Fine-Mapping Loss of Gene Architecture at the CDKN2B (p15<sup>INK4b</sup>), CDKN2A (p14<sup>ARF</sup>, p16<sup>INK4a</sup>), and MTAP Genes in Head and Neck Squamous Cell Carcinoma

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Objective: To identify the extent and the smallest region of loss for CDKN2B<sup>INK4b</sup>, CDKN2A<sup>ARF, INK4a</sup>, 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) (p15<sup>INK4b</sup>), CDKN2A (p14<sup>ARF</sup>, p16<sup>INK4a</sup>) 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 CDKN2B<sup>INK4b</sup>, 7 for CDKN2A<sup>ARF, INK4a</sup>, 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 at the DNA level for CDKN2A.

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 CDKN2A<sup>ARF, INK4a</sup> starting at p16<sup>INK4a</sup> exon 1a to include exons 2 and 3. Homozygous loss was indicated for CDKN2B<sup>INK4b</sup> and CDKN2A<sup>ARF, INK4a</sup> in UMSCC-11A, and UMSCC-81A. Cell line UMSCC-81B indicated retention of all 9p loci except for exon 1a (p16<sup>INK4a</sup>). 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 1a (p16<sup>INK4a</sup>) as the smallest region of loss in the CDKN2A<sup>ARF, INK4a</sup> gene. The frequency and precise loss of CDKN2B<sup>INK4b</sup>, CDKN2A<sup>ARF, INK4a</sup>, 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.

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. Deletion of MTAP was the most frequent alteration in genomewide profiling studies of oral squamous cell carcinoma. Because deletion of CDKN2A (p16) and CDKN2B (p14) causes dysregulation of the 2 pathways, Rb and p53, important in most cancers, loss of MTAP activity is thought to be incidental and not of pathogenic consequence.

The goal of this study was to fine map precise architectural loss of CDKN2A (p14, p16), CDKN2B (p15), and MTAP to identify the extent to which this locus is affected by deletion, offering additional therapeutic targets of consideration in HNSCC.
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 UMSSC-81A) (Figure 2B), and gain of a copy number is correspondingly denoted by an increase in peak height.

### Abbreviations:
- **CDKN2A/B**: cyclin-dependent kinase 2A/B, ELAVL2, embryonic lethal, abnormal vision, Drosophila, homolog-like 2; FLJ00026, alias, DOCK8, dedicator of cytokinesis 8; IFNB1, interferon 1, fibroblast; IFNW1, interferon α1; 0000000XAA1354, IFNA5 gene for interferon α5; MLT3, myeloid lymphoid or mixed-lineage leukemia (trithorax [Drosophila] homolog), translocated to 3; MTAP, methylthioadenosine phosphorylase; PCR, polymerase chain reaction; TEK, endothelial-specific receptor tyrosine kinase.

*See Figure 1.

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

<table>
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<th>Length of PCR</th>
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### Figure 2. Fine-mapping multiplex ligation-dependent probe amplification assay illustrated for cell lines UMSSC-81A/B. A, Note 38 individual peaks ranging from 130 to 472 base pairs in the male control sample (Table). The 3 cyclin-dependent kinase 2B (CDKN2B) probes are depicted in orange (probes 1, 2, and 3 in Figure 1); the 2 CDKN2A p14 probes are in blue (probes 4 and 5 in Figure 1); the 2 CDKN2A probes between p14 and p16 are in purple (probes 6 and 7 in Figure 1); the 3 CDKN2A p16 probes are in green (probes 8, 9, and 10 in Figure 1); and the 3 methylthioadenosine phosphorylase (MTAP) probes are in red (probes 11, 12, and 13 in Figure 1). B, Note the complete loss of the CDKN2B/A genes (a total of 10 probes) in UMSSC-81A compared with the normal male genomic DNA. Note the retention of all 3 MTAP probes. C, The UMSSC-81B cell line indicated retention of all 9p21 loci except for exon 1α (p16INK4a), making the latter the smallest region of overlap for loss in the CDKN2A/B region. Note the retention of all 3 MTAP probes. Asterisk indicates the minimal region of loss (CDKN2A exon 1α).

Cell line DNA, starting with approximately 20 to 50 ng, is interrogated for the 38 genes in 1 reaction tube. Briefly, DNA is diluted with water to a total volume of 5 µL and then is 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 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.
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, 2^n). 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.

Primers were designed in intron 1e 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’ GGCAAGGAGGACCATATTCTGAGCT 3’; 5’ nucleotide 187697: 5’ GAGCCAAGTCTCGGTGACTCACT 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).

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 1e 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.23 Other genes being targeted for gene therapy include the B-cell leukemia/lymphoma 2 (BCL2) gene homologue BCL2X, which inhibits BCL2 function and has been shown to have altered the sensitivity of tumor cells to chemotherapeutic agents by restoring sensitivity to apoptosis.24 Several strategies directed against epidermal growth factor receptor overexpression have included the use of monoclonal antibodies targeted against epidermal growth factor receptor.25 A chimeric anti–epidermal growth factor receptor monoclonal antibody (C225) has been tested in combination with either cisplatin26 or radiation,27 and the results are promising.

Genetic alterations at the 9p21 locus have been linked to malignant progression in HNSCC.2,3 Currently, recombinant adenovirus capable of directing a high level of p16 protein expression (Ad5-p16) demonstrated a significant antitumor effect of Ad5-p16 against human HNSCC in vivo.13 Other proteins produced at this 9p21 locus—p15 (CDKN2B), p14 (CDKN2A), and MTAP—each have the potential to independently serve as therapeutic targets in HNSCC.

In this study, 7 HNSCC cell lines, including 6 that indicated loss and retention of the CDKN2A/B locus by an MLPA genomewide 112-probe panel,21 were examined using a fine-mapping 9p21 probe set. We found homozygous loss at the CDKN2A locus in 5 of 6 HNSCC cell lines: UMSCC-11A, UMSCC-17A/B, UMSCC-81A, and UMSCC-81B. The UMSCC-81B cell line indicated retention of all 9p loci except for exon 1 (p16INK4a), making the latter the smallest region of overlap for loss in the UMSCC-81B cell line. The UMSCC-81B cell line indicated retention of all 9p loci except for exon 1 (p16INK4a), making the latter the smallest region of overlap for loss in the UMSCC-81B cell line. The latter would explain the observation of an intact 9p21 region in UMSCC-11B and offers a rationale, presumably, for selection of a tumor subpopulation that escapes chemotherapy likely aided by an intact 9p21 DNA repair and survival repertoire.

In UMSCC-11A, of the 3 MTAP MLPA probes (1 at the 3‘ end, 1 in intron 6, and 1 at the 5‘ end), homozygous loss was indicated for only the 3‘ end of the MTAP gene, pointing to selective loss of the carboxy terminal region of the gene (Figure 1B).

Toohey,28 a quarter of a century ago, first recognized that certain murine malignant hematopoietic cell lines lacked MTAP activity. The function of MTAP is to cleave methylthioadenosine, a by-product of polyamine metabolism, to adenosine and 5′-methylthioribose-1-phosphate, which are recycled to adenosine nucleotides and methionine, respectively. The MTAP, the first enzyme in the pathway, seems to be expressed in all normal human tissues,30,31 which suggests that the entire salvage pathway is present in all of the cells in the human body, designating MTAP an important housekeeping gene in malignancy.

Genetic studies show high rates of MTAP loss in non–small cell lung cancer, melanoma, bladder cancer, pancreatic cancer, osteosarcoma, and endometrial cancer.32-35 Tumors that lack MTAP are expected to be sensitive to inhibitors of purine synthesis or methionine starvation. Genomewide profiling studies of oral SCC17 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 CDKN2AINK4a (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.18 However, there are several reasons proposed to suggest that this may not be the case.14 First, homozygous deletion is an unusual mechanism for inactivation of a tumor suppressor gene. Most tumor suppressor genes are inactivated by point mutation of one allele followed by loss of the other allele (loss of heterozygosity). This is rarely observed for CDKN2A.36 A likely hypothesis for this observation is that homozygous deletion can remove more than 1 gene from the region, whereas point mutation followed by loss of heterozygosity cannot.14 Second, in certain cancers, loss of MTAP has been observed in cells that retain p16. Schmid et al32 found in a study of non–small cell lung cancer that homozygous deletion of MTAP occurred in 38% (19 of 50) of the samples compared with only 18% (9 of 50) for p16. In another study,37 it was found that 3 of 7 primary astrocytomas were deleted for MTAP, but only 2 of 7 were deleted for p16. The fact that MTAP is lost independently of p16 hints that loss of MTAP may have some functional basis in tumor biology.14 In UMSCC-11A, despite homozygous loss of CDKN2A/B genes, only the 3‘ end of MTAP was lost. The latter may point to the 3‘ end as a selective target for gene deletion, suggesting the carboxy terminal of the gene as a critical region.

Tumors that lack MTAP are expected to be sensitive to inhibitors of purine synthesis or methionine starva-
related growth-inhibitory actions of methotrexate in enhanced the potency and efficacy of the antipurine-shown that the co-addition of an inhibitor of MTAP en-
cers. Genomewide profiling studies of oral squamous use as adjunct therapy for a variety of different can-
set of patients with MTAP-deficient malignancies (including HNSCC) and treating them with such agents.
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 al 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-Difluoro-
methylornithine is currently undergoing clinical trials for use as adjunct therapy for a variety of different cancers.

There is a large gap in our knowledge and understanding of the frequency and precise loss of p15, p14, p16, 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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