Epigenetic Events of Disease Progression in Head and Neck Squamous Cell Carcinoma

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Objective: To examine the promoter methylation status of the 22 cancer genes and their contribution to disease progression in 6 head and neck squamous cell carcinoma (HNSCC) cell lines.

Design: A panel of 41 gene probes, designed to interrogate 35 unique genes with known associations to cancer including HNSCC, was interrogated for alterations in gene copy number and aberrant methylation status (22 genes) using the methylation-specific multiplex ligation-dependent probe amplification assay.

Subjects: Primary (A) and recurrent or metastatic (B) HNSCC cell lines UMSCC-11A/11B, UMSCC-17A/17B, and UMSCC-81A/81B are described.

Results: Nine genes, TIMP3, APC, KLK10, TP73, CDH13, IGSF4, FHIT, ESR1, and DAPK1, were aberrantly methylated. The most frequently hypermethylated genes were APC and IGSF4, observed in 3 of 6 cell lines, and TP73 and DAPK1, observed in 2 of 6. For KLK10 and IGSF4, TIMP3 and FHIT, and TP73, in UMSCC-11B, UMSCC-17B, and UMSCC-81B, respectively, promoter hypermethylation was a disease progression event, indicating complete abrogation of tumor suppressor function for KLK10, IGSF4, and TIMP3 and gene silencing of 1 of 2 copies of TP73. Hypermethylation of IGSF4, TP73, CDH13, ESR1, DAPK1, and APC were primary events in UMSCC-17A.

Conclusions: Gene silencing through promoter hypermethylation was observed in 5 of 6 cell lines and contributed to primary and progressive events in HNSCC. In addition to genetic alterations of gains and losses, epigenetic events appear to further undermine a destabilized genomic repertoire in HNSCC.

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genes) related to head and neck cancer to examine the contribution of aberrant methylation in the context of changes in gene copy number. To accurately identify epigenetic (gene silencing via promoter hypermethylation) and non-epigenetic (gene loss and gain) alterations, we used the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay.27

**METHODS**

**HNSCC CELL LINES AND DNA EXTRACTION**

Tumor sample acquisition, tissue culture, and karyotype analysis methods have been detailed elsewhere.28,29 The tumor samples were derived as follows: the UM-SCC-11A and UM-SCC-11B cell lines were obtained from tumor tissue from the primary tumor site (larynx) before and after chemotherapy, respectively. The UM-SCC-17A (supraglottis) and UM-SCC-17B (neck soft tissue) cell lines were taken from tumor tissue simultaneously from primary (A) and metastatic (B) sites. The UM-SCC-81A (larynx) and UM-SCC-81B (tonsillar pillar) cell lines were derived from tumor tissue obtained from primary (A) and second primary (B) sites.

DNA was extracted using the QIAamp Kit (Qiagen Inc, Chatsworth, Calif) at passages 83 and 90 for UM-SCC-11A and UM-SCC-11B, respectively; 138 and 184 for UM-SCC-17A and UM-SCC-17B, respectively; and 24 and 129 for UM-SCC-81A and UM-SCC-81B, respectively.

**THE MS-MLPA ASSAY**

The MS-MLPA assay allows for the simultaneous detection of changes in methylation status as well as copy number changes of approximately 41 different DNA sequences in a single reaction requiring only 20 ng of human DNA.27 The standard use of the technique to observe quantitative changes in copy number has been outlined in other studies.30,31 In MS-MLPA, for 26 of the 41 probes, the recognition sequence detected by the MLPA probe is contained within a restriction site for the methyl-sensitive enzyme, HhaI (Figure 1). The remaining 15 genes lack an HhaI site and serve as undigested controls.

The 41 gene probe panel (Figure 2) interrogates 36 unique genes implicated in cancer including HNSCC for losses and gains in a separate reaction in the absence of the methyl-sensitive enzyme HhaI. Because there are 2 probes each for VHL, CDKN2A, BRCA1, and BRCA2 and 3 probes for MLH1, a normal control DNA sample will generate 41 individual peaks in the absence of a HhaI recognition site (Figure 3). A concurrently run reaction with the 41 gene probe set in the presence of HhaI is designed to detect aberrant promoter hypermethylation by taking advantage of an HhaI site in 22 of the 35 unique genes (note that 1 of the 2 BRCA1 and BRCA2 probes is designed to recognize a region outside the HhaI recognition site) (Figure 2). On digestion of the sample DNA with HhaI, probes designed to recognize HhaI sites within unmethylated regions will not generate a signal because these sequences have become cut by HhaI and cannot bind to the probe. Conversely, an MLPA probe will bind to an intact methylated site, spared by HhaI, and generate an amplification signal, producing 15 separate peaks in a normal control DNA sample (Figure 4).

To detect both aberrant methylation and changes in copy number, each sample requires 2 MLPA reactions. Approximately 20 ng of DNA are used for each of the 2 reactions, one without HhaI and the other with HhaI. Briefly, DNA from normal control subjects (male and female) and DNA from HNSCC cell lines for each of the 2 MLPA reactions is diluted with water to a total volume of 5 µL. DNA is denatured and fragmented by heating for 5 minutes at 98°C in a thermocycler. Binary MLPA probes are added and allowed to hybridize to their targets during a 16-hour incubation at 60°C. Dilution buffer and a special ligase-65 enzyme (MRC-Holland, Amsterdam, the Netherlands) are added to the vial. During 15-minute incubation at 60°C, the 2 parts of a probe can be ligated to each other and become an amplifiable molecule provided that the complementary sequence is present in the sample (Figure 1). This is followed by the addition of polymerase chain reaction (PCR) primers, deoxynucleotide triphosphates, and Taq polymerase, followed by the following cycles: 1 cycle at 95°C for 1 minute; 10 cycles at 95°C for 30 seconds, 70°C for 30 seconds, and 72°C for 1 minute; and 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. All (ligated) probes are amplified by the same primer pair, one of which is tagged with a fluorescent dye. The relative amounts of PCR product obtained reflect the relative amounts of ligated probes at the start of the PCR reaction. Amplification products are analyzed on an ABI 310/ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif), and the products are normalized and quantified.
INTERPRETATION

To quantitative gene copy number, loss or gain, is determined through a process of normalization. The latter address variations in the surface area of a peak (intensity) due to fluctuations in the assay run such as amount of DNA, ploidy variations, and PCR conditions. Briefly, the peak area for each probe is expressed as a percentage of the total surface area of all peaks of a sample in an assay run (process of normalization, Figure 3). The relative copy number for each probe is obtained as a ratio of the normalized value for each locus (peak) of the sample to that of the normal control. A difference is significant only if the ratio is less than 0.7 (loss) or higher than 1.3 (gain). Complete loss or 0 copies is indicated by absence of a peak for that particular locus (illustrated for CDKN2A in UMSCC-11A; Figure 3). A relative copy number of 2 is considered normal, 1 or 0 copies is considered loss, and 3 copies or more is considered gain (illustrated for MEN1 in UMSCC-11A and GSTP1 in UMSCC-11A and UMSCC-11B; Figure 5).

Figure 2. Multiplex ligation-dependent probe amplification copy number without Hha. bp Indicates base pair; PCR, polymerase chain reaction.
Aberrant methylation is identified as the appearance of a signal peak that is otherwise absent in normal DNA samples (Figure 4). Probe signals to methylated sequences yield peaks in the presence of HhaI that are much stronger (higher) than those generated in the absence of HhaI because there are fewer regions to amplify after HhaI digestion (15 sequences vs 41 sequences, Figure 4 and Figure 3, respectively). The latter observation has been validated for the same control DNA samples in more than 8 separate reactions with HhaI and without HhaI. Based on the latter, for aberrantly methylated peaks, when the gene copy number is 1 or if both copies of a gene become hypermethylated, the peaks for those genes are invariably either higher or of equal height to its corresponding peak generated in the absence of HhaI (Figures 6, 7, and 8). Because aberrantly methylated gene loci generate signals that are not present in normal controls, quantification of gene copy number that becomes aberrantly methylated cannot be determined through the usual normalization process. Therefore, to quantify whether one, both, or more copies of a specific gene locus becomes aberrantly hypermethylated, a mathematical algorithm devised to obtain the percentage ratio of the peak height of a probe signal generated in the presence of HhaI, which is absent in the normal control, to that of its counterpart peak generated in the absence of HhaI (Figures 6, 7, and 8).

\[
\text{Percentage Ratio} = \frac{\text{Peak Height (Methylated With HhaI)}}{\text{Peak Height (Unmethylated, Without HhaI)}} \times 100
\]

In normal control DNA samples, we have calculated this percentage ratio to be greater than 100%. Based on this, a statistical algorithm determined the following cutoff percentage ratios: 60% or higher indicates methylation of either the sole copy or both copies of a gene, illustrated for KLK10 (single copy) and IGSF4 (2 copies); lower than 60% but 30% or higher as methylation of only 1 of the 2 copies for a particular gene, illustrated for TP73 in UMSCC-17A (Figure 7) and UMSCC-81B (Figure 8), CDH13 and ERS1 in UMSCC-17A, and FHIT in UMSCC-17B (Figure 7). When the percentage ratio is lower than 30%, it is relegated to background noise.

**RESULTS**

Gain and loss of gene loci were observed in all of the 6 cell lines (Figure 2). Common losses included CDKN2A, CDKN2B, and BCL2 (illustrated for UMSCC-11A/B; Figure 3 and Figure 2). Homozygous loss was noted for CDKN2A in UMSCC-11A and UMSCC-81A and for CDKN2B in UMSCC-81A (Figure 2). Common gains were noted at the 11q13 region for MEN1 and GSTP1 (illustrated for UMSCC-11A/B; Figure 4 and Figure 2).

Aberrant methylation was observed for 9 genes (Table and Figures 6, 7, and 8) in 5 of 6 HNSCC cell lines based on the appearance of signal peaks not present in the control, indicating that the promoter regions of these genes were hypermethylated and, thus, able to escape digestion by the methyl-sensitive HhaI enzyme. The 9 genes that were hypermethylated are as follows: APC (5q21), TIMP3 (22q12.3), KLK10 (19q13.3), TP73 (1p36), CDH13 (16q24.2), IGSF4...
Figure 4. Methylation-specific multiplex ligation-dependent probe amplification indicated aberrant methylation peaks for KLK10 and IGSF4 in UMSCC-11B. No aberrant methylation was observed in UMSCC-11A. PCR indicates polymerase chain reaction.

Figure 5. Multiplex ligation-dependent probe amplification gain of gene loci. Note gain of MEN1 (3 copies) and GSTP1 (5 copies) in UMSCC-11A. UMSCC-11B also indicated gain of GSTP1 (3 copies). Additional gains are illustrated in Figure 2. PCR indicates polymerase chain reaction.
(11q23), ESR1 (6q25.1), FHIT (3p14.2), and DAPK1 (9q34.1).

The most frequently hypermethylated genes were APC and IGSF4 observed in 3 of 6 cell lines, and TP73, ESR1, and DAPK1 observed in 2 of 6 cell lines. The UMSCC-11A cell line did not show aberrant methylation (Figure 4 and Figure 6). The UMSCC-11B cell line showed aberrant methylation of KLK10 and IGSF4 (Figure 4 and Figure 6), silencing the sole KLK10 copy and both copies of IGSF4 (Figures 2, 5, and 6; and Table). Hypermethylation of IGSF4, DAPK1, ESR1, and APC were present in both UMSCC-17A and UMSCC-17B (Figure 7). In UMSCC-17A, aberrant methylation was indicated for both copies of APC and 1 of 2 copies of IGSF4 in both UMSCC-17A and UMSCC-17B (Figure 7 and Table). DAPK1, present in 1 copy in UMSCC-17A, was hypermethylated, as were both copies in UMSCC-17B (Figure 7 and Table). Both copies of ESR1 were hypermethylated in UMSCC-17B, whereas UMSCC-17A had hypermethylation of only 1 of the 2 ESR1 copies. Hypermethylation of 1 of the 2 copies of CDH13 occurred only in UMSCC-17A, and only UMSCC-17B showed hypermethylation of 1 of 2 copies of FHIT and the sole TIMP3 copy (Figure 7 and Table). Both copies of APC in UMSCC-81A and 1 of 2 copies of TP73 in UMSCC-81B were hypermethylated (Figure 8 and Table).

Epigenetic mechanisms involve DNA and histone modifications resulting in the heritable silencing of genes without a change in their coding sequence. The major form of epigenetic information in mammalian cells is DNA methylation or the covalent addition of a methyl group to the fifth position of cytosine within CpG dinucleotides predominantly located in the promoter region, which typically remain unmethylated in normal cells. The consequence of CpG island hypermethylation, especially for those islands associated with TSG promoters, is the loss of TSG function, which contributes to tumorigenesis. Gene silencing, as a consequence of promoter hypermethylation, can be partially relieved by demethylation of the promoter region. Recent work has revealed that DNA methylation is an important player in many processes, including DNA repair, genome instability, and regulation of chromatin structure.

In HNSCC, methylation of p16, RARβ, and MGMT suggested early events, with incidences of methylation in HNSCC cell lines and primary tumors being similar. Promoter hypermethylation pattern of the p16, MGMT, GSTP1, and DAPK genes have been used as molecular markers for cancer cell detection in the paired serum and tumor DNA, and almost half of the patients with...
HNSCC with methylated tumors were found to display these epigenetic changes in the paired serum.22

In our study, we identified genetic and epigenetic alterations of disease progression using a candidate gene approach. Genomic alterations of gains and losses of gene loci were observed in all of the 6 cell lines and confirmed previously reported31 common losses of CDKN2A, CDKN2B, and BCL2. Common gains were noted at the 11q13 region for MEN1 and GSTP1. Epigenetic alterations of aberrantly methylated promoter regions were indicated for 9 genes, APC (5q21), TIMP3 (22q12.3), KLK10 (19q13.3), TP73 (1p36), CDH13 (16q24.2), IGSF4 (11q23), ESR1 (6q25.1), FHIT (3p14.2), and DAPK1 (9q34.1), all of which have been identified both as TSGs and as being implicated in malignancy,35-40 though their relationship to HNSCC is still relatively unknown.

The most frequently hypermethylated genes were APC and IGSF4, observed in 3 of 6 cell lines, and TP73 and DAPK1, observed in 2 of 6 cell lines. For KLK10 and IGSF4, TIMP3 and FHIT, and TP73, in UMSCC-11B, UMSCC-17B, and UMSCC-81B, respectively, promoter hypermethylation was a disease progression event, indicating complete abrogation of tumor suppressor function for KLK10, IGSF4, and TIMP3 and gene silencing of 1 of 2 copies of TP73. Hypermethylation of IGSF4, TP73, CDH13, ESR1, DAPK1, and APC were primary events in UMSCC-17A. Hypermethylation of APC was a frequent finding in esophageal, cardiac, and gastric adenocarcinomas41,42 and in Barrett metaplasia and dysplasia.42 In the latter, hypermethylation of APC, CDKN2A, and ESR1 were usually found in a large contiguous field, suggesting either a concerted methylation change associated with metaplasia or a clonal expansion of cells with abnormal hypermethylation. Our findings reiterate APC and ESR1 as primary events in HNSCC.

IGSF4 is a novel immunoglobulin-like intercellular adhesion molecule first characterized as a tumor suppressor of non–small cell lung cancer and termed TSLC1,43,44 in which silencing was primarily achieved by allelic loss and promoter methylation. TSLC1 is located at the region of 11q23.2 and spans more than 300 kilobases.44 Promoter hypermethylation of TSLC1 has been reported in nasopharyngeal carcinomas.45 In esophageal squamous cell carcinomas, TSLC1 expression or loss correlated with the promoter methylation status and TSLC1 expression could be restored by a demethylating agent in certain cell lines.46

In nasopharyngeal carcinomas (NPC), a high frequency of promoter hypermethylation of multiple cancer-related genes implicates methylation as the most common mechanism of inactivating genes.46 The incidence of promoter methylation in NPC samples was reported as 84% for RASSF1A, 80% for RARβ2, 76% for DAPK, 46%
The epigenetic silencing of multiple cancer-related genes, including RASSF1A, RARβ2, DAPK, p16, p15, p14, and MGMT, and 3% for GSTP1.\(^47\) The epigenetic silencing of multiple cancer-related genes, including RASSF1A, RARβ2, DAPK, p16, p15, p14, and MGMT implicate corresponding disruption of the Ras signaling pathway, the retinoid signaling pathway, the metastasis-related process, cell cycle, p53 network, and DNA repair, respectively, in NPC.\(^47\)

In esophageal adenocarcinoma a positive methylation status for multiple genes predicts poor prognosis. Methylation frequencies of the genes analyzed were APC, 68%; E-cadherin (CDH1), 66%; MGMT, 56%; ER, 51%; p16, 39%; DAPK, 19%; and TIMP3, 19%.\(^48\) In patients with head and neck cancer, DAPK, which produces a protein that acts as a positive mediator of apoptosis induced by

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Abbreviations: n, copy number without HhaI; m, methylated copy number after HhaI digestion.
interferon γ, showed a positive correlation between methylation and the presence of lymph node metastases. 22

Hypermethylation of TP73, also reported in nasopharyngeal carcinomas with a frequency of 20%, was part of a methylation gene profile that included CDH1 (50%), CDKN2B (50%), THBS1 (50%), RASSF1A (46%), MLH1 (40%), MGMT (28%), CDKN2A (23%), caspase-8 (7%), ARF (3%), and VHL (0%). 20 Similar to TP53, TP73 is another gene that maps to the short arm of chromosome 17 and shares a strong homology to p53. 30 Overexpression of the p73 protein has been shown to activate the transcription of p53-responsive genes and inhibit cell growth in a p53-like manner by inducing apoptosis 30-32, however, unlike TP53, no mutation of TP73 has been found in human tumors.

In our study, gene silencing through promoter hypermethylation was observed in 5 of 6 cell lines and contributed to primary and progressive events in HNSCC. Homozygous loss of CDKN2A obviated gene-silencing events for p14 and p16, and UMSCC-81A and for p15 (CDKN2B) in UMSCC-81A. Of the 9 genes, APC, TIMP3, KLK10, TP73, CDH1, IGSF4, ESR1, FHIT, and DAPK, aberrant methylation has been reported in squamous cell carcinomas for all except KLK10 and FHIT.

Our approach using the MS-MLPA assay, 32 a modification of the novel MLPA assay, permitted identification of genomic alterations of losses and gains in addition to assessing epigenetic status. This method works very well with DNA from formalin-fixed paraffin tumor tissue and precursor lesions as well as for DNA from fresh frozen tumor samples. 21 The latter precludes some degree of normal tissue admixture and further reduces methylation signatures as positive signals unlike loss of heterozygosity, which requires a relatively pure amount of tumor DNA. Thus, progressive alterations of epigenetic events, as well as genic gains and losses, appear to underlie a molecular continuum in HNSCC progression.

In summary, genic and epigenetic events appear to further undermine a destabilized genomic repertoire in HNSCC. Successful integration of information from these approaches should provide a more complete picture of the ways in which gene deregulation occurs in solid tumors, how multigene deregulation leads to specific tumor phenotypes, and how this information can be used to improve the treatment of patients with cancer.

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