Epigenetic Inactivation of RASSF1A in Head and Neck Cancer

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INTRODUCTION

HNSCC occurs in >50,000 Americans each year, and the incidence of this type of tumor is expected to rise as a result of the increasing number of female and adolescent smokers. Despite the great emphasis on early diagnosis and the efforts to improve surgical and radiation treatment, ~50% of HNSCC patients do not survive for >5 years after diagnosis (1). The growing epidemiological problem and lack of progress in head and neck oncology emphasizes the need for basic studies on the molecular biology of HNSCC. Over the past decade, molecular and cytogenetic studies have contributed significantly to the identification of genetic and epigenetic changes associated with this cancer.

Tobacco and alcohol consumption are well-established risk factors of HNSCC (2). Loss of chromosomal areas and mutations in oncogenes are crucial events in tumor formation (3–7). In addition, inactivation of TSGs by promoter methylation is also common. However, a small proportion (15–20%) of HNSCC occurs in nonsmokers and nondrinkers, suggesting the presence of other risk factors. Recent epidemiological and molecular data suggest that HPV infection of the upper airway may promote head and neck tumorigenesis (8–10). HPV-positive oropharyngeal cancers display distinct pathological molecular features as well as a different clinical course from HPV-negative oropharyngeal cancers, whose etiology is linked to smoking and drinking (9).

Allelic loss on chromosomal arm 3p is common in head and neck cancers (11–15) and other cancers, including lung, kidney, breast, and bladder (14–16). Dammann et al. (20) described the cloning and characterization of a human RAS effector homologue, RASSF1, located in the 120-kb region of minimal homozygous deletion at 3p21.3 in lung cancer (17). The RASSF1A isoform was frequently inactivated in several types of tumors (18–20). Huang et al. (21) also provided evidence that promoter hypermethylation and allelic loss are the major mechanisms for inactivation of RASSF1A in nasopharyngeal tumorigenesis.

In this study, we investigated whether RASSF1A alterations might play a role in the tumorigenesis of head and neck cancer. We also tested for HPV status based on previous evidence that HPV and RASSF1A alterations rarely occur together in cancer cell lines.

ABSTRACT

Purpose: RASSF1A, a recently identified candidate tumor suppressor gene, was found to be inactivated in lung cancer and other tumor types. We sought to understand the role of RASSF1A in head and neck cancer.

Experimental Design: We analyzed the status of RASSF1A and presence of high-risk human papilloma virus (HPV) in head and neck cancer squamous cell carcinoma (HNSCC) cell lines and primary tumors. We used methylation-specific PCR to detect promoter hypermethylation and direct sequence analysis to detect point mutations in primary tumors and cell lines. 5-aza-2-deoxycytidine was used to demethylate the RASSF1A promoter in cell lines.

Results: Promoter methylation of RASSF1A was detected in 42.9% (3 of 7) cell lines and 15% (7 of 46) primary tumors but not in the normal control DNA. Direct sequence analysis revealed a point mutation in a cell line and another in a primary HNSCC. After treatment with 5-aza-2-deoxycytidine, re-expression and demethylation of RASSF1A gene were detected in cell lines with promoter hypermethylation. HPV DNA was detected in 34.7% (16 of 46) primary HNSCC. We found a significant inverse correlation between RASSF1A promoter methylation and HPV infection (P = 0.038).

Conclusions: Our results suggest that RASSF1A is inactivated in a subset of HNSCC primary tumors. Moreover, an inverse correlation between RASSF1A and HPV supports a biological mechanism in which both RASSF1A promoters methylation and HPV infection abrogate the same pathway in tumorigenesis.
MATERIALS AND METHODS

Samples. Forty-six head and neck primary tumors from the Johns Hopkins Hospital were selected to include a high proportion of oropharyngeal tumors with a high frequency of HPV positivity. Seven head and neck cancer squamous cell lines (06, 011, 013, 019, 023, 028, and 029) were also included. DNA was purified by phenol-chloroform extraction and ethanol precipitation and dissolved in distilled water as described previously (22).

DNA Sequence Analysis. Samples from 26 primary tumors and seven cell lines were subjected to DNA sequencing for screen of RASSF1A gene mutation. All six exons of RASSF1A gene were examined using the 10 pairs of primers, as described previously (21). After detection of a PCR product, direct PCR sequencing reactions were performed using the Amplicycle Sequencing Kit (Perkin-Elmer, Branchburg, NJ).

Bisulfite Treatment. DNA from primary tumor and cell lines was subjected to bisulfite treatment, as described previously (23). Briefly, 1 μg of DNA was denatured by sodium hydroxide and modified by sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp.), again treated with sodium hydroxide, precipitated with ethanol, and resuspended in water.

MSP. Genomic DNAs, modified by bisulfite treatment and sodium hydroxide, were used as a template for MSP. The RASSF1A gene primer pairs specific for methylated and unmethylated DNA were listed as described previously (21).

Each PCR product was directly loaded on 4% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Microsatellite Analysis of DNA. A panel of four dinucleotide microsatellite repeats PCR primers (D3S1588, D3S1621, D3S1568, and D3S3582; Research Genetics, Huntsville, AL) was used to identify losses at the 3p21.3 region. PCR conditions and criteria for LOH and homozygous deletion were described previously (22).

Expression Analysis of RASSF1 Isoforms and RNA Analysis. Isoform-specific RT-PCR assays were used for analysis of RASSF1A and RASSF1C expression. Primers for RASSF1A and RASSF1C included R182 and either NF or NOX182 as described previously (19). Total RNA was isolated from the head and neck cancer cell lines by TRizol extraction (Life Technologies, Inc., Rockville, MD). Five μg of total RNA were reverse transcribed by use of Super Script First Strand cDNA kit (Invitrogen Co.). To induce expression of RASSF1A after exposure to 5-aza-2-deoxycytidine, a drug that inhibits DNA methylation, subconfluent cultures of either RASSF1A-expressing or nonexpressing cell lines, were exposed to 1 μM 5-aza-2-deoxycytidine for 7 days. After isolation of total RNA using RNAeasy extraction kit (Qiagen), multiplex RT-PCR was performed for GAPDH and either RASSF1A or RASSF1C. RT-PCR of GAPDH transcripts was performed with PCR primers described previously (19). RT-PCR products were separated by 4% agarose gel electrophoresis and visualized after staining with ethidium bromide.

Analysis of p53. Using the Affymetrix GeneChip p53 reagent kit, genomic DNA from tumor or cell line samples was PCR amplified for p53 exons 2–11. Next, the amplified DNA was fragmented with calf intestine alkaline phosphatase (Roche). The fragmented DNA was fluorescently end-labeled using Enzo’s BioArray Terminal Labeling Kit. The samples were hybridized to the GeneChip p53 array and washed then visualized using a GeneArray scanner. A reference DNA sample (provided in the Affymetrix kit) was also run to use as a baseline sequence for analysis. Samples read as mutated with the GeneChip p53 array were manually sequenced for confirmation.

Analysis of HPV. Purified genomic DNA was amplified by PCR for the HPV-16 and HPV-18 E7 genes as well as for an internal reference gene, β-globin. Oligonucleotide primers and PCR conditions were described previously (24).

Statistical Analysis. Statistical analysis was performed by use of χ2 and Fisher’s exact tests to test for differences between groups. All analyses were performed using SPSS Window version 9.0 (SPSS, Inc., Chicago, IL).

RESULTS

To investigate epigenetic silencing of RASSF1A in HNSCC, we first tested for promoter methylation in seven head and neck HNSCC cell lines (06, 011, 013, 019, 023, 028, and 029). Using MSP, 42.9% (3 of 7) of cell lines demonstrated RASSF1A methylation. To determine the precise pattern of CpG methylation within the RASSF1A CpG islands, we directly sequenced all three MSP products from the methylated cell lines. All three samples demonstrated methylation of all 16 CpG sites within the amplified fragment. RT-PCR analysis of the three HNSCC cell lines with RASSF1A methylation demonstrated complete transcriptional silencing, whereas RASSF1A expression was detected in the other four cell lines without RASSF1A methylation. To confirm that promoter hypermethylation contributes to the lack of expression of RASSF1A in the HNSCC cell lines, we assessed the effect of 5-aza-2-deoxycytidine, a drug that inhibits DNA methylation. We exposed the RASSF1A-nonexpressing HNSCC cell lines (013, 019, and 028) to 5-aza-2-deoxycytidine for 3 days and found re-expression of RASSF1A by these cell lines but little or no change in the expression of the housekeeping gene GAPDH (Fig. 1) or in the expression of the other isoform RASSF1C (data not shown).

We then tested 46 HNSCC primary tumors for allelic loss using microsatellite markers, which map close to the RASSF1A gene. We found allelic loss in 43.5% (20 of 46) of the HNSCC primary tumors in at least one closely mapped marker. We then investigated the frequency of RASSF1A promoter methylation in
all 46 HNSCC primary tumors by MSP (Fig. 2). Aberrant promoter methylation of RASSF1A was found in 15% (7 of 46) primary tumors but not in the paired normal DNA (Table 1). Only three cases with RASSF1A promoter methylation demonstrated an allelic imbalance in the 3p21.3 region by microsatellite analysis.

To determine whether the RASSF1A gene was altered by mutation in a subset of 26 primary tumors (including all four with RASSF1A promoter methylation and absence of LOH) and seven cell lines, we performed extensive mutational analysis for all six exons (including intron/exon boundaries) of the RASSF1A gene using direct PCR sequencing. We found two missense mutations, one mutation in a cell line and another in a primary HNSCC without evidence of promoter hypermethylation of the RASSF1A gene. The mutation was at codon 133 (GCT to TCT) that predicts Ala to Ser in the 011-cell line and at codon 281 (TTT to TCT) that predicts Phe to Ser in HNSCC primary tumor. The latter mutation was somatic, i.e., not present in the matched lymphocyte DNA. The identical mutation at codon 133 was detected previously in a breast tumor by Burbee et al. (19).

We then screened for the presence of HPV DNA in these tumors by PCR for both E6 and E7 genes, 34.7% (16 of 46) of the primary tumors demonstrated the presence of HPV-16 genes, and HPV was not present in any of the cell lines. There was an inverse correlation between RASSF1A promoter methylation and the presence of HPV in the 46 primary HNSCCs (P = 0.038 by $\chi^2$).

We further investigated the correlation between p53 gene mutation and the presence of HPV from our data bank. Among the 46 head and neck cancers, a result of p53 sequence infor-

tification of genes such as p16, MGMT, GST-π, and DAP-kinase, whose expression is down-regulated in HNSCC samples compared with normal tissue (28–29). Mapping studies of chromosome 3 deletions provide evidence for the presence of three discrete 3p deleted regions (3p14, 3p21, and 3p24-p25) in lung and head and neck cancers (13, 15). It has been suggested that the short arm of chromosome 3 harbors several TSGs, which may be of diagnostic and therapeutic importance (12, 30). Extensive LOH studies in carcinoma of the lung, breast, cervix, kidney, and head and neck suggest that either a single TSG or a group of different ones reside on 3p and contribute to the pathophysiology of these cancers (31, 32). More recently, homozgyous deletions of 3p21.3 have been reported in several breast cancer and lung cancer cell lines, and deletion may be a major mechanism of inactivation for RASSF1A in these cancers (12, 15).

Ectopic expression of RASSF1A decreases in vitro colony formation, suppresses anchorage-dependent growth, and dramatically reduces tumorigenicity in vivo (17, 19). With these tumor suppression effects, the presence of a RAS association domain suggests that RASSF1A may function as an effector molecule in the RAS-activated growth inhibition signaling pathways. Recent studies suggest that RASSF1A inhibits accumulation of native cyclin D1, and RASSF1A-induced cell cycle arrest can be relieved by ectopic expression of cyclin D1 or of other downstream activators of the G1-S phase transition (33). There is strong evidence that RASSF1A functions as a bonafide TSG that undergoes epigenetic inactivation in cancer by methylation of the CpG islands in the promoter region (16–21, 34). We have confirmed the presence of RASSF1A methylation in 15% of primary HNSCCs and higher frequency in cell lines.

After treatment of 5-aza-2-deoxycytidine in HNSCC cell lines with RASSF1A gene promoter hypermethylation, re-expression and demethylation of the promoter region of RASSF1A gene were demonstrated. Our results suggest that aberrant hypermethylation of the RASSF1A promoter region is directly responsible for transcriptional inactivation of its expression in HNSCC cell line as shown in other tumor types (18, 19). Half (50%) of primary HNSCCs showed LOH at the 3p21.3 region. These results are consistent with reports that methylation and LOH are the major loss function pathways for RASSF1A inactivation because we detected only one primary tumor with a somatic mutation. Other studies have also reported RASSF1A methylation in head and neck and other tumors and the observation that point mutations of this gene are rare (18–21, 35–38). Inactivation in one case (primary tumor #3) by point mutation of one allele and promoter hypermethylation in the other provides additional support for a complete abrogation of RASSF1A function in tumor cells.

HPV has also been implicated in the etiology of HNSCC. Recent observations suggest that patients with HPV-associated HNSCC may display a clinical course different from those of patients with HNSCC whose etiology is linked to smoking and drinking (9). HPV was detected in primary HNSCC tumors without RASSF1A promoter methylation status. This result points to a strong inverse correlation between HPV infection and RASSF1A silencing/promoter hypermethylation. This correlation could also reflect a functional role of the cellular RASSF1A and viral proteins in the G1 cell cycle check point.
P16, cyclin D1 (and rarely, Rb) are critical regulators of the G1 checkpoint and play important roles in the tumorigenesis of head and neck cancer. The E7 papillomavirus protein can bypass Rb family-dependent cell cycle regulation by directly inhibiting the interaction of Rb protein with E2F transcription factors and other pocket-binding proteins. H1299 and HeLa CCL2 cell lines are known to be insensitive to RASSF1A-induced cell cycle arrest. This is likely attributable to the expression of the papillomavirus E7 protein that can bypass the Rb family cell cycle arrest. This is likely attributable to the expression of the papillomavirus E7 protein that can bypass the Rb family cell cycle arrest. This is likely attributable to the expression of the papillomavirus E7 protein that can bypass the Rb family cell cycle arrest. This is likely attributable to the expression of the papillomavirus E7 protein that can bypass the Rb family cell cycle arrest.

Recently, Agathanggelou et al. (20) also described the absence of RASSF1A promoter methylation in primary cervical
cancers. Although the report did not demonstrate the presence of HPV in cervical cancers, we can postulate a low frequency of RASSF1A promoter methylation because of a high rate HPV infection in these tumors. Thus, an inverse correlation between RASSF1A and HPV in cancers may be common.

There was no association between RASSF1A inactivation and p53 status. The inverse association between p53 mutations and HPV presence in head and neck cancer continues to support two parallel pathways of HNSCC development (9, 39, 40). Our results further suggest that there may be important overlapping pathways in the development of head and neck cancers between HPV and RASSF1A. Although the E6 protein inactivates p53, E7 may block Rb family-mediated cell cycle arrest in oropharyngeal tumors. Thus, RASSF1A inactivation now represents another important genetic feature of HPV-negative tumors.

Our study provides evidence that promoter hypermethylation is the major mechanism for inactivation of RASSF1A in HNSCC and indicates this gene may be a key tumor suppressor target at 3p21.3. Some sites were underrepresented in our study (e.g., larynx) and could harbor a higher frequency of RASSF1A methylation. The inverse correlation between HPV infection and RASSF1A silencing in the development of head and neck cancers is based on a limited number of tumors and remains to be fully explored.

REFERENCES


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