Fine-Mapping Loss of Gene Architecture at the CDKN2B (p15INK4b), CDKN2A (p14ARF, p16INK4a), and MTAP Genes in Head and Neck Squamous Cell Carcinoma

Maria J. Worsham, PhD; Kang Mei Chen, MD; Nivedita Tiwari, MS; Gerard Pals, PhD; Jan P. Schouten, PhD; Seema Sethi, MD; Michael S. Benninger, MD

Objective: To identify the extent and the smallest region of loss for CDKN2BINK4b, CDKN2AARF,INK4a, and MTAP. Homozygous deletions of human chromosome 9p21 occur frequently in malignant cell lines and are common in squamous cell carcinoma of the head and neck (HNSCC). This complex region encodes the tumor suppressor genes cyclin-dependent kinase 2B (CDKN2B) (p15INK4b) and CDKN2A (p14ARF, p16INK4a) and the housekeeping gene methylthioadenosine phosphorylase (MTAP).

Design: A targeted probe panel designed to finely map the region of 9p21 loss comprised 3 probes for CDKN2BINK4b,7 for CDKN2AARF,INK4a,6,8 and 3 for MTAP and was interrogated using the multiplex ligation-dependent probe amplification assay (MLPA). The MLPA genomic copy number alterations for CDKN2A were validated using real-time polymerase chain reaction.

Subjects: Six HNSCC primary (A) and recurrent or metastatic (B) cell lines were examined: UMSCC-11A/11B, UMSCC-17A/17B, and UMSCC-81A/81B.

Results: Cell line UMSCC-11B retained all 9p loci tested in the region. Cell lines UMSCC-17A/B indicated homozygous deletion of CDKN2AARF,INK4a starting at p16INK4a exon 1α to include exons 2 and 3. Homozygous loss was indicated for CDKN2BINK4b and CDKN2AARF,INK4a in UMSCC-11A, and UMSCC-81A. Cell line UMSCC-81B indicated retention of all 9p loci except for exon 1α (p16INK4a). Selective loss of the 3’ end of MTAP was observed in UMSCC-11A. Genomic alterations by fine-mapping MLPA were validated at the DNA level for CDKN2A.

Conclusions: We identified exon 1α (p16INK4a) as the smallest region of loss in the CDKN2AARF,INK4a gene. The frequency and precise loss of CDKN2BINK4b, CDKN2AARF,INK4a, and MTAP in the prognosis of 9p21-deleted HNSCC may provide impetus for use of these targets as therapeutic biomarkers in head and neck cancer.


T HE MOST COMMON CHROMOSOME rearrangements associated with squamous cell carcinomas of the head and neck (HNSCCs) are unbalanced translocations leading to chromosomal deletions.1 Chromosome 9p21 has been reported to be a critical region of loss in various cancers. Genetic alterations at the 9p21 locus have been linked to malignant progression in HNSCC.2-3 The cyclin-dependent kinase 2A (CDKN2A) and CDKN2B genes map to 9p21 and are in tandem, spanning a region of approximately 80 kilobases (kb), with CDKN2B located 25 kb centromeric to CDKN2A.4,5 (Figure 1). The CDKN2A locus controls the Rb pathway (which regulates G1/S-phase transition) and the p53 pathway (which induces growth arrest or apoptosis in response to either DNA damage or inappropriate mitogenic stimuli by generating 2 gene products).6,7 This regulatory function is achieved through the p16 protein product, which functions upstream of Rb, and the p14 protein, which blocks MDM2 inhibition of p53 activity, preventing p53 degradation, and thus permitting p53-induced apoptosis or growth arrest.8 The CDKN2D/p14 gene has a unique first exon 1β that, under the control of its own promoter, splices onto exon 2 of CDKN2A/p16 in an alternative reading frame, allowing production of the 2 totally unrelated proteins, p14 and p16, respectively (Figure 1).8

Whereas CDKN2A/p16 mutations selectively inactivate the Rb pathway, deletion of the CDKN2A locus impairs the Rb and p53 pathways.6-8 Deletion of the CDKN2A locus also frequently affects the CDKN2B locus, which encodes p15, an important mediator of the antiproliferative effect of transforming growth factor β.9 Inactivation of the CDKN2B (p15), CDKN2A (p14), and CDKN2A (p16) genes is a fre-
The human methylthioadenosine phosphorylase (MTAP) gene at 9p21, approximately 100-kb telomeric to CDKN2A, is an essential enzyme for the salvage of adenine and methionine (Figure 1). Cells that lack this enzyme become sensitive to purine synthesis inhibitors or methionine starvation and can be therapeutically exploited for selective therapy.

Although inactivation of p16 has been reported as the most common genetic alteration in HNSCC, making it an ideal target for gene replacement, the extent to which p16 is independently altered, or the frequency with which p14 and p16 are affected, has not been examined thoroughly. Because these proteins are completely unrelated, bearing no amino acid homology to one another, each of these targets has the potential to independently serve as therapeutic interventions in HNSCC.

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Table. 9p21 Fine-Mapping Multiplex Ligation-Dependent Probe Amplification Gene Probe Panel

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Abbreviations: CDKN2A/B, cyclin-dependent kinase 2A/B; ELAV2, embryonic lethal abnormal vision; DOCK8, Drosophila homolog-like 2; FLJ00026, alias; MLLT3, myeloid lymphoid or mixed-lineage leukemia (trithorax [Drosophila] homolog), translocated to 3; MTAP, methylthioadenosine phosphorylase; PCR, polymerase chain reaction; TEK, endothelium-specific receptor tyrosine kinase.

*See Figure 1.

indicated by a reduction in peak height for that particular gene probe, homozygous loss is indicated by the absence of a peak (illustrated for CDKN2A and CDKN2B probes in UMSCC-81A) (Figure 2B), and gain of a copy number is correspondingly denoted by an increase in peak height.

Cell line DNA, starting with approximately 20 to 50 ng, is interrogated for the 38 genes in 1 reaction tube. Brieﬂy, DNA is digested with water to a total volume of 5 µL and then denatured by heating for 5 minutes at 98°C in a thermocycler. Binary MLPA probes are added and allowed to hybridize to their targets during 16 hours of incubation at 60°C. Dilution buffer and a special Ligase-65 enzyme (MRC-Holland) are added to the vial. During 15 minutes of incubation at 60°C, the 2 parts of a probe molecule can be ligated to each other and become an amplifiable molecule provided that the complementary sequence is present in the sample. This is followed by the addition of PCR primers, deoxynucleotide triphosphates, and Taq polymerase, followed by the following cycles: 1 cycle for 1 minute at 95°C; 10 cycles for 30 seconds at 95°C, 30 seconds at 70°C, and 1 minute at 72°C; and 30 cycles for 30 seconds at 95°C, 30 seconds at 60°C, and 1 minute at 72°C. All (ligated) probes are ampliﬁed by the same primer pair, one of which is tagged with a ﬂuorescent dye. Ampliﬁcation products (38 individual probe peaks in the control [Figure 2A]) are analyzed using an ABI 310/ABI 3100 DNA sequencer, and the products are quantiﬁed, normalized, and interpreted.
LightCycler by measuring the fluorescence of SYBR green I during the temperature. DNA melting curves were acquired using the LightCycler software (version 5.32) to plot the negative derivative of fluorescence over temperature vs temperature. For PCR products generated on the LightCycler, melting curve analysis was performed immediately after amplification.

Primers were designed in intron 1 of CDKN2A between nucleotides 187602 and 187697 using Oligo 6 (Molecular Biology Insights Inc, Cascade, Colo). The primer pairs were as follows: 5’ nucleotide 187602: 5’ GCC AAG GAG GAC CAT AAT TC 3’; 3’ nucleotide 187697: 5’ GGA CCA AGA CTG CGC TGA C 3’. Detection and quantification of target DNA was performed using the double-stranded DNA specific dye SYBR green I, which is similar to ethidium bromide in that it binds preferentially to double-stranded DNA, emitting a fluorescent signal that is proportional to the amplified target concentration. The signal is measured in channel 1 (at 530 nm) at the end of the elongation phase once per cycle and is monitored in real time. The ubiquitous β-globin gene was used as an internal control (housekeeping gene).

REAL-TIME PCR

Real-time PCR was used to confirm MLPA DNA copy number loss and retention of CDKN2A in cell lines UMSCC-11A/B, UMSCC-17A/B, and UMSCC-81A/B. In real-time PCR, the amount of product formed (detected by binding of the fluorescent dye SYBR green I) is plotted as a function of the number of cycles. Each cycle represents a doubling of DNA (ie, as measured by fluorescence of SYBR green I bound to double-stranded DNA), so the accumulation of DNA product is exponential across consecutive cycles (ie, 2n). It is the ability to identify the exponential phase of product formation “in real time” that makes the method quantitative because the amount of product is exponentially related to the amount of template (the unknown quantity) during this phase. Therefore, during the exponential phase of PCR product formation, the amount of product doubles during each cycle. The beginning of the exponential phase of amplification (so-called crossing points or threshold cycle [Ct]) is considered the most reliable point of the PCR related to sample concentration. Two samples that reach the threshold within 1 cycle of each other (eg, Ct1 = 10, Ct2 = 11) differ by 2-fold in the amount of amplified product (target gene).

Melting curve analysis is an exact and fast method for checking PCR specificity. Every DNA fragment melts at a characteristic temperature, called the melting temperature, defined as the temperature at which 50% of the DNA is single stranded. The most important criteria that determine the melting temperature are the G+C content and the length of the fragment. The LightCycler Instrument (Roche Diagnostic Corp, Indianapolis, Ind) monitors the fluorescence continuously while raising the temperature gradually. When the temperature in the capillary reaches the melting temperature of the fragment under study, there is a sharp decrease in fluorescence because SYBR green I dye is released from the amplicon. Plotting fluorescence vs temperature generates melting curves. When melting curves are displayed as the first negative derivative of fluorescence vs temperature, a peak is generated at the melting temperature. DNA melting curves were acquired using the LightCycler by measuring the fluorescence of SYBR green I during a linear temperature transition from 65°C to 97°C at 0.1°C/s. Fluorescence data were converted into melting peaks by using the LightCycler software (version 5.32) to plot the negative derivative of fluorescence over temperature vs temperature. For PCR products generated on the LightCycler, melting curve analysis was performed immediately after amplification.

RESULTS

FINE MAPPING

The UMSCC-11B cell line retained all 9p loci tested in the region. Homozygous loss was indicated for CDKN2A(B) and CDKN2A(ARF, INK4a) in UMSCC-81A and UMSCC-11A (Figure 1B and Figure 2, UMSCC-81A). Cell lines UMSCC-17A/B indicated homozygous deletion of CDKN2A(ARF, INK4a) starting at p16 exon 1a to include exons 2 and 3. Cell line UMSCC-81B indicated retention of all 9p loci except for exon 1a (p16INK4a, probe 8) (Figure 1A), making the latter the smallest region of overlap for loss in the CDKN2A(ARF, INK4a) region (Figure 1B and Figure 2).

In UMSCC-11A, all 3 MTAP MLPA probes—1 at the 3’ end (probe 13, exon 7) (Figure 1A), 1 in exon 6 (probe 12) (Figure 1A), and 1 at the 5’ end (probe 11)—homozygous loss was indicated only for the 3’ end of the MTAP gene.

VALIDATION OF GENOMIC LOSS OF THE CDKN2A LOCUS

Genomic loss and retention of CDKN2A copy number with real-time PCR for UMSCC-11A/B, UMSCC-17A/B, and UMSCC-81A/B was concordant with confirming retention of the CDKN2A intron 1a sequences in UMSCC-11B and UMSCC-81B and loss in UMSCC-11A, UMSCC-17A/B, and UMSCC-81A (illustrated for UMSCC-11A/B in Figure 3). Here, normal copy number was indicated for β-globin in UMSCC-11A/B and for CDKN2A in UMSCC-11B. In UMSCC-11A, however, loss of CDKN2A is indicated by a delayed Ct. Melting curve analysis confirmed lack of a specific melting peak for CDKN2A. The MLPA fine mapping confirmed homozygous loss of this region (Figure 1B).

COMMENT

Genetic alterations provide a means of identifying tumor cells and defining changes that presumably deter-
mine biological differences from their normal counterparts. The underlying hypothesis is that behavior of tumor cells is determined by genetic changes that alter cell growth, cell differentiation, programmed cell death, and cell migration. Knowledge of the genetic mechanisms that drive cancer growth and development can provide better diagnostic and prognostic information and more appropriate selection of therapy.

The precise role of the clinical application of molecular prognostic markers in HNSCC remains elusive. Studies of consistent genetic changes in HNSCC have been instrumental in the initiation of gene therapy trials in vitro and in experimental animals. The transfer of wild-type p53 into HNSCC tumor cells with mutant p53 was shown to induce growth arrest and tumor regression. Other genes being targeted for gene therapy include the B-cell leukemia/lymphoma 2 (BCL2) gene homologue BCL2XS, which inhibits BCL2 function and has been shown to have altered the sensitivity of tumor cells to chemotherapeutic agents by restoring sensitivity to apoptosis. Several strategies directed against epidermal growth factor receptor overexpression have included the use of monoclonal antibodies targeted against epidermal growth factor receptor. A chimeric anti–epidermal growth factor receptor monoclonal antibody (C225) has been tested in combination with either cisplatin or radiation, and the results are significant.

Studies of consistent genetic changes in HNSCC have been instrumental in the initiation of gene therapy trials in vitro and in experimental animals. The transfer of wild-type p53 into HNSCC tumor cells with mutant p53 was shown to induce growth arrest and tumor regression. The precise role of the clinical application of molecular prognostic markers in HNSCC remains elusive. The UMSCC-81B cell line indicated retenzygous loss at the CDKN2A locus in 5 of 6 HNSCC cell lines: UMSCC-11A, UMSCC-11B, UMSCC-81A, and UMSCC-81B. The UMSCC-81B cell line indicated retention of all 9p loci except for exon 1α (p16INK4a), making the latter the smallest region of overlap for loss in the CDKN2ARF, INK4a region (Figure 1B and Figure 2). Studies reporting homozygous deletions of CDKN2A in HNSCC may, therefore, underestimate the frequency of this region depending on the extent to which deletion mapping was performed.

Homozygous loss for CDKN2B (p16) and CDKN2AARF (p14) genes causes dysregulation of the 2 pathways important in most cancers (Rb and p53), loss of MTAP activity is thought to be incidental and not of pathogenic consequence. Because deletion of the CDKN2AINK4a (p16) and CDKN2AAARF (p14) genes causes dysregulation of the 2 pathways important in most cancers (Rb and p53), loss of MTAP activity is thought to be incidental and not of pathogenic consequence. Genetic studies show high rates of MTAP loss in non–small cell lung cancer, melanoma, bladder cancer, pancreatic cancer, osteosarcoma, and endometrial cancer. Tumors that lack MTAP are expected to be sensitive to inhibitors of purine synthesis or methionine starvation. Genomewide profiling studies of oral SCC found frequent deletion of MTAP, however, mapping of precise loss of MTAP and its association with concomitant or independent loss with CDKN2A/B genes was not addressed.

Because deletion of the CDKN2AARF (p16) and CDKN2AAARF (p14) genes causes dysregulation of the 2 pathways important in most cancers (Rb and p53), loss of MTAP activity is thought to be incidental and not of pathogenic consequence. Because deletion of the CDKN2AARF (p16) and CDKN2AAARF (p14) genes causes dysregulation of the 2 pathways important in most cancers (Rb and p53), loss of MTAP activity is thought to be incidental and not of pathogenic consequence. Therefore, this study did not address genetic alterations at the 9p21 locus, which inhibit BCL2 function and are predicted to have altered the sensitivity of tumor cells to chemotherapeutic agents by restoring sensitivity to apoptosis. Several strategies directed against epidermal growth factor receptor overexpression have included the use of monoclonal antibodies targeted against epidermal growth factor receptor.
tion. It has been demonstrated that the antipurine-related growth-inhibitory action of the antifolate agent methotrexate was more pronounced in subsets of pancreatic carcinoma cell lines that were p16−, MTAP− than in pancreatic carcinomas or normal keratinocyte epithelial cells that were p16+, MTAP+. Furthermore, it was shown that the co-addition of an inhibitor of MTAP enhanced the potency and efficacy of the antipurine-related growth-inhibitory actions of methotrexate in MTAP− but not MTAP+ cell lines. Thus, MTAP+ cells are less sensitive to the inhibitory effects that antifolates such as methotrexate have on purine de novo synthesis. In contrast, because MTAP-deficient malignant cells cannot recycle the purine moiety of methylthioadenosine, tumor cells are more dependent on purine de novo biosynthesis and are more sensitive than MTAP-containing cells to the antipurine actions of antifolates.

To examine the effects of MTAP in tumorigenesis, Christopher et al13 reintroduced MTAP into MCF-7 breast adenocarcinoma cells. Although MTAP expression does not affect the growth rate of cells in standard tissue culture conditions, it severely inhibits their ability to form colonies in soft agar or collagen. In addition, the study showed that MTAP-expressing cells are suppressed for tumor formation when implanted into severe combined immunodeficient (SCID) mice. Because MTAP expression causes a significant decrease in intracellular polyamine levels and alters the ratio of putrescine to total polyamines, consistent with this observation, the study also showed that the polyamine biosynthesis inhibitor α-difluoromethylornithine inhibited the ability of MTAP-deficient cells to form colonies in soft agar, whereas addition of the polyamine putrescine-stimulated colony formation in MTAP-expressing cells, suggesting that MTAP-negative tumors may be particularly sensitive to α-difluoromethylornithine. The results of this study indicate that MTAP has tumor suppressor activity and suggest that its effects may be mediated by altering intracellular polyamine pools.

There are a variety of potential implications of these findings for clinical cancer chemotherapy, including HNSCC. If normal cells, which are uniformly MTAP+, are intrinsically less sensitive to the antipurine actions of antifolates such as methotrexate, then this might in part be the basis for the relatively selective action that these agents have against certain malignancies. If the corollary is correct that MTAP-deficient malignant cells are especially sensitive to certain antifolates that act in whole or in part by inhibiting purine de novo synthesis, one would theoretically obtain an improved therapeutic index by identifying the subset of patients with MTAP-deficient malignancies (including HNSCC) and treating them with such agents. α-Difluoromethylornithine is currently undergoing clinical trials for use as adjunct therapy for a variety of different cancers. Genomewide profiling studies of oral squamous cell carcinoma, which found frequent deletions of MTAP and loss of MTAP in 1 of 6 cell lines in this study, provide a strengthening rationale for a role of MTAP in HNSCC. Stratifying the prognosis of 9p21-deleted HNSCC for MTAP status may provide a rationale to examine this locus in larger studies to assess the feasibility of polyamine biosynthesis inhibitors as novel treatment interventions in MTAP-deleted HNSCC tumors.

There is a large gap in our knowledge and understanding of the frequency and precise loss of p15, p14, and MTAP in HNSCC tumorigenesis. Better understanding of the contribution of these 4 gene products in the prognosis of 9p21-altered HNSCC can provide impetus for exploitation of these targets as therapeutic biomarkers in head and neck cancer.

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Correspondence: Maria J. Worsham, PhD, Department of Otolaryngology–Head and Neck Surgery, Henry Ford Hospital, 1 Ford Pl, 1D, Detroit, MI 48202 (mworsham@hfhs.org).

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lase as target for chemoselective treatment of T-cell acute lymphoblastic leukemia.


