Short Communication

Ataxia-Telangiectasia-Mutated (ATM) Gene in Head and Neck Squamous Cell Carcinoma: Promoter Hypermethylation with Clinical Correlation in 100 Cases

Lingbao Ai,1,3 Quynh N. Vo,4 Chunlai Zuo,1 Liwen Li,1 Wenhua Ling,2 James Y. Suen,2 Ehab Hanna,2 Kevin D. Brown,4 and Chun-Yang Fan1

Departments of 1Pathology and 2Otolaryngology, University of Arkansas for Medical Sciences and John L. McClellan Memorial Veterans’ Hospital, Little Rock, Arkansas; 3College of Public Health, Sun-Yat Sen University, Guangzhou, People’s Republic of China; and 4Department of Biochemistry and Molecular Biology and the Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana

Abstract

The Ataxia-telangiectasia-mutated (ATM) gene product is a well-characterized tumor suppressor that plays a key role in maintenance of genomic stability. We have recently documented that the ATM promoter is a target for epigenetic silencing in cultured tumor cells. Here we show that aberrant methylation of the ATM promoter occurs in a significant percentage (25%) of head and neck squamous cell carcinomas. The presence of methylated ATM promoter shows a statistically significant correlation with an earlier age of initial diagnosis and decreased overall survival, particularly in early-stage tumors. These findings indicate that ATM promoter hypermethylation occurs in head and neck squamous cell carcinoma, and this feature is a potentially useful prognostic marker in this tumor type.

Introduction

Head and neck tumors account for ~5% of the total cancer burden in the United States, and 80–90% of these tumors are squamous cell carcinoma (SCC). It is widely recognized that development of head and neck SCC (HNSCC), as with all cancers, is a multistep process characterized by the progressive accumulation of genomic aberrations. Such genetic changes can ultimately lead to a selective growth advantage and drive tumor formation and progression by up-regulating oncogene and/or down-regulating tumor suppressor activity (1).

Ataxia-telangiectasia (A-T) is an autosomal recessive dis-order, principally characterized by progressive neurodegeneration, immunodeficiency, and marked predisposition to the development of malignancies. The ataxia-telangiectasia-mutated (ATM) gene was mapped to 11q22–23 (2), and later positionally cloned by Savitsky et al. (3). We now recognize that ATM is a protein kinase that functions as a tumor suppressor by triggering appropriate cellular response to genome damage resulting from ionizing radiation or chemical carcinogen exposure (for review see Ref. 4).

Despite the general role that ATM plays in maintaining genome integrity, inactivating somatic mutations in this gene have been principally characterized in hematological tumors (5–7). However, several groups have identified the 11q22–23 locus as a common deletion site in HNSCC (8–10). It is currently unknown whether ATM is lost in HNSCCs displaying this deletion; nevertheless, in light of the role this protein plays in maintenance of genomic homeostasis, ATM remains an attractive target for inactivation in HNSCC and other cancer types.

Our group recently showed that aberrant hypermethylation of the ATM gene promoter results in markedly reduced expression of this gene (11). Aberrant promoter methylation results in lost or greatly diminished expression of a number of tumor suppressors including BRCA-1 (12, 13), p16INK4a (14, 15), and pVHL (16). Such observations have resulted in the widely held view that epigenetic silencing through promoter hypermethylation is a common mechanism for tumor suppressor inactivation during tumorigenesis (17). In this report, we investigated whether the ATM gene is a target for promoter hypermethylation in HNSCC. We observed that a significant percentage (25%) of the 100 HNSCCs examined displayed aberrant methylation of the ATM promoter. Further, we found that ATM promoter hypermethylation displays a significant correlation with decreased overall patient survival, suggesting that this event has potential use as a prognostic factor.

Materials and Methods

Tumor Acquisition. One hundred consecutive untreated cases of HNSCC were collected from the Anatomical Pathology file (from 1993 to 1998) cataloged by the Department of Pathology, John L. McClellan Memorial Veterans’ Hospital, Little Rock, AR. Both paraffin blocks and H&E-stained slides of tumors from each case were available for study. Case selection was based on the following criteria: (a) primary surgical resection with curative intent or diagnostic biopsy for the purpose of adjuvant therapy; and (b) no prior history of HNSCC and adjuvant therapy. The histology of each case was reviewed and representative tissue blocks containing invasive SCC were selected for DNA extraction and promoter methylation analysis. Clinical follow-up was available for all of the cases up to May, 2002. Pertinent patient information was retrieved from the...
Computerized Patient Record System, Department of Veterans Affairs.

The patient population consisted of all males ranging in age from 45 to 85 years (mean, 65.8 years). The mean follow-up period for all of the patients was 51.2 months. Median survival based on all causes of death was 49.2 months. Of 31 surviving patients, the mean survival was 67.2 months (range, 8.4–144 months). Of the 47 patients with local recurrence or a second primary tumor, 43 (92%) died and 41 of these patients died of their cancer. Nine patients had more than one recurrence, and all died of their disease. Of the 53 patients without local recurrence or a second primary tumor, 26 (49%) died and 8 died of their disease. History of tobacco and alcohol use was positive in 55 and 47 patients but negative in 12 and 20 patients, respectively (see Table 1). History of smoking and drinking was not properly documented in 34 and 33 patients, respectively (see Table 1).

As a control, we analyzed ATM promoter methylation status in nonneoplastic tissue obtained from tonsillectomy pro-
cedures conducted on patients less than 70 years old. Noncancerous tissue was harvested from paraffin-embedded tissue sections using a Pixcell II LCM microscope (Arcturus Engineering, Mountain View, CA). DNA was subsequently extracted and analyzed as outlined below.

**DNA Extraction from Paraffin Blocks.** Five tissue sections (5 μm thick) from each selected paraffin tissue block were deparaffinized by washing twice in 100% xylene followed by two washes in 100% ethanol. The deparaffinized tissues were dried at room temperature for 30 min and then boiled in 1× citrate buffer (Cat. No. 00-5000; Zymed Lab. Inc., San Francisco, CA) for 30 min. We found that this step dramatically improved the conversion efficiency of the non-CpG cytosines (i.e., CpC, CpA, CpT) to uracil on sodium bisulfite treatment in DNA samples obtained from formalin-fixed tissues. Subsequently, DNA was isolated using the EX-WAX DNA Extraction kit (Intergen Co., New York, NY), according to the manufacturer’s instruction. Human placental DNA (Sigma) was used as a negative control and CpGenome universal methylated human DNA (Intergen) was used as methylation-positive control DNA.

**MSP.** Genomic DNA harvested from HNSCC and tonsillectomy specimens, as well as negative and positive control DNA samples, were subjected to bisulfite modification before methyl-
ylation-specific PCR (MSP) using CpGenome DNA modification kit (Intergen).

MSP analysis of the ATM promoter region was carried out in 25-μl PCR reactions containing 1 μl of bisulfite-treated genomic DNA, dNTPs (200 μM each), oligonucleotide primers (25 pmol each/reaction), 2.5 mM MgCl₂, and 0.625 units of Hotstar Taq (Qiagen, Valencia, CA) in 1× PCR buffer. MSP primers for analysis of the ATM gene promoter are: 5'-GTCTTGGAGTTTGGTGAAGGGT-3' (sense) and 5'- AAC-TACCCTACTCCCACCTCCCA-3' (antisense) for amplification of the promoter existing in an unmethylated state, and 5 '- GGAGTTTGAGTTGAAGGGT-3' (sense) and 5'-CTAC-CTACTCCGCTTCCGA-3' (antisense) for amplification of the promoter existing in a methylated state. Thermocycling conditions used were: initial denaturation and hot start at 95°C for 15 min; 40 cycles consisting of 30 s at 95°C, 30 s at 56°C (unmethylated reactions), or 59°C (methylated reactions), and 1 min at 72°C. After thermocycling, reactions were subjected to a 5-min, 72°C incubation. Positive and negative control reactions were run in parallel with each set of MSP reactions. MSP reactions were analyzed by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining.

**Statistical Analysis.** Overall patient survival was calculated using the Kaplan-Meier method. The association among various factors such as ATM promoter methylation, and clinical and pathological parameters were analyzed with cross-table χ² test, and the listed Ps in Table 1 were obtained after correction for multiple comparisons within each prognostic group. Overall patient survival was calculated using the Kaplan-Meier method and differences in survivor function due to ATM promoter methylation status were calculated by the log-rank test. Ps for multiple log-rank tests were adjusted with a Wilcoxon (Gehan) method. Multivariate analysis was performed using a Cox regression model. All Ps given are two-sided. SPSS Version 11.0 for PC software (SPSS Inc., Chicago, IL) was used for all statistical analyses.

**Results**

To analyze the methylation status of the ATM promoter in genomic DNA extracted from HNSCC samples, we designed a
set of MSP primers that amplify a segment of the ATM promoter existing in either a methylated or nonmethylated state (18). Specifically, this involved designing primers that anneal to the promoter based strictly on the modification status of CpG dinucleotides after sodium bisulfite modification. Bisulfite modification leads to the deamination of unmethylated cytosines to uracil, but methylated cytosines are unmodified by this treatment. The primers designed to amplify unmethylated ATM promoter sequence yield an amplicon from nt −404 to −159, and the methylated primer set yield an amplicon from nt −399 to −161 (numbering system based on location of the ATM translational start site). Thus, the predicted size of the unmethylated amplicon is 246 bp, and the methylated amplicon is 239 bp. A schematic diagram of the ATM proximal promoter region with the location of the ATM MSP primer sets is shown in Fig. 1A.

To initially verify our designed primers, we tested these oligonucleotides in PCR reactions using human placental and methylated human DNA (Fig. 1B). We observed that PCR reactions conducted on this non-bisulfite-modified DNA failed to result in accumulation of either an unmethylated (U) or methylated (M) amplicon (Fig. 1B, Lanes 1 and 2). This result indicates that productive amplification using either primer set requires bisulfite-modified template DNA. Next, we tested bisulfite-modified placental DNA and observed the accumulation only of a 246-bp amplicon in reactions using the unmethylated amplicon-specific primers (Fig. 1B, Lane 3 and 4). PCR conducted on bisulfite-modified methylated human DNA showed only accumulation of the 239 methylated amplicon (Fig. 1B, Lanes 5 and 6). The identity of these amplicons was confirmed by DNA sequencing.

As a further verification step, we tested this MSP primer set using genomic DNA harvested from the colorectal tumor line HCT-116. We previously demonstrated, using a restriction enzyme-based approach, that this line displays aberrant methylation of the ATM proximal promoter region and that this correlated with markedly decreased ATM protein abundance (11). Analysis of bisulfite-modified HCT-116 DNA with our MSP primer set showed accumulation of the methylated-specific amplicon (Fig. 1C, Lanes 1 and 2), consistent with our previous findings. Further, MSP conducted on genomic DNA isolated from HCT-116 cells cultured in the presence of the global demethylating agent 5-azacytidine (Fig. 1C, Lanes 3 and 4) as well as genomic DNA from normal human foreskin fibroblasts (Fig. 1C, Lanes 5 and 6) showed accumulation of the unmethylated amplicon, consistent with the unmethylated nature of the ATM promoter in these cells (11). Taken together, our verification experiments indicate that these MSP primers amplify genomic DNA in a manner that clearly ascertain the methylation status of the ATM promoter.

These MSP primers were subsequently used to analyze genomic DNA extracted from fixed HNSCCs embedded in paraffin. To date, we have analyzed 100 consecutive untreated cases of HNSCC selected on the basis of criteria outlined in “Materials and Methods.” Fig. 2A displays representative MSP data obtained from five HNSCC samples. In tumor samples 1, 10, and 78, we observed amplification with the unmethylated-specific primers indicating a lack of ATM promoter hypermethylation in these tumor samples. Tumor samples 23 and 26 show amplification of both unmethylated and methylated-specific amplicons. Amplification of both unmethylated and methylated-specific MSP products is a common finding during analysis of tumor samples (19) and could be attributable to contamination of the tumor sample with normal cells. Alternatively, these results could stem from methylation of a single ATM allele within individual tumor cells, heterogeneous ATM promoter methylation within the tumor cell population, or both.

The 239-bp fragment amplified with the methylated-specific primer set contains a total of 25 CpG dinucleotides. To resolve the density of CpG methylation within the ATM promoter, we cloned and sequenced methylation-specific amplicons from HNSCC DNA. A representative DNA sequence chromatogram derived from a cloned, methylated-specific amplicon is shown (Fig. 2B). In this clone, sequencing revealed that two of the three CpG dinucleotides within the 35-bp region of the ATM promoter displayed are methylated. Moreover, sequencing of 17 cloned methylated-specific MSP amplicons derived from seven HNSCCs revealed that >80% of the CpGs within this region of the ATM promoter are methylated in these tumors. Taken together, these findings indicate that a subset of HNSCCs display dense methylation of the ATM promoter.

To examine ATM promoter hypermethylation in normal oral epithelium, we analyzed eight noncancerous tonsillectomy tissues by MSP. The results from five tonsillectomy specimens are shown (Fig. 2C), and no indication of ATM promoter methylation in these, or the other three tissues analyzed, was observed. Although we cannot conclude, with certainty, that ATM promoter methylation does not occur in normal tissues, these findings clearly indicate that aberrant methylation of the ATM promoter occurs in HNSCC.

MSP data collected on 100 HNSCCs indicated that the
ATM promoter was hypermethylated in 25 (25%) of these tumors. Association of ATM promoter hypermethylation with various clinical, pathological, and treatment parameters was analyzed using a cross-table χ² test (Table 1). Of note, we found a statistically significant correlation between ATM promoter hypermethylation in primary HNSCC tumors and initial diagnosis at age 65 or younger (P = 0.05). However, no significant correlation was noted with other parameters such as tumor site and size, lymph node status, clinical stage, history of tobacco or alcohol use, and chemoradiation therapy.

Applying a Kaplan-Meier survival test on the entire patient population, we found that ATM promoter hypermethylation was significantly correlated with decreased overall patient survival (P = 0.03; Table 2; Fig. 3A) but not with disease-free survival (P = 0.76). The overall 5-year survival is 22% in tumors with ATM promoter hypermethylation and 46% in those without ATM promoter hypermethylation (Table 2). Multivariate analysis was also performed on this data set using the Cox regression model. This test indicated that ATM promoter hypermethylation could predict decreased overall patient survival, independent of other potential prognostic factors, such as tumor size, nodal status, clinical stage, and history of tobacco and alcohol use (P = 0.03). ATM promoter hypermethylation may also correlate with decreased cause-specific survival but this correlation did not reach statistical significance (P = 0.09).

Finally, association of ATM promoter hypermethylation with overall patient survival was further stratified by tumor size (T stage) and lymph node status (N stage; Table 2). ATM promoter hypermethylation was found to be significantly correlated with decreased overall survival in tumors of small size (T₁, P = 0.01; Table 2; Fig. 3B) and tumors without lymph node spread (N₀, P = 0.03; Table 2; Fig. 3C). No significant statistical correlation was observed between ATM promoter hypermethylation and overall survival rate in larger tumors (T₂, T₃, or T₄; P = 0.29), or tumors with nodal metastasis (N₁, N₂, P = 0.38; Table 2).

Discussion

We have previously demonstrated that the ATM gene is a target for epigenetic silencing through aberrant methylation of its proximal promoter region (11). However, whether aberrant methylation of the ATM promoter occurs in tumors was not established in this previous study. We report here an extension of our previous work by using methylation-specific PCR to examine ATM promoter methylation in HNSCC. Using a stringently verified set of MSP primers, we documented that the ATM promoter is a target for aberrant hypermethylation in HNSCC. Given the important role that ATM plays in maintaining genome integrity and the causative role that genome instability plays in cancer onset and progression, reduced ATM function through epigenetic silencing is an attractive candidate mechanism contributing to HNSCC and, perhaps, other tumor types.

We documented ATM promoter methylation correlates with reduced ATM protein expression in cultured cells (11). Furthermore, we have observed that ATM promoter methylation correlates with reduced ATM mRNA expression in
ATM – 11q22 promoter hypermethylation in 25% of 100 HNSCC

ATM genetic alterations (e.g., with the loss of tumor suppressor function stemming from genomic instability, absent or reduced HNSCCs. Nevertheless, in addition to allowing increased quantification and analysis of these cancers, efforts are focusing on using quantitative reverse transcription-PCR to measure ATM mRNA abundance in fresh tumors and paraffin-embedded tissue. Thus, whereas we have no direct evidence for diminished ATM expression in HNSCCs displaying ATM promoter methylation, it is altogether reasonable to suspect that these tumors express reduced levels of the ATM protein. Current efforts are focusing on using quantitative reverse transcription-PCR to measure ATM mRNA abundance in fresh HNSCCs. Nevertheless, in addition to allowing increased genomic instability, absent or reduced ATM expression leads to heightened sensitivity to the cytotoxic effects of ionizing radiation (4, 11). Thus, ATM promoter methylation may be a potentially valuable molecular marker to predict the efficacy of organ-preservation radiation therapy for patients with HNSCC.

In cancer, epigenetic silencing often cooperates with genetic changes (gene mutation and deletion) in silencing a number of tumor suppressor genes such as p16Ink4a, E-cadherin, pVHL, BRCA-1, and MLH1 (for review, see Ref. 17). Promoter hypermethylation of one allele is frequently accompanied by deletion of the opposite allele (16, 20), mimicking the type of loss of heterozygosity usually seen with the loss of tumor suppressor function stemming from genetic alterations (e.g., point mutation). We have detected ATM promoter hypermethylation in 25% of 100 HNSCC analyzed. Several studies documented allelic loss at 11q22–23 in ~30% of cases of HNSCC (8–10). Even though it remains to be determined whether the ATM locus (located at 11q22–23) is lost by chromosomal rearrangement in HNSCC, our data suggest that the ATM gene may be inactivated in a subset of HNSCCs by promoter hypermethylation. Hence, it is plausible that, as seen in other tumor types, ATM expression is lost in HNSCC through a combination of chromosomal alteration and epigenetic events.

One hundred untreated HNSCC cases were analyzed for ATM promoter hypermethylation, and the relevance of this aberrant methylation event was correlated with various pertinent clinical and patient parameters. The goal of this analysis was to determine the prevalence and prognostic significance of ATM promoter hypermethylation in HNSCC. ATM promoter hypermethylation was detected in a significant percentage (25%) of the HNSCCs tested in this study. Furthermore, using the entire patient population as the basis for analysis, we found that ATM promoter hypermethylation correlates significantly with decreased overall patient survival. Moreover, this correlation was found to be independent of other potential prognostic factors, such as tumor size, lymph node status, clinical stage, and history of tobacco and alcohol use. Thus, this data seemingly indicates that hypermethylation of the ATM promoter can be used as a prognostic factor in HNSCC.

The proportion of HNSCC patients surviving 5 years postdiagnosis is 46% when the ATM promoter is not methylated compared with 22% when the ATM promoter is methylated. Again, when these data were stratified by tumor size and nodal status, a significant correlation persists in T1 and N0 tumors. The overall 5-year survival rate is 68% (T1 tumor) and 57% (N0 tumor) when the ATM promoter is not methylated, and 20% (T1 tumor) and 25% (N0 tumor) when the ATM promoter is methylated. Each of these criteria was found to be statistically significant. Taken together, these data suggest that ATM promoter methylation has a more prominent impact on patient survival in early-stage tumor development.

It is noteworthy that ATM promoter hypermethylation displays a significant correlation with decreased overall patient survival (P = 0.03) but not with disease-free survival (P = 0.76). Disease-free survival was defined as the time from tumor resection until the first evidence of tumor recurrence or the development of secondary primary tumor. Over-

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<th>ATM MSP results</th>
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a MSP, methylation-specific PCR; T, tumor; N, lymph node.
b All survival data were presented as cumulative probability.
c Log-rank test is a test of equality of survivor function across groups; adjusted Ps for multiple log-rank tests were obtained using a Wilcoxon (Gehan) method.
d Stage was determined by pathological examination.

5 Q. N. V. and K. D. B., unpublished observations.
All patient survival was defined as the time from tumor resection to the time of death due to all causes. It is unknown why ATM promoter hypermethylation has a more profound impact on overall patient survival than on disease-free survival. The negative impact of ATM promoter hypermethylation on disease-free recurrence may be because the number of tumor recurrences was much smaller (47 of 100; 37 without and 10 with ATM promoter hypermethylation) than that of total death (69 of 100; 51 without and 18 with ATM promoter hypermethylation). Study of a larger panel of HNSCC patients is likely required to more accurately assess the impact of ATM promoter methylation on tumor recurrence.

In summary, we have extended our previous studies to further characterize ATM promoter methylation using a highly specific and sensitive methylation-specific PCR approach. We determined that ATM promoter hypermethylation occurs in a significant number of HNSCCs, and that this event correlates with overall patient survival, particularly in early-stage tumors. These studies indicate that ATM promoter methylation may be a useful prognostic marker in this disease.

References


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