsatellite markers and analysis on a 377 ABI PRISM automatic sequencer (30). In this study, we have also examined an additional marker on 2p16 (D2S2259). LOH for the latter was identified in 26 of 60 (43%) informative cases. The comparative analysis of expression of hMLH1 and hMSH2 with allelic imbalance data of four markers on chromosome 3p (Table 3) showed that hMLH1 reduced expression correlated with allelic imbalance at the D3S1289 locus on 3p21 (Fisher’s exact test, P = 0.00019). No such correlation was found with the loci D3S1304 (3p26; P = 0.14), D3S1266 (3p24; P = 0.1), and D3S1300 (3p14; P = 0.57). However, an inverse correlation was found between LOH at locus D3S1300 (Fisher’s exact, P = 0.016) and expression of hMSH2 protein (Table 3). In addition, a trend was observed between a higher expression level of the hMSH2 protein and LOH at loci D3S1266 (P = 0.063) and D3S1289 (P = 0.059), but not with locus D3S1304 (P = 0.55). No association was found between hMSH2 expression and LOH at the D2S391 (P = 0.28) and D2S2259 (P = 0.24) loci. However, a correlation was found between hMLH1 reduced expression and LOH at the D2S391 (P = 0.048) but not at the D2S2259 locus (P = 0.14).

**DISCUSSION**

The hMLH1 and hMSH2 DNA MMR genes are known to be implicated in human cancer, with colon cancer being the most well studied model. However, the information of the status of these two genes in lung cancer is limited. In this study, we have investigated the expression of the hMLH1 and hMSH2 DNA MMR genes in NSCLC lesions. The immunohistochemical analysis demonstrated that 59% of the examined tumors had reduced expression of hMLH1 and 58% had reduced expression of hMSH2, whereas 34% demonstrated reduction of expression in both of these genes (Fig. 3). It is of note that 82% of all examined lung tumors showed reduced expression of at least one of the two investigated genes. This is the first report on the protein expression levels of the above mentioned genes in NSCLC, and the results suggest a critical role for these DNA MMR genes in lung carcinogenesis.

It is of note that the reduction of expression of these two genes is associated with the histological subtypes; in adenocarcinomas hMSH2 expression was more frequently reduced than that of hMLH1, while the converse was observed in SqCCL (Fig. 2). Because both genes are considered to be inactivated in a two-hit model (4), we investigated the relationship between MMR gene expression levels and allelic imbalance (LOH) on 3p and 2p chromosome arms, the locations of these genes. The results indicated that reduced hMLH1 expression correlated with LOH at the D3S1289 (3p21) locus (P = 0.00019). This suggests that loss of one allele of the hMLH1 gene may be one of the major genetic events involved in its inactivation. This may explain the finding that hMLH1 expression is more frequently reduced in SqCCL than in adenocarcinomas, because the former have demonstrated a greater incidence of LOH on chromosome 3p (29–30, 32). Hypermethylation of the hMLH1 promoter has been demonstrated in human tumors (10–14), and this most likely also contributes to changes in the gene’s expression. However, such analysis was not performed on this set of our samples and remains to be elucidated in future studies. The mutational analysis of the hMLH1 promoter region and the hot spot exons did not reveal any mutations, which is in agreement with previous reports (8, 33) and suggests that mutations are unlikely to be a major cause of hMLH1 inactivation in lung carcinogenesis. A correlation was found between the reduced expression of hMLH1 and LOH at the D2S391 (2p16) locus, which may suggest that hMLH1 expression regulatory gene(s) are located in this region but further studies are required to clarify this aspect.

It is of particular note that an inverse relationship between LOH at the D3S1300 locus and hMSH2 expression was identified where hMSH2 reduced expression was more prevalent in samples retaining heterozygosity at this locus. This may imply the presence of a negative hMSH2 regulatory gene on 3p, suggestive of negative feedback mechanism; however, additional studies are required to elucidate the nature of this relationship. This inverse correlation provides a possible explanation for the lower incidence of reduced hMSH2 expression in normal samples with reduced expression of only one of the examined proteins, did not show additional associations with any of the clinicopathological parameters examined.

**Mutational Analysis of hMLH1 and hMSH2 Promoter Regions and Hot Spot Exons.** Mutational analysis using SSCP and HA was performed on the promoter regions of hMLH1 and hMSH2 genes in 120 samples and in exons shown to have mutational hot spots in HNPCC and sporadic colorectal carcinomas (exons 9, 13, and 16 of hMLH1 and exons 5, 7, and 8 of hMSH2). No mutations or polymorphisms were detected in any of the examined regions. We have also performed automated sequencing analysis for both genes in 20 randomly selected samples to check possible SSCP false negatives, however, no mutations were found.

**Table 3 Expression levels of hMLH1 and hMSH2 proteins in lung tumors in relation to allelic imbalance on chromosomes 3p and 2p. Only informative cases (heterozygous status in normal) were included.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression level</th>
<th>D3S1304 H</th>
<th>D3S1304 L</th>
<th>D3S1266 H</th>
<th>D3S1266 L</th>
<th>D3S1289 H</th>
<th>D3S1289 L</th>
<th>D3S1300 H</th>
<th>D3S1300 L</th>
<th>D2S391 H</th>
<th>D2S391 L</th>
<th>D2S2259 H</th>
<th>D2S2259 L</th>
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</thead>
<tbody>
<tr>
<td>hMLH1</td>
<td>Normal</td>
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<td>24</td>
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</tr>
<tr>
<td></td>
<td>Reduced</td>
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</table>

* H, heterozygous; L, LOH.
SqCCL compared with adenocarcinomas (Fig. 2). This is possibly due to the relatively higher incidence of LOH on 3p found in SqCCL (29–30, 32).

We found no association between hMSH2 expression and LOH at the D2S391 and D2S2259 (2p16) loci, which suggests that allelic loss at these loci is not the main event contributing to the reduction of hMSH2 expression in NSCLC. Our results indicated no mutations in the promoter and the hot spot exons of hMSH2, which is in agreement with previous reports (33–35). Furthermore, no hypermethylation of hMSH2 promoter has been demonstrated in certain human tumors (13–15), thus, inactivation of the hMSH2 gene may rely on alternative mechanisms involving changes in its upstream regulatory genes. Recent reports have revealed p53-binding sites on the hMSH2 promoter (36) and a possible hMSH2 expression regulatory role of p53 in leukemias (37).

hMLH1 reduced expression correlated with both higher daily tobacco uptake and total tobacco exposure (pack-years), indicating that tobacco carcinogens are implicated in hMLH1 inactivation and, moreover, that they may have an additive effect. The lack of association with the current/former smoking status argues that smoking-related MMR inactivation may be irreversible and it is in agreement with the fact that smoking cessation does not reduce risk for lung cancer development in chronic smokers to the baseline of nonsmokers. Moreover, hMLH1 expression levels did not seem to differ between smokers and nonsmokers. Although only six nonsmokers were included in this study and a “passive smoker” status is difficult to assess, the above finding indicates that carcinogens apart from those found in tobacco may also affect hMLH1 expression. Reduced expression of hMLH1 in SqCCL correlated with nodal metastasis, suggesting that the hMLH1 gene may contribute to a more aggressive tumor phenotype in this histological subtype. Thus, hMLH1 expression may be a useful molecular marker for the clinician when developing a treatment regimen.

The comparative analysis of hMLH1 and hMSH2 expression in the tumors in this study did not reveal any associations between the expression of these two genes. Furthermore, tumor specimens with combined reduced expression of both hMLH1 and hMSH2 proteins did not correlate with any clinicopathological parameters. Thus, no complementary role of these two proteins was demonstrated. The frequencies of the reduced expression of these two genes are different in SqCCL and adenocarcinomas, and this may be due to the different incidence of LOH on chromosome 3p in these tumor subtypes. Also, smoking seems to affect hMLH1 but not hMSH2, whereas hMLH1, but not hMSH2, correlates with nodal metastasis in SqCCL. All of the above findings support distinct roles for the two genes in lung carcinogenesis. The results suggest that at least some of the environmental and endogenous factors involved in their inactivation pathway are different. Further investigation is required to elucidate the complete pathways of inactivation of these two genes in NSCLC and reveal additional factors implicated in their regulation.

ACKNOWLEDGMENTS

We are indebted to all of the clinical staff at the Cardiothoracic Center of Broadgreen (Liverpool, Merseyside, United Kingdom) for access to their patients.

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