Methylation of Multiple Genes as Diagnostic and Therapeutic Markers in Primary Head and Neck Squamous Cell Carcinoma

Kangmei Chen, MD; Raja Sawhney, MD; Mumtaz Khan, MD; Michael S. Benninger, MD; Zizheng Hou, MD; Seema Sethi, MD; Josena K. Stephen, MD; Maria J. Worsham, PhD

Objective: To examine epigenetic events of aberrant promoter methylation as diagnostic markers in primary head and neck squamous cell carcinoma using a novel multigene approach. Promoter methylation-mediated silencing is a hallmark of several established tumor suppressor genes. Changes in DNA methylation have been reported to occur early in carcinogenesis and therefore are potentially important early indicators of existing disease.

Design: A multicandidate gene probe panel interrogated DNA for aberrant methylation status in 22 cancer genes using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay. Aberrant promoter hypermethylation was confirmed using methylation-specific polymerase chain reaction after bisulfite treatment.

Setting: Primary care medical center.

Subjects: We examined fresh-frozen primary head and neck tumor specimens from 28 patients, including 21 late-stage (19 stage IV and 2 stage III) and 7 early-stage (6 stage II and 1 stage I) tumors.

Results: Promoter hypermethylation was observed in 14 of the 28 patients (50%). Genes for RARB, APC, and CHFR were most frequently hypermethylated, occurring in 11 (39%) for RARB, 7 (25%) for CHFR, and 6 (21%) for APC. Aberrant methylation of CHFR was solely a stage IV event. Methylation-specific polymerase chain reaction after bisulfite treatment with conventional and real-time polymerase chain reaction confirmed aberrant methylation for RARB and CHFR.

Conclusions: Promoter methylation profiling of primary head and neck squamous cell carcinoma using multiple target genes identified RARB, APC, and CHFR as frequent epigenetic events. The clinical implications of these genes as diagnostic and treatment biomarkers are highly relevant as attractive targets for cancer therapy, given the reversible nature of epigenetic gene silencing.


HEAD AND NECK SQUAMOUS cell carcinoma (HNSCC) carries a high mortality rate, despite advances in chemotherapy and radiation therapy. This is mainly because the disease, at the morphologic and genetic levels, is highly heterogeneous. A current shortcoming in the prognosis and treatment of HNSCC is a lack of methods that adequately address the complexity and diversity of the disease.

Historically, the molecular pathogenesis of cancer has been teased out 1 gene at a time. The development of several new high-throughput analytical methods for the analysis of DNA, messenger RNA, and proteins within a cell have permitted a more detailed molecular characterization of the cancer genome. In HNSCC, recent comprehensive high-throughput methods have underscored the contribution of genetic and epigenetic events, often working together, in the development and progression of HNSCC.

Epigenetic mechanisms involve DNA and histone modifications, resulting in the heritable silencing of genes without a change in their coding sequence. Gene transcriptional inactivation via hypermethylation at the CpG islands within the promoter regions is an important mechanism. In HNSCC, methylation of p16, RARB, and MGMT suggested early events, with incidences of methylation in HNSCC cell lines and primary tumors being similar.

Aberrant DNA methylation patterns in HNSCC have served as powerful diagnos-
tic, sensitive detection, and risk assessment biomarkers. The promoter hypermethylation patterns of \( p16 \), \( MGMT \), \( GSTP1 \), and \( DAPK \) have been used as molecular markers for cancer cell detection in serum DNA, and almost half of the patients with HNSCC and methylated tumors were found to display these epigenetic changes in the paired serum.9

Most of the published epigenetic data in HNSCC comes from methylation-specific polymerase chain reaction (MSP) after bisulfite treatment.16 The success of MSP has been attributed to its increased sensitivity; however, it generally relies on a preselected number of genes, assessed 1 gene at a time, compared with high-throughput microarray-based methylation analysis17 and multicandidate gene meth-

### Table 1. HNSCC Study Cohort

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; MD, moderately differentiated; NA, not available; PD, poorly differentiated; WD, well differentiated.

![Figure 1](Methylation-specific multiplex ligation-dependent probe amplification (MLPA) assay, described by Worsham et al.14 PCR indicates polymerase chain reaction.)
ylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) applications.

We evaluated aberrant methylation status in 28 primary HNSCCs using a multicandidate gene assay (MS-MLPA) and confirmed aberrant promoter methylation using conventional MSP (gel electrophoresis separation of products) and real-time polymerase chain reaction (PCR) after bisulfite treatment.

METHODS

SUBJECTS

The study cohort consisted of fresh-frozen, untreated primary HNSCC specimens from 28 patients undergoing surgery as the primary treatment. The patients were selected serially during a 32-month period (April 9, 2003, to January 4, 2006). The age at tumor presentation ranged from 43 to 85 years. Nineteen of the 28 patients (68%) were white; 7 (25%), African American; 1 (4%), Hispanic; and 1 (4%), East Indian. Twenty-three patients were men and 5 were women. Most tumors (n=16) were located in the larynx; 9 were in the oral cavity, including 6 tongue sites; 2 were in the pyriform sinus; and 1 was in the pharynx. Twenty-one tumors were late stage (19 stage IV and 2 stage III) and 7 were early stage (6 stage II and 1 stage I). Histologically, most tumors were moderately differentiated with evidence of keratinization (Table 1). Tissue was collected according to institutional review board protocols.

DNA EXTRACTION

Fresh-frozen primary HNSCC tissue specimens were cut into small pieces and homogenized (VirTis homogenizer; VirTis Inc, Gardiner, New York) in a 1.5-mL microcentrifuge tube containing 100 µL of phosphate-buffered saline solution before DNA extraction. The DNA from the tissue and corresponding peripheral blood lymphocytes were extracted using a DNA purification kit (QIAamp DNA Mini Kit; Qiagen Inc, Valencia, California).

DNA ANALYSIS

The DNA was interrogated for alterations in aberrant methylation status using the novel, multigene (22 genes) MS-MLPA assay and confirmed with results of conventional MSP (gel electrophoresis separation of products) and real-time PCR after bisulfite treatment.

MS-MLPA ASSAY

The MS-MLPA assay allows for the detection of changes in methylation status and in copy number of 41 different genes in a single reaction and has been previously described (Figure 1). The standard use of the MLPA technique to observe quantitative changes in copy number has been outlined in other studies. Briefly, the MS-MLPA 41-gene probe panel (ME001; MRC-Holland, Amsterdam, the Netherlands) interrogates 35 unique genes related to head and neck cancer, 22 of which are tumor suppressor genes designed to detect aberrant promoter hypermethylation in the presence of HhaI by taking advantage of an HhaI site in 22 of the 35 unique genes (Table 2). The remaining 15 genes lack an HhaI site and serve as undigested controls. Because there are 2 probes each for MLH1, RASSF1, TNFRSF1A, and BRCA2, a normal (control) DNA sample will generate 41 individual peaks in the absence of HhaI (Figure 2A and Figure 3A). On digestion of the sample DNA with HhaI, probes that recognize the unmethylated regions will not generate a signal because these sequences have become cut by HhaI and cannot bind to the probe (Figure 1). Conversely, an MLPA probe will bind to an intact methylated site, spared by HhaI, and generate an amplification signal, producing in a mathematical algorithm cutoff ratio for peak height of 30% or higher.

BISULFITE MODIFICATION AND MSP ASSAY

We used a DNA methylation kit (EZ kit; Zymo Research, Orange, California) to modify genomic DNA (150 ng) from tumor and control universal methylated and control unmethylated DNA.
During the process, methylated DNA is protected and unmethylated cytosine is converted to uracil. The modified DNA served as a template using primers specific for the methylated or the modified unmethylated sequences. Appropriate negative and positive controls were included in each PCR.

The DNA methylation patterns in the CpG islands of RARB using conventional MSP were obtained using a commercially available kit (WIZ Amplification Kit; Chemicon International, Inc, Temecula, California). For PCR amplification, 3 µL of bisulfite-modified DNA was added in a final volume of 25-µL PCR mix containing 1× PCR buffer, 2.5 mM deoxynucleotide triphosphate, 1 mM magnesium chloride, 1 U Amp gold Taq DNA polymerase, and 0.3 µM primer followed by 40 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 1 minute. The PCR generated an 84–base pair (bp) methylated product, a 94-bp unmethylated product, and a 92-bp wild-type PCR product (Figure 4).

In addition, real-time PCR was performed with the same primers and controls with the CpG WIZ Amplification kit with 2 µL of modified DNA using a commercially available kit (LightCycler FastStart DNA Master SYBR green 1; Roche Applied Science, Indianapolis, Indiana) (Figure 5). For CHFR, using conventional MSP, methylation-specific primers included sense primer 5’TCTCGTACTCGTAGGCGAC and antisense primer 5’TGGATTAACATACGAGCG. Unmethylated DNA-specific primers included sense primer 5’TCTCGTACTCGTAGGCGAC and antisense primer 5’ACAATTAACATACGAGCG. The PCR amplification consisted of 40 cycles at 95°C for 45 seconds, 57°C for 45 seconds, and 72°C for 1 minute for methylation primers and 95°C for 45 seconds, 50°C for 45 seconds, and 70°C for 1 minute for unmethylation primers. The CHFR PCR product size generated by methylation and unmethylation primers was 155 bp (Figure 6).

The resultant PCR products of conventional MSP were separated on 2% agarose gel stained with ethidium bromide and visualized under UV illumination (Figures 4 and 6).

Results

Of the 28 primary HNSCCs, promoter hypermethylation by MS-MLPA was identified in 7 genes in 13 cases (Table 3). Conventional and real-time PCR MSP analyses confirmed RARB and CHFR MS-MLPA results in all occurrences. In addition, MSP detected methylation of CHFR in case 1 and RARB in case 4, which were not observed by MS-MLPA (Table 3 and Figures 4-6), bringing the total number of cases with at least 1 aberrantly methylated gene to 14 (Table 3). Promoter hypermethylation of RARB and the adenomatosis polyposis coli gene (APC) was observed in early and late stage tumors. The most frequently hypermethylated gene was RARB, in 11 of 28 cases (39%), including 8 stage IV, 2 stage III, and 1 stage II HNSCCs. Aberrant methylation of APC occurred in 6 of 28 cases (21%), including 2 stage II, 1 stage III, and 3 stage IV HNSCCs (Table 3). Aberrant methyl-
ation of CHFR was solely a stage IV event, occurring in 7 of 28 cases (25%). Promoter hypermethylation of CDKN2B and DAPK1 was observed in 1 stage II tumor (case 16) (Table 3) and was absent in late-stage HNSCC.

**COMMENT**

Identification of aberrant methylation in promoter regions of cancer genes yields important tumor biomarkers, underscoring a role for epigenetics in tumor pathogenesis. Methylation of DNA serves to assess the epigenetic state of a locus, is preserved in purified isolated DNA, and is amenable to PCR-based methylation assays using whole genomic DNA from fresh-frozen tissue, cell lines, and DNA from formalin-fixed paraffin tumor tissue. The last precludes some degree of normal tissue admixture and further underscores methylation signatures as positive signals (Figures 4 and 6), unlike loss of heterozygosity, which requires a relatively pure amount of tumor DNA.

The MS-MLPA promoter methylation profiling of 22 tumor suppressor genes, many of which are involved in head and neck cancer, identified RARB, APC, and CHFR as frequent epigenetic events. The clinical implications of these genes as diagnosis and treatment biomarkers in HNSCC are highly relevant.

The regulation of cell growth and differentiation of normal, premalignant, and malignant cells by retinoids, known to possess antiproliferative, differentiative, immunomodulatory, and apoptosis-inducing properties, are thought to result from their direct and indirect effects on gene expression. These properties are mediated by the nuclear receptors, including retinoic acid receptors alpha, beta, and gamma and retinoid X receptors alpha, beta, and gamma.

The direct role of RARB in regulating gene expression and its retinoid-mediated properties as therapeutic targets in HNSCC have been well documented. Treatment with certain retinoids have been shown to suppress premalignant oral lesions and prevent the development of second primary cancers among patients with head and neck and lung cancers. Decreased expression of RARB has been associated with increased keratinizing squa-
promoter hypermethylation in RARB was the most frequent, occurring in 11 of 28 specimens (39%) of the HNSCC study cohort characterized histologically by moderately differentiated squamous cell carcinoma with evidence of keratinization.

Chromosomal segregation at mitosis is preceded by a series of steps, including condensation of the chromosome and separation of the centrosome, chromosomal alignment, and sister-chromatid separation.21 To ensure fidelity of the replicated genetic material, mitosis is carefully choreographed and monitored by several checkpoint systems.30,31 Missteps in any one of these processes can result in aneuploidy or genetic instability, setting off any number of deleterious events such as unregulated cell growth, leading to neoplastic transformation and tumor progression. A new mitotic checkpoint that delays chromosomal condensation in response to mitotic stress induced by paclitaxel or nocodazole was shown to involve a gene (named checkpoint with FHA and ring finger [CHFR]) associated with delaying prophase in human cells.32 Mitotic checkpoint function is impaired in a significant proportion of human cancer cell lines,33,34 often by genetic alterations,33,35,36 supporting a possible link between the impaired mitotic checkpoint and oncogenesis.

The examination of the prevalence and pattern of CHFR inactivation in human tumors found CpG methylation–dependent silencing of CHFR expression in 40% of primary colorectal cancers, 53% of colorectal adenomas, and 30% of primary head and neck cancers.37 Treatment with the methyltransferase inhibitor 5-aza-2’-deoxycytidine induced reexpression of CHFR.38 In addition, because cancer cells that lack CHFR expression have shown to be more susceptible to the microtubule inhibitor paclitaxel,37 silencing of CHFR by methylation can serve as a marker for predicting sensitivity to particular chemotherapeutic agents.

In this pilot cohort of 28 HNSCCs, we found aberrant methylation of CHFR in only stage IV tumors (7 of 19). Confirmation of this finding in a larger cohort would support a role of CHFR promoter hypermethylation in tumor progression with potential utility as an epigenetic biomarker of late-stage disease.

The tumor suppressor gene APC was originally implicated in colon cancer. Promoter hypermethylation of APC has been reported in 25% of oral cancers38 and in Barrett metaplasia and dysplasia.39 In our cohort, APC, like RARB, was hypermethylated in early- and late-stage tumors, suggesting DNA methylation of APC and RARB as earlier epigenetic events when compared with methylation of CHFR.

In this study, the objective of using MSP was primarily to serve as a validation of the novel, candidate multigene MS-MLPA panel to interrogate and detect promoter hypermethylation in multiple genes simultaneously. Results of MSP for CHFR and RARB (although not performed for APC) concurred with our MS-MLPA results. Aberrant methylation of CHFR in case 1 and RARB in case 4 was detected by MSP but not scored by MS-MLPA. The latter observation is likely owing to the exclusion of these MS-MLPA methylation peaks because they did not meet the cutoff ratio of 30% or greater set by the mathematical algorithm designed to distinguish legitimate methylation peaks from background noise.14 Although a dis-
In summary, promoter methylation profiling of a pilot primary HNSCC cohort that targeted multiple cancer genes identified RARB, APC, and CHFR as frequent epigenetic events. These preliminary findings of promoter hypermethylation of RARB and APC in early- and late-stage tumors and of CHFR by MS-MLPA and MSP assays only in late-stage tumors appear to suggest an epigenetic progression continuum, with CHFR as a late event and a putative diagnostic biomarker for late-stage disease. Validation of these findings in larger HNSCC cohorts would further support these genes as relevant epigenetic biomarkers of cancer therapy given the reversible nature of epigenetic gene silencing.

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Correspondence: Maria J. Worsham, PhD, Department of Otolaryngology—Head and Neck Surgery, Henry Ford Health System, 1 Ford Pl, Ste 1D, Detroit, MI 48202 (mworsha1@hfhs.org).

Author Contributions: Dr Worsham had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Chen and Worsham. Acquisition of data: Chen, Sawhney, Benninger, Hou, Sethi, Stephen, and Worsham. Analysis and interpretation of data: Chen, Sawhney, Benninger, Hou, Sethi, Stephen, and Worsham. Drafting of the manuscript: Chen, Khan, and Worsham. Critical revision of the manuscript for important intellectual content: Chen, Sawhney, Benninger, Hou, Sethi, Stephen, and Worsham. Statistical analysis: Worsham. Obtained funding: Worsham. Administrative, technical, and material support: Chen, Sawhney, Khan, Benninger, Hou, Sethi, Stephen, and Worsham. Study supervision: Worsham.

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