

Homozygous Deletion of *CDKN2A* and Codeletion of the Methylthioadenosine Phosphorylase Gene in the Majority of Pleural Mesotheliomas

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ABSTRACT

Purpose: Homozygous deletions at chromosome region 9p21 targeting the *CDKN2A* gene have been reported as a common cytogenetic abnormality in mesothelioma. *MTAP*, a gene ~100-kb telomeric to *CDKN2A*, encodes methylthioadenosine phosphorylase, an enzyme essential in the salvage of cellular adenine and methionine, and its codeletion with *CDKN2A* has been reported in other tumors. The aim of this study was to define the prevalence of homozygous deletion of *CDKN2A* alone or in combination with *MTAP* in a large series of pleural mesothelioma.

Experimental Design: We used a fluorescent *in situ* hybridization assay for *CDKN2A* and *MTAP* on interphase nuclei in imprints of frozen tissue from 95 cases of pleural mesothelioma. Histologically, the cases were classified as epithelial (71), biphasic (19) and sarcomatous (5). In each experiment, a 9p21 locus specific probe and a chromosome 9 centromeric probe were used and fluorescent *in situ* hybridization signals for both probes were simultaneously recorded in at least 100 nuclei. Cases were considered homozygously deleted if both 9p21 signals were lost in at least 20% of nuclei.

Results: Overall, 70 cases (74%) had homozygous deletion of *CDKN2A*. *MTAP* was codeleted in 64 of these cases (91%). No case with *MTAP* deletion without *CDKN2A* deletion was identified. Homozygous loss of *CDKN2A* was seen in 49 of 71 epithelial (70%), 16 of 19 biphasic (89%), and 5 of 5 sarcomatous (100%) mesotheliomas.

Conclusions: Homozygous deletion of *CDKN2A* is seen in the majority of pleural mesotheliomas, and *MTAP* is codeleted in most of these cases. Previous cell line studies have shown that loss of *MTAP* renders cells dependent on *de novo* synthesis of purine derivatives. Thus, the particularly

high prevalence of *MTAP* codeletion in mesothelioma makes it an ideal candidate for trials of targeted therapy using inhibitors of *de novo* AMP synthesis (e.g., L-alanosine).

INTRODUCTION

Malignant mesothelioma is an aggressive neoplasm of the serosal membranes of the body cavities. The majority of cases occur in patients exposed to asbestos. The diagnosis is often difficult because in most cases mesothelioma has an insidious onset, and the patients present with nonspecific symptoms. Recent advances in multimodality management have resulted in moderately improved survival but mainly for patients with stage I disease (1–3).

The molecular pathogenesis of malignant mesothelioma appears to involve a still poorly understood combination of exposure to environmental (asbestos) and infectious (SV40) agents and somatic genetic alterations (most commonly in *CDKN2A* and *NF1*), as recently reviewed elsewhere (4). Cytogenetic and molecular studies have identified several frequent genetic alterations in mesothelioma (5, 6), of which one of the most common is homozygous deletion of the 9p21 locus within a cluster of genes that includes *CDKN2B*, *CDKN2A*, and *MTAP*. *CDKN2B* and *CDKN2A* encode cell cycle regulatory proteins, whereas *MTAP* encodes methylthioadenosine phosphorylase, an enzyme essential in the salvage pathway of AMP synthesis and in methionine synthesis. Results from several groups suggest that the prevalence of *CDKN2A* deletion in malignant mesothelioma is up to 72% among primary tumors and may be even higher in mesothelioma cell lines (7–9). In addition, as in other cancer types, studies of mesothelioma have described *CDKN2A* promoter methylation as an alternative mechanism of *CDKN2A* inactivation in some nondeleted cases (10).

CDKN2A encodes two important cell cycle regulatory proteins, the p16 protein (11) and, in an alternative reading frame, the p14ARF protein (12). P16, a cyclin-dependent kinase inhibitor, acts through CDK4/CDK6 and blocks the phosphorylation of the RB protein, and p14ARF binds MDM2, thus preventing the latter from binding p53 and targeting it for degradation (reviewed in Ref. 13).

MTAP converts methylthioadenosine, a product of polyamine synthesis, to adenine and methylthioribose-1-phosphate. The former is used for AMP and the latter for methionine synthesis (Fig. 1). Thus, tumor cells that lack *MTAP* are completely dependent on *de novo* synthesis of purine derivatives for the generation of AMP and, therefore, are potentially sensitive to inhibitors of the *de novo* purine synthesis pathway (e.g., L-alanosine).

On the basis of studies of several tumor types, it appears that the 9p21 deletions targeting *CDKN2A* are usually large and often also include *CDKN2B* and *MTAP* (14–16). However, there have been no systematic studies of the frequency of

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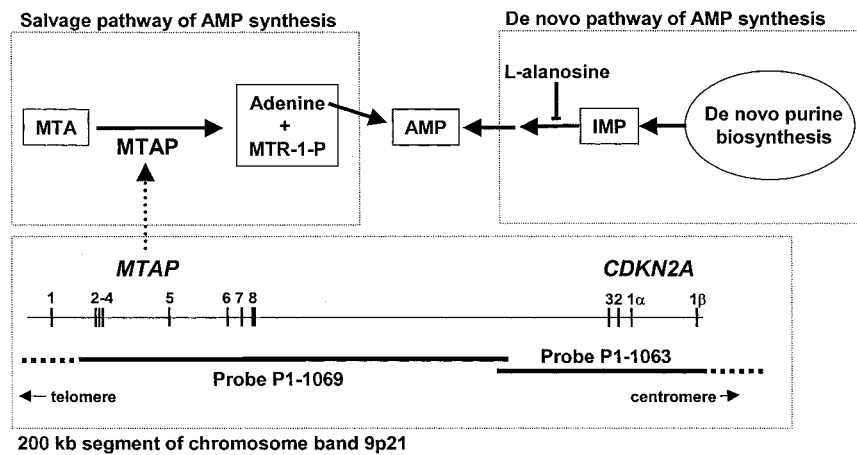


Fig. 1 Top panels show the *de novo* and salvage pathways of AMP synthesis and the effects of L-alanosine and loss of MTAP. AMP deficiency impairs DNA synthesis and cellular energy production, which require dATP and ATP, respectively. A more detailed version of these metabolic pathways is available elsewhere (34). Bottom panel shows a schematic gene map of chromosome 9p21 representing the 200-kb portion containing the *CDKN2A* and *MTAP* genes and the areas covered by the P1-1063 and P1-1069 FISH probes. The P1-1063 probe covers the *CDKN2A* and *CDKN2B* genes (latter not shown). The telomeric extent of the P1-1069 probe includes at least exon 2 of the *MTAP* gene. The intronic and intergenic dimensions are to scale and are based on human genome data online.⁴ The positions and sizes of the P1 clones are approximate. MTA, methylthioadenosine; MTR-1-P, methylthioribose-1-phosphate; AMP, adenine monophosphate.

codeletion of *CDKN2A* and *MTAP* in malignant mesotheliomas. To address this issue, we have used FISH³ with probes corresponding to *CDKN2A* or *MTAP* to determine the frequency of *CDKN2A-MTAP* codeletion in 95 cases of primary pleural malignant mesothelioma. Our results confirm the high prevalence of *CDKN2A* deletion in mesotheliomas and suggest that this tumor may be an especially attractive candidate for *MTAP*-directed therapy.

MATERIALS AND METHODS

We studied 96 frozen tissue specimens from 95 patients (17 females, mean age: 55.8 years, range: 30–76 years; 78 males, mean age: 63.2, range: 32–77 years) collected between 1992 and 2000. Samples were procured at MSKCC under an approved Institutional Review Board protocol. The diagnosis in each case was confirmed by histological examination and electron microscopy or immunohistochemistry if necessary. Histologically, the cases included 71 epithelioid, 19 biphasic, and 5 sarcomatoid subtypes. We also studied three mesothelioma cell lines (JMN, NCI-H2052, NCI-H28; gifts of Frank Sirotak, MSKCC) and used a Ewing's sarcoma cell line (A673; American Type Culture Collection, Manassas, VA) with known homozygous 9p21 deletion (17) and normal peripheral blood lymphocytes as controls.

Slide Preparation. Tissue imprints of the frozen tissue were air dried followed by fixation in modified Carnoy's fixative (methanol:glacial acetic acid = 3:1) for 30 min and then by air drying. The slides were stored on -20°C until hybridization. The first and last imprints were stained with Diff Quik to

evaluate the presence of tumor cells. Pretreatment with collagenase H and postfixation with formalin were performed as described previously (18).

Probe Preparation. Plasmid DNA from clones P1-1063 and P1-1069 (Ref. 11; gift of Alex Kamb, Myriad Genetics, Salt Lake City, UT) containing a fragment of human genomic DNA ~ 100 kb from the *CDKN2A* and *MTAP* regions, respectively (Fig. 1), was isolated from large scale bacterial cultures using standard methods (Qiagen plasmid maxi kit; Qiagen, Inc., Valencia, CA). Labeled probe was prepared by nick translation (Nick translation kit; Vysis, Inc., Downers Grove, IL) using spectrum orange- or spectrum green-labeled dUTP (Vysis, Inc.), following the manufacturer's instructions. The chromosome 9 centromere probe used for two- or three-color FISH, labeled with spectrum green or spectrum aqua, respectively, was CEP-9 (Vysis, Inc.). Probes were stored at -20°C .

FISH. In each experiment, dual color FISH was performed using a spectrum green-labeled CEP9 probe and a spectrum orange-labeled *CDKN2A* (P1-1063) or *MTAP* (P1-1069) probe and Ewing's sarcoma cell line A673 (with known homozygous deletion of *CDKN2A*; Ref. 17) and normal peripheral blood lymphocytes served as positive and negative controls, respectively. The slides and probe DNA was denatured separately in 70% formamide/ $2\times$ SSC (pH 7.4, $73-75^{\circ}\text{C}$, 5 min). After denaturation, the slides were dehydrated in an ice-cold graded ethanol series, and the probe mix was applied. Slides were coverslipped, sealed with rubber cement, and overnight hybridization was performed in a humid chamber at 37°C . Posthybridization wash was performed in $1\times$ SSC/ 0.3% NP40 ($72-73^{\circ}\text{C}$, 2 min). The air-dried slides were then stained with 4',6-diamidino-2-phenylindole-II (Vysis, Inc.).

Scoring. Slides were examined and images were obtained using an epifluorescent microscope (Olympus BX40; Olympus, Melville, NY) equipped with appropriate filters and

³ The abbreviations used are: FISH, fluorescent *in situ* hybridization; MSKCC, Memorial Sloan-Kettering Cancer Center.

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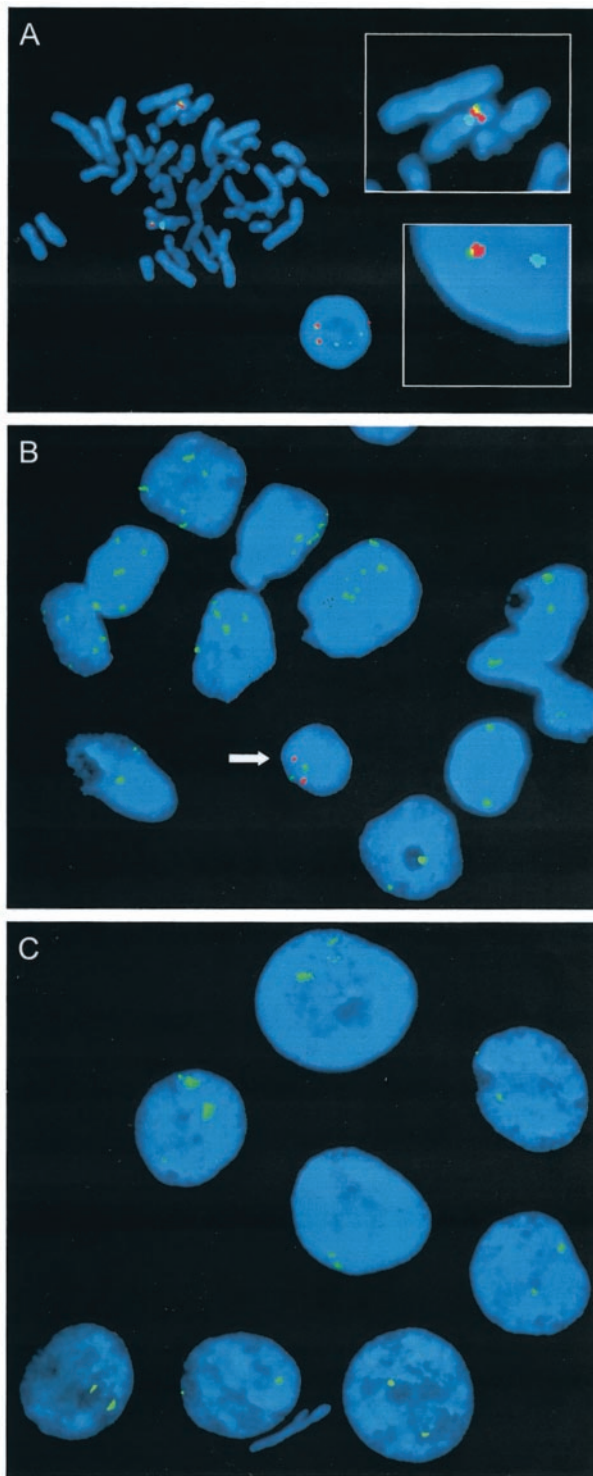


Fig. 2 A, Three-color FISH of normal peripheral blood lymphocytes with probes for chromosome 9 centromere (Vysis CEP9, spectrum aqua), *CDKN2A* (P1-1063, spectrum orange) and *MTAP* (P1-1069, spectrum green). Insets show higher magnification of metaphase and interphase signals. B and C, two-color FISH of frozen mesothelioma tissue imprints showing homozygous deletion of 9p21 genes; B, homozygous deletion of *CDKN2A* (P1-1063, spectrum orange) with chromosome 9 aneuploidy (Vysis CEP9, spectrum green). The arrow marks a normal nucleus (lymphocyte). C, homozygous deletion of *MTAP* (P1-1069, spectrum

an image analysis system (Applied Imaging, Santa Clara, CA). Signal number for both probes was recorded simultaneously in at least 100 nuclei. Cases with >20% of nuclei lacking both signals for the locus-specific probe (*CDKN2A* or *MTAP*) and showing at least one signal for the CEP-9 probe were considered homozygously deleted.

PCR. To confirm the identity of the P1 clones P1-1063 and P1-1069, used as FISH probes, we verified by PCR that they contained *CDKN2A* and *MTAP*, respectively. For *CDKN2A*, we used primers for exon-1 α (5'-GAAGAAAGAGGAGG-GGCTG-3' and 5'-GCGCTACCTGATTCCAATTC-3') and exon-1 β (5'-CCCAGTCTGCAGTTAAGG-3' and 5'-GTCTA-AGTCGTTGTAACCCG-3'; gift of Paola Capodici, MSKCC), in separate reactions. The annealing temperature for *CDKN2A* exon-1 α primers was 55°C and for *CDKN2A* exon-1 β primers was 55°C (35 cycles) using an iCycler (Bio-Rad, Hercules, CA). For *MTAP*, we used primers for exons 2–7 (gift of Richard Gorlick, MSKCC), as described elsewhere (19).

RESULTS

We first confirmed by PCR on DNA from P1 clones 1063 and 1069 that *CDKN2A* and *MTAP* were respectively contained within the aliquots used as FISH probes (results not shown). Probe specificity was also verified by triple color FISH (CEP-9 spectrum aqua, P1-1063 spectrum orange, and P1-1069 spectrum green) on metaphase spreads of normal peripheral blood lymphocytes (Fig. 2A). As expected from human genome data and previous publications, P1-1069 is telomeric to P1-1063 on 9p. Thus, *CDKN2A* and *MTAP* deletion could be studied separately, using these clones individually.

The FISH results on the mesothelioma frozen tissues are summarized in Table 1. Using probe P1-1063, homozygous deletion was detected in 70 of 95 (74%) cases. By histological subtype, homozygous deletion of *CDKN2A* was identified in 49 of 71 (69%) cases with epithelioid histology (Fig. 2, B and C), in 16 of 19 (84%) cases with biphasic histology, and in 5 of 5 (100%) cases with sarcomatoid histology. One patient with epithelioid mesothelioma had two specimens from two consecutive surgeries and both had homozygous deletion and were counted as one case.

Using the same scoring criteria, homozygous deletion of *MTAP* was identified using probe P1-1069 in 64 of 95 cases (67%). No *MTAP* deletion was seen without *CDKN2A* deletion. Thus, 64 of 70 (91%) cases with *CDKN2A* deletion were also codeleted for *MTAP*.

Chromosome 9 copy number was abnormal in 42 cases (44%), including 28 of 71 (39%) epithelioid, 9 of 19 (47%) biphasic, and 5 of 5 (100%) sarcomatoid cases. Cases with both monosomy and polysomy 9 were identified, however, gains were more common than losses (Fig. 2B).

Homozygous deletion of *CDKN2A* and *MTAP* was detected in two of three mesothelioma cell lines (JMN and NCI-H2052), whereas neither gene was deleted in the third cell line (NCI-H28; results not shown).

orange) with normal chromosome 9 copy number (Vysis CEP9, spectrum green). No normal nuclei are present in this field.

Table 1 Summary of FISH data on 95 cases of pleural mesothelioma

Histological type	Homozygous deletion				Chromosome 9 aneuploidy	
	CDKN2A		MTAP			
Epithelioid	49/71	69%	45/71	63%	28/71	39%
Biphasic	16/19	84%	15/19	79%	9/19	47%
Sarcomatoid	5/5	100%	4/5	80%	5/5	100%
Total	70/95	74%	64/95	67%	42/95	44%

DISCUSSION

Published studies suggest that homozygous deletion of *CDKN2A* is one of the most frequent genetic alterations in malignant mesothelioma (7–9). The present analysis of 95 cases of pleural mesothelioma demonstrates a prevalence of *CDKN2A* homozygous deletion of 74%. In the largest previous study of primary tumors, homozygous *CDKN2A* deletion was found by a similar FISH assay in 36 of 50 cases (72%; Ref. 8). Detection of deletions by FISH has the advantage of being able to identify cases with hemizygous *versus* homozygous deletion and cases with heterogeneous tumor cell populations. In addition, admixed benign cells (stromal cells, lymphocytes, and so forth) do not raise the risk of false negative results because of the *in situ* nature of the analysis, a problem commonly encountered with methods using DNA or RNA extracted from bulk tumor tissue (e.g., PCR, Southern blot, or Northern blot). Thus, in a study that included both primary tumor material and corresponding cell lines from 21 patients, *CDKN2A* deletion was detectable in all 21 cell lines but in only 5 of the matching primary tumors (7). In retrospect, some studies that used PCR-based techniques to detect homozygous *CDKN2A* deletion in primary tumors may have underestimated the proportion of deleted cases because of the contribution of admixed benign cells to the DNA used for the PCR assays. Thus, the *CDKN2A* deletion rate in mesothelioma is among the highest of any studied tumor type. Other tumors with frequent 9p21 deletion include high-grade gliomas (11, 20, 21), acute lymphoblastic leukemia (22, 23), pancreatic adenocarcinomas (24, 25), and bladder carcinomas (14), among others. We also identified 5 cases with hemizygous loss of *CDKN2A*, of which 4 also showed loss of one copy of *MTAP*. *CDKN2A* promoter methylation analysis in these 5 cases showed methylation in one case (P. B. I., M. L., unpublished data).

By histological subtype, we found that homozygous deletion of *CDKN2A* was somewhat more common in cases with sarcomatous elements (biphasic or pure sarcomatoid) than in cases with epithelioid histology, but this did not achieve statistical significance (88 *versus* 69%; $P = 0.10$). However, this trend is of interest because in a smaller series ($n = 50$), Xiao *et al.* (8) found a similar difference in the *CDKN2A* deletion rates in these two histological groups (100 *versus* 52%; $P < 0.001$).

We also found that both copies of *MTAP* were lost in 67% of cases. Because no *MTAP* deletion was identified without concurrent *CDKN2A* deletion, this represented a 91% codeletion rate. Two of three mesothelioma cell lines that we studied showed homozygous deletion of both *CDKN2A* and *MTAP*, and one cell line showed no loss of either genes. Specific published data on *MTAP* deletion or codeletion in mesothelioma are lim-

ited, but a reexamination of previous studies reveals results consistent with the present data. Olopade *et al.* (26) found *MTAP* and *CDKN2A* codeletion in 5 of 5 mesothelioma cell lines. Prins *et al.* (9) mapped 9p21 deletions in 12 mesothelioma cell lines. STS marker 1063.7 (known to map between *CDKN2A* and *MTAP*) was preserved in 5 cell lines and lost in 7, suggesting that *MTAP* codeletion may have occurred in the latter 7 cell lines (58%). Because Xiao *et al.* (8) used a probe mixture of P1-1063 and P1-1069 in their FISH analysis for *CDKN2A* deletions, their percentage of 72% deleted cases also applies to *MTAP*. Comparable rates of *MTAP* deletion or codeletion have only been detected in high-grade gliomas and leukemias (26). Moreover, it is possible that the proportion of mesotheliomas with *MTAP* inactivation may be even higher than detected in the present series because a minority of cases have been reported to show deletion of only the last four exons of *MTAP* (27, 28), which may leave enough of the *MTAP* gene to result in a hybridization signal with the P1-1069 FISH probe. Immunohistochemistry or a combination of microdissection and PCR might help to identify these few additional cases among the 9% of *CDKN2A*-deleted cases lacking FISH evidence of *MTAP* codeletion.

Our finding that *MTAP* was only deleted in the presence of *CDKN2A* deletion is in agreement with the majority of published studies in other tumor types (14–16, 22, 23, 29). In contrast, some studies have reported a minority of cases with evidence of *MTAP* deletion without *CDKN2A* loss among other tumor types (19, 21, 28). The issue remains unresolved because the latter studies have been based on PCR analysis of DNA extracted from tumor tissue and none, to our knowledge, have identified *MTAP* loss without *CDKN2A* loss in cell lines or by FISH.

L-Alanosine, the L-isomer of alanosine, is an inhibitor of *de novo* AMP synthesis that was the subject of a National Cancer Institute-sponsored study between 1978 and 1985 (Refs. 30–32; reviewed in Ref. 33). In Phase I and Phase II studies, ~300 patients (with renal cell carcinoma and malignant melanoma) were treated, but the results were discouraging. However, these tumors were not tested for 9p21 deletions. In addition, subsequent retrospective analysis of the cases failed to identify *MTAP* deletions in these tumor types (33). Recent *in vitro* cytotoxicity studies of pediatric T-cell acute lymphoblastic leukemias and adult T-cell leukemia have demonstrated that, as expected, *MTAP*– leukemia cells are more sensitive to the toxicity of L-alanosine than are *MTAP*+ leukemic cells (34, 35). Moreover, normal lymphocytes are rescued from L-alanosine toxicity by the *MTAP* substrate, 5'-deoxyadenosine (34–36). In these independent studies, L-alanosine alone or in combination with a

salvage agent was shown to be clinically active in tumors with homozygous *MTAP* deletions. In addition, reintroduction of the *MTAP* cDNA in *CDKN2A*-/*MTAP*-pancreatic carcinoma cell lines restored the *MTAP*-dependent adenine and methionine salvage pathways, decreased the rates of *de novo* synthesis, and decreased cellular sensitivity to the antipurine-related growth inhibitory actions of methothrexate and azaserine (37).

Malignant mesothelioma is a highly malignant tumor with an aggressive course. Most cases are diagnosed at an advanced stage and have a short survival time (6–10 months), and current chemotherapeutic agents are not effective in the majority of cases. The high deletion rate of the 9p21 locus including the *MTAP* gene makes mesothelioma a strong candidate for L-alanosine and other inhibitors of *de novo* AMP synthesis. The very high prevalence of *CDKN2A* deletion also makes this tumor an interesting target for gene therapy approaches that restore the function of p16CDKN2A or p14ARF (38, 39) or that target tumor cells with genetic or functional defects in the p53 pathway (40). Finally, the very high prevalence of *CDKN2A* deletion in mesothelioma can also be used a diagnostic marker in the distinction between reactive and neoplastic mesothelial cells in pleural effusions (41).

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