Inactivation of Human MutL Homolog 1 and MutS Homolog 2 Genes in Head and Neck Squamous Cell Carcinoma Tumors and Leukoplakia Samples by Promoter Hypermethylation and Its Relation With Microsatellite Instability Phenotype

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BACKGROUND. A subset of head and neck squamous cell carcinoma (HNSCC) exhibits a microsatellite instability (MIN) phenotype. The authors correlated alterations in the mismatch-repair genes human mutL homolog 1 (hMLH1) and human mutS homolog 2 (hMSH2) in primary head and neck squamous cell carcinoma (HNSCC) tumors and in samples of leukoplakia with the MIN phenotype.

METHODS. One hundred twenty-three paired HNSCC normal and tumor tissues and 27 leukoplakia samples were examined for hypermethylation of hMLH1 and hMSH2 promoters. The hypermethylation status of the tissues was confirmed by expression studies. Sixty-three of 123 randomly selected tumors and all 27 leukoplakia samples were genotyped with 8 microsatellite markers to determine MIN.

RESULTS. Fifty percent of HNSCC tumors and 63% of leukoplakia samples harbored hypermethylation at either or both hMLH1 and hMSH2 promoters. Normal tissues adjacent to methylation-positive tumors also demonstrated hypermethylation of both promoters at a high frequency (25%). A positive correlation between tobacco habit and promoter hypermethylation was observed (P = .001). A correlation was observed between MIN and the frequency of promoter hypermethylation in the leukoplakia samples, but no such trend was observed in the HNSCC tumors. It is noteworthy that patients who had a high frequency of MIN-positive tumors exhibited hypermethylation in both the affected tissues and the adjacent normal tissues (P = .007). Patients with a tobacco habit who had promoter hypermethylation at both the affected tissues and the adjacent normal tissues had tumors that mostly were MIN positive (P = .047).

CONCLUSIONS. The current results suggested that tobacco-addicted individuals are more susceptible to promoter hypermethylation of hMLH1 and hMSH2 and that, if such hypermethylation occurs in the normal squamous epithelium of the head and neck region, then those tissues are likely to develop into tumors that involve the MIN pathway. Cancer 2007;109:703–12.

KEYWORDS: promoter hypermethylation, hMLH1, hMSH2, microsatellite instability, head and neck squamous cell carcinoma.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world1 and accounts for 30% to 40% of all cancers in India.2 The major risk factors for this type of cancer are tobacco intake, both in the form of smoking and chewing, alcohol consumption, and human papillomavirus infection.3,4
HNSCC is characterized by complex, nonrandom changes in its genome and often shows different types of genomic instabilities. Previous investigators reported that a substantial fraction of HNSCC tumors exhibits instability at the simple repeat sequences (microsatellites) that are distributed throughout the genome known as microsatellite instability (MIN). In colorectal and other tumors, MIN occurs because of defects in mismatch-repair (MMR) genes, such as human mutL homolog 1 (hMLH1); human mutS homolog 1, 3, and 6 (hMSH2, hMSH3, and hMSH6); and postmeiotic segregation increased 1 and 2 (PMS1 and PMS2). However, involvement of MMR genes in the development of MIN in HNSCC tumors is uncertain.

In approximately 90% of patients with hereditary nonpolyposis colorectal cancer (HNPCC), a familial form of colorectal cancer, mutations are observed mainly in hMLH1 and hMSH2. A small proportion of HNPCC families also show mutations in PMS1, PMS2, hMSH3, and hMSH6. There also are reports of hMLH1, hMSH2, hMSH6, and hMSH3 inactivation by mutation in sporadic colorectal cancers, hematologic malignancies, and endometrial cancers. Most sporadic colorectal, gastric, endometrial, esophageal, urothelial, and hematologic malignancies, however, suffer promoter hypermethylation in hMLH1. A PMS2 defect has been identified in a small proportion of sporadic colorectal cancers, but promoter hypermethylation events were not observed. In HNSCC, there are a few reports on hMLH1 and hMSH2 inactivation by promoter hypermethylation leading to MIN phenotype.

For the current report, we investigated the nature of defects in 2 MMR genes, hMLH1 and hMSH2, in patients with HNSCC and in patients with leukoplakia from eastern India. We did not detect any mutation in these 2 genes, but a large number of tumors exhibited hypermethylation of the promoter of both genes. We correlated the promoter hypermethylation of the 2 genes with MIN status of the tumors in the background of tobacco habit and hypermethylation in normal tissues adjacent to the tumors.

**MATERIALS AND METHODS**

**Tumor Samples**

One hundred twenty-three patients with head and neck cancer and 27 patients with dysplastic leukoplakia were included in the study. Freshly operated tumor and histologically matched, adjacent normal tissues were collected from patients who underwent surgery prior to receiving any treatment from Chittaranjan National Cancer Institute and Thakurpukur Cancer Welfare Home and Research Institute (both located in Kolkata, India). Dysplastic leukoplakia tissue samples along with histologically matched, adjacent normal tissues or peripheral blood leukocytes were collected from Dr. R. Ahmed Dental College and Hospital in Kolkata. Written consent was obtained from the patients before sample collection. The institutional review committee on research using human subject cleared the project after due deliberation. The samples were frozen immediately after collection and stored at −80°C for future use. All tumors were diagnosed histopathologically as squamous cell carcinoma. For RNA isolation, part of the freshly operated tissues was collected directly in TRI-ZOL reagent (Invitrogen, Carlsbad, CA). Information on sex, age, site, International Union Against Cancer tumor, lymph node, metastasis (TNM) classification system. The samples were frozen immediately after collection and stored at −80°C for future use. All tumors were diagnosed histopathologically as squamous cell carcinoma. For RNA isolation, part of the freshly operated tissues was collected directly in TRI-ZOL reagent (Invitrogen, Carlsbad, CA). Information on sex, age, site, International Union Against Cancer tumor, lymph node, and metastasis (TNM) classification, and tobacco habit of the patients who were included in the study is listed in Table 1.

**Microdissection and DNA Isolation**

The frozen primary tumor tissues were subjected to microdissection to remove contaminating normal cells that were present in the tumor sections. The
normal tissues also were microdissected to remove the infiltrated tumor cells. Genomic DNA was extracted by using conventional proteinase K digestion followed by equilibrated phenol-chloroform-isoamyl alcohol extraction.

**MIN Analysis**

Eight highly polymorphic microsatellite markers were used in this study (D1S2893, D2S123, D3S1611, D5S346, D8S261, D9S168, D11S4177, and D18S34) for genotyping the paired tumor normal DNA for MIN analysis, as described previously. These markers were selected based on the recommendation of the National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition and our previous reports, which indicated high instability. A tumor was scored as microsatellite unstable (MIN-positive) if it showed allelic alterations in ≥1 marker, and MIN-positive results were subdivided further into MIN H and MIN L groups depending, on whether they showed alteration in ≥2 markers and 1 marker, respectively.

**Promoter Hypermethylation Analysis**

The methylation status of the CpG islands of hMLH1 and hMSH2 promoters was determined by using methylation-sensitive restriction digestion and polymerase chain reaction (PCR) analyses. The promoter region of hMLH1 (gene identification [GI] no. 51464027) contains 4 HpaII restriction enzyme sites at nucleotide positions −567, −527, −347, and −341; whereas 3 sites are found at nucleotide positions −393, −366, and −99 of hMSH2 (GI no. 51460714). The flanking primers would amplify a 608-base pair (bp) fragment for hMLH1 or a 520-bp fragment for hMSH2. In addition, D3S1561 and D2S123 loci were used as positive controls for PCR amplification. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

**RNA Isolation, Complementary DNA Synthesis, and Semiquantitative Reverse Transcriptase-PCR**

The tumor tissues and adjacent normal squamous epithelial counterparts were homogenized in TRIZOL reagent, and total RNA was isolated according to the manufacturer’s instructions. One microgram of DNaseI-treated RNA was reverse transcribed with random hexamer primer using the MMLV Reverse Transcriptase Kit (Promega, Madison, WI) in a total volume of 20 μL and stored at −20°C until use. Gene-specific primers for the hMLH1, hMSH2, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) genes were used to PCR amplify the corresponding complementary DNAs. PCR was performed for 30 cycles, and the products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining.

**Immunohistochemistry**

Five-micrometer to 10-μm frozen tissue sections were fixed with ice-cold methanol for 10 minutes followed by 0.5% Triton-X treatment for 30 seconds. The fixed sections were incubated with anti-MLH1 and anti-MSH2 antibodies (BD Pharmingen, San Diego, CA) followed by fluorescein isothiocyanate-conjugated goat antimouse secondary antibody (Sigma, St. Louis, MO; dilution, 1:2000). Finally, the slides were visualized under a fluorescent microscope (Olympus-BX-40), and photomicrographs were taken.

**RESULTS**

**hMLH1 and hMSH2 Promoter Hypermethylation of HNSCC Tumors and Leukoplakia Tissues**

One hundred twenty-three tumor and normal paired primary HNSCC samples and 27 leukoplakia tissues were screened to detect the promoter hypermethylation (Me) status of hMLH1 and hMSH2. Hypermethylation positive (Me-positive) DNA samples that could not be cleaved by methylation-sensitive HpaII produced the expected 608-base pair (bp) and 520-bp amplicon for hMLH1 and hMSH2 promoters, respectively (Figs. 1A, 2A). These samples did not show any amplification signal for hMLH1 or hMSH2 promoters when genomic DNA was digested with the methylation-insensitive isochizomer MspI (Figs. 1A, 2A). The amplification of the microsatellite markers D3S1561 and D2S123 (devoid of any CCGG sequence), which were upstream of hMLH1 and hMSH2, respectively, always were used as internal controls to exclude the
possibility that failure of \textit{hMLH1} and \textit{hMSH2} promoter amplification was not caused by the failure of PCR amplification (Figs. 1A, 2A). We determined the expression of both genes in normal tissues and in tumor tissues to confirm that observed differences in methylation patterns also reflected expression levels in the corresponding genes. Thirty primary tumor tissues and 20 paired, adjacent normal tissues were screened for the expression of \textit{hMLH1} and \textit{hMSH2} by semiquantitative reverse transcriptase (RT)-PCR. Me-positive tissues showed weak expression levels of the corresponding genes compared with the expression levels in Me-negative tissues (Figs. 1B, 2B). The RT-PCR expression levels were normalized with the expression of G3PDH as an internal control (Figs. 1B, 2B). Moreover, 11 primary tumor tissue specimens and 9 paired normal tissue counterparts were subjected to immunohistochemistry to confirm the presence or absence of \textit{hMLH1} and \textit{hMSH2} proteins in Me-negative or Me-positive samples, respectively. All Me-negative samples showed expression of the corresponding proteins, and Me-positive samples did not show any expression of the proteins (Figs. 1C, 2C).

Overall 61 of 123 HNSCC tumors (50%) showed hypermethylation of either 1 or both \textit{hMLH1} and \textit{hMSH2} (Fig. 3A). Among the Me-positive tumors, 28 of 123 tumors (23%) were hypermethylated exclusively at the \textit{hMLH1} promoter, 16 of 123 tumors (13%) were hypermethylated exclusively at the \textit{hMSH2} promoter, and 17 of 123 tumors (14%) were hypermethylated at promoters of both \textit{hMLH1} and \textit{hMSH2} (Fig. 3A). Similarly, 4 of 27 leukoplakia tissue samples (15%) and 5 of 27 leukoplakia tissue samples (18%) exhibited hypermethyl-

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**FIGURE 1.** Detection of promoter hypermethylation of the human mutL homolog 1 gene (\textit{hMLH1}) in primary head and neck squamous cell carcinoma tumors. (A) Genomic DNA from paired tumor (T) and normal (N) samples, as indicated above each lane, were digested separately with HpaII (H) and MspI (M), as indicated below each lane, and were amplified by polymerase chain reaction (PCR) analysis. Lane U indicates \textit{hMLH1} promoter amplification of undigested DNA; lane –ve, negative control; bp, base pairs. (B) Semiquantitative reverse transcriptase-PCR analysis of \textit{hMLH1} in the same paired tumor and normal tissues. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) expression was used as an internal control in all analyses. (C) The presence of \textit{hMLH1} protein in hypermethylation-negative samples is indicated by strong green immunohistochemical signal. Lane –ve indicates negative control immunostaining without incubation in primary antibody.
Alterations exclusively of the hMLH1 and hMSH2 promoters, respectively (Fig. 3B). Simultaneous promoter hypermethylation of both genes was observed in 8 of 27 leukoplakia samples (30%), and 10 of 27 leukoplakia samples (37%) did not show hypermethylation in the promoter of either gene (Fig. 3B). It is noteworthy that adjacent normal tissues from 15 of 28 hMLH1 Me-positive HNSCC tumors (54%) and from 3 of 16 hMSH2 Me-positive HNSCC tumors (19%), respectively, were also identified as hypermethylated at these promoters (Fig. 3C). Similarly, 12 of 17 normal tissue samples (71%) adjacent to Me-positive HNSCC tumors for both promoters also were hypermethylated (Fig. 3C). However, a similar analysis could not be done for the leukoplakia samples, because adjacent normal tissues were available from only 7 of the patients with leukoplakia.

**Mutation Analysis of hMLH1 and hMSH2 in HNSCC Tumors**

Sequence analysis of the mutational hotspot exons of hMLH1 (exons 1, 10, 13, 15, 16, and 19) and hMSH2 (exons 5, 7, 10, 12, 13, and 15) in 10 MIN-positive HNSCC tumors and 10 MIN-negative HNSCC tumors revealed no sequence variation in the samples. Hence, no mutations in hMLH1 and hMSH2 genes were identified in these limited numbers of tumors.
Comparison Between Promoter Hypermethylation Pattern with Clinicopathologic Parameters and Tobacco Habit

The correlations between hypermethylation pattern and age, sex, site, TNM classification, and tobacco habit are illustrated in Table 2. No statistically significant correlation was observed with site and TNM classification of the tumors. HNSCC tumors from stages I and II exhibited a high frequency of hypermethylation in both promoters that remained constant in the subsequent stages (stages III and IV). This observation suggests that hypermethylation of these promoters is probably an early event, and this hypermethylated state is maintained during the tumor progression. Involvement of promoter hypermethylation in early stages of HNSCC development was supported further by the observation that dysplastic leukoplakia tissue, which is considered a premalignant condition for a subtype of HNSCC tumors (oral cavity), also exhibited a high frequency of hypermethylation of both promoters. In analyzing the correlation between hMLH1 and hMSH2 promoter hypermethylation and tobacco habit, we observed that a significant number of patients with HNSCC and leukoplakia who had a tobacco habit had hypermethylation of both promoters \( (P = .001; \text{chi-square test}) \). It is noteworthy that all leukoplakia samples analyzed in the current study were from tobacco-addicted individuals, which may be the reason for the observation of high frequency of hypermethylation in these tissues. In addition, patients in the older age group (aged \( \geq 50 \) years) had a higher frequency of hypermethylation \( (P = .018; \text{chi-square test}) \) compared with patients in the younger age group (aged \( < 50 \) years). It also is noteworthy that patients with HNSCC who had a tobacco habit \( (n = 91) \) in the older age group (aged \( \geq 50 \) years; \( n = 55 \)) more frequently had hypermethylation of both promoters (Me-positive vs Me-negative, 37 patients vs 18 patients; \( P = .01; \text{chi-square test} \)) compared with younger patients (aged \( < 50 \) years; \( n = 36 \); Me-positive vs Me-negative, 15 patients vs 21 patients; \( P \) value not significant).

Association Between hMLH1 and hMSH2 Promoter Hypermethylation and MIN in HNSCC Tumors and Leukoplakia Samples

We determined the MIN status of a subset of 63 tumors, which were selected randomly from 123
HNSCC tumors, and all 27 leukoplakia tissues by using 8 microsatellite markers. In that analysis, we observed that 19 of 27 leukoplakia samples (70%) had instability of \( /C21 \) marker (MIN-positive), and 6 of 27 leukoplakia samples (22%) had instability of \( /C21 \) markers (MIN H). Figure 4A shows the distribution of methylation-positive and methylation-negative leukoplakia samples that exhibited instability in 0 markers (MIN-neg-ative), 1 marker (MIN L), and MIN H samples with adjacent normal tissue methylation (Me+ [T & N]) and without adjacent normal tissue methylation (Me+ [T only]).

HNSCC tumors, and all 27 leukoplakia tissues by using 8 microsatellite markers. In that analysis, we observed that 19 of 27 leukoplakia samples (70%) had instability of \( \geq 1 \) marker (MIN-positive), and 6 of 27 leukoplakia samples (22%) had instability of \( \geq 2 \) markers (MIN H). Figure 4A shows the distribution of methylation-positive and methylation-negative leukoplakia samples that exhibited instability in 0 markers (MIN-negative), 1 marker (MIN L), and \( \geq 2 \) markers (MIN H). It is apparent from Figure 4 that, with the increase in the level of instability (MIN-neg-ative to MIN L to MIN H), the relative proportion of leukoplakia samples that harbored promoter hypermethylation of \( hMLH1 \) and \( hMSH2 \) increased, although the increase was not statistically significant, probably because of the small number of samples.

Similar analysis in HNSCC tumors revealed that 38 of 63 tumor tissues (60%) were unstable at \( \geq 1 \) marker (MIN-positive), and 20 of 63 tumors (32%) were unstable in \( \geq 2 \) markers (MIN H). Figure 4B shows the distribution of methylation-positive and methylation-negative tumors among the MIN-negative, MIN L, and MIN H groups of HNSCC tumors. This analysis did not reveal any specific correlation between MIN and promoter hypermethylation. Careful analysis of Me-positive HNSCC tumors and leukoplakia samples combined together revealed the following noteworthy observations: Fifteen of 36 Me-positive patients (42%) showed hypermethylation exclusively in the affected tissues, whereas 21 of 36 Me-positive patients (58%) exhibited hypermethylation also at the adjacent normal tissues. Moreover, we observed that patients who had hypermethylation of both affected tissues and adjacent normal tissues mostly had the MIN-positive phenotype \( (P = .007; \) chi square test) (Fig. 4C). Conversely, affected tissues with no hypermethylation in the adjacent normal tissues exhibited the MIN-negative phenotype at greater frequency (Fig. 4C). Considering such distribution within the MIN H group, most patients who had hypermethylation of both the affected tissues and the adjacent normal tissues also had the MIN-positive phenotype \( (P = .03; \) chi square test) (Fig. 4C). Because we observed a correlation between tobacco habit and hypermethylation status of these 2 promoters, we compared MIN status with hypermethylation in normal tissues from patients who had a tobacco habit and demonstrated hypermethylation in normal tissues from patients who had a tobacco habit and demonstrated hypermethylation in their affected tissues. Table 3 shows that most MIN-positive samples had hypermethylation in adjacent normal tissues, whereas most MIN-negative samples were negative for normal tissue hypermethylation, and the distribution was statistically significant \( (P = .047; \) Fisher exact test). These results suggest that patients with HNSCC and leukoplakia who were addicted to tobacco and had \( hMLH1 \) and/or \( hMSH2 \) promoter hypermethylation in both tumor and normal tissues were more likely to have the MIN-positive phenotype. It is worth noting that 27 of 90 patients (30%) comprised of both MIN-positive dysplastic leukoplakia and HNSCC who were Me-negative were aged \( \leq 50 \) years (data not shown).

### Table 3

<table>
<thead>
<tr>
<th>Tumor class</th>
<th>Positive</th>
<th>Negative</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIN positive</td>
<td>10</td>
<td>3</td>
<td>.047</td>
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<tr>
<td>MIN negative</td>
<td>4</td>
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MIN indicates microsatellite instability.

* Fisher exact test.
DISCUSSION

The results of this study indicated that the inactivation of \textit{hMLH1} and \textit{hMSH2} in HNSCC was caused primarily by promoter hypermethylation and not genetic alterations. A high percentage of the HNSCC tumors (61 of 123 tumors; 50\%) and dysplastic leukoplakia samples (17 of 27 samples; 63\%) showed promoter hypermethylation of either or both \textit{hMLH1} and \textit{hMSH2}. In 33 of 130 patients (25\%), hypermethylation of these 2 promoters in the corresponding adjacent, normal tissues also was observed. All stages of tumor development, including precancerous dysplastic leukoplakia, exhibited hypermethylation of the promoter of these 2 genes. It is noteworthy that a higher incidence of promoter hypermethylation was observed in tobacco-addicted patients. Tobacco-addicted patients in the group aged \textgreater 50 years (ie, those with a longer period of carcinogen exposure) had a higher frequency of promoter hypermethylation than patients in the group aged \textless 50 years who had a tobacco habit or patients who had no tobacco habit at all.

Only few studies on \textit{hMLH1} and \textit{hMSH2} alterations have been reported in HNSCC, and its involvement in the generation of MIN phenotype in these tumors is not understood clearly.\textsuperscript{10} Consistent with other reports,\textsuperscript{10} we did not observe the mutation of these 2 genes in any tumors. However, the frequency of \textit{hMLH1} promoter hypermethylation observed in the current study was much greater than that reported in another study from India.\textsuperscript{48} To our knowledge, no report on \textit{hMSH2} promoter hypermethylation in Indian patients with HNSCC was available before the current study. Similar to the current results, high frequency of promoter hypermethylation of both \textit{hMLH1} and \textit{hMSH2} in HNSCC tumors has been reported in the Turkish population.\textsuperscript{41} High frequency of \textit{hMLH1} promoter hypermethylation also has been reported in the Polish population for laryngeal cancer\textsuperscript{38} and in the Korean population for oral cancer.\textsuperscript{39} We observed a positive correlation between tobacco addiction and the hypermethylation of the 2 genes. The high frequency of \textit{hMLH1} and \textit{hMSH2} promoter hypermethylation observed in the current study may have been caused by the greater numbers of patients reported with tobacco addiction. A similar positive correlation has been reported between tobacco addiction and hypermethylation of the promoter of other tumor-suppressor genes in oral and different cancers.\textsuperscript{49,50}

Consistent with our previous findings,\textsuperscript{7,42} we observed a high frequency of MIN in the tumor tissues from the patients with HNSCC. In addition, we also report a high frequency of MIN in dysplastic leukoplakia tissues. This suggests that the MIN phenotype is an early event in the development of HNSCC. We made a few interesting observations while correlating the MIN phenotype with the promoter hypermethylation of \textit{hMLH1} and \textit{hMSH2} in these tissues. There was a correlation between the level of MIN and the frequency of promoter hypermethylation in the dysplastic leukoplakia samples, but no such trend was observed for the HNSCC tumors. It is noteworthy that, considering only samples that were positive for promoter hypermethylation, MIN was associated with tobacco-addicted patients having hypermethylation of the affected tissues along with the adjacent normal tissues. Another intriguing observation was that 30\% of the promoter Me-negative tissues comprised of both dysplastic leukoplakia and HNSCC tumors also had a MIN-positive phenotype. Currently, the reason behind the MIN phenotype in these promoter Me-negative samples remains unclear. Wang et al. also made a similar observation in patients with HNSCC from a younger age group who had no tobacco habit.\textsuperscript{10} The finding that the promoter Me-negative but MIN-positive HNSCC tumors and dysplastic leukoplakia samples in the current study also were from patients ages \textless 50 years who had less exposure to tobacco suggests that the MIN phenotype demonstrated in these patients was caused by defects in some other pathways.

There are many reports of silencing tumor suppressor genes, even in normal tissues, by promoter hypermethylation. It has been observed that histologically normal tissues adjacent to tumors and premalignant lesions contain high levels of methylation of several genes, suggesting that methylation is an early event.\textsuperscript{51,52} It is noteworthy that such phenomena are more pronounced in individuals with a tobacco habit.\textsuperscript{52,53} Recently, genome-wide analysis of de novo methylation in cancer cells suggested that an instructive mechanism may be operating in incipient tumor tissues.\textsuperscript{54} Thus, it may be hypothesized that individuals who have a tobacco habit for a considerable period could sustain promoter hypermethylation of the genes involved in the MMR pathway in their normal head and neck squamous epithelium, leading to the gradual accumulation of mutations and, eventually, selection of the rapidly growing malignant cells. Future experiments that directly demonstrate the tobacco-induced methylation of mismatch-repair pathway genes and the consequent development of the MIN phenotype would validate this hypothesis.

REFERENCES


