Methylthioadenosine Phosphorylase Gene Deletions Are Common in Osteosarcoma

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ABSTRACT

Purpose: Methylthioadenosine phosphorylase (MTAP) is an enzyme essential in the salvage of cellular adenine and methionine synthesis. The MTAP gene is located in the 9p21 chromosomal region and its loss is frequently associated with deletion of the tumor suppressor genes p15INK4b and p16INK4a. The aim of this study was to investigate the frequency of molecular alterations in MTAP in osteosarcoma.

Experimental Design: Samples from patients with high-grade osteosarcoma (n = 96) and three osteosarcoma cell lines (HOS, SaOS-2, and U2OS) were analyzed. Genomic DNA was analyzed for MTAP gene deletions by PCR, RNA expression was measured by semiquantitative reverse transcription-PCR, and the protein levels were measured by immunohistochemistry.

Result: Deletion of at least one MTAP exon was found in 36 of 96 (37.5%) osteosarcoma patient samples and in one of the three cell lines (HOS). In all cases in which an MTAP gene deletion was observed, there was absence of detectable mRNA and protein. Furthermore, in four osteosarcoma patients, an MTAP deletion which was not evident at diagnosis was detected in subsequent tumor samples.

Conclusions: The MTAP gene is commonly deleted in osteosarcoma patient samples, leading to an absence of mRNA and protein expression; these results indicate that inhibitors of de novo purine synthesis or methionine depletion may be effective as treatments for osteosarcoma patients whose tumors fail to express MTAP.

INTRODUCTION

MTAP5 (MeSAdo phosphorylase) is a ubiquitous enzyme that catalyzes the phosphorolysis of the nucleoside MTA, which is generated during the synthesis of polyamines spermidine and spermine (Ref. 1; Fig. 1). MTAP is an essential enzyme in the salvage pathway of adenine and in methionine synthesis (2–5). The gene that encodes this enzyme is mapped to chromosome locus 9p21, which is 100 kb telomeric to the genes encoding the cyclin-dependent kinase inhibitors p15INK4b and p16INK4a, which are often deleted in tumor cells (6–9).

Homozygous deletions of the MTAP gene have been observed in hematological neoplasias, i.e., non-Hodgkin lymphomas (10); or the acute form of lymphoblastic leukemia, particularly T cell (11, 12) but not the chronic form (12). In addition, MTAP deletions have been described in small cell (13) and non-small cell (14) lung cancer, bladder cancer (15), pancreatic carcinoma cell lines (3), endometrial adenocarcinoma (16), ovarian cancer (17), melanoma (18), glioma (19), head and neck cancer (20), and in myxoid chondrosarcoma and in soft-tissue sarcoma (20–23). Despite the frequency of INK4 deletions in this locus, no study of MTAP gene deletions in osteosarcoma has been reported to date.

The major aim of this study was to analyze osteosarcoma patient samples and cell lines for MTAP gene deletions. Samples were analyzed for MTAP gene deletions by PCR for exons 2–7. The exons were screened for MTAP mutations using a SSCP method. To support the presence or absence of MTAP deletions, MTAP mRNA expression was analyzed by quantitative RT-PCR, and protein expression was analyzed by immunohistochemistry and Western-blot. The MTAP status was related to patient clinical features to identify any potential association.

MATERIALS AND METHODS

Patient Samples. Samples from 96 high-grade osteosarcoma tumors were obtained from patients who underwent surgery at Memorial Sloan-Kettering Cancer Center. The majority of the patients were treated on the institutional T12 or the Children’s Cancer Group 7921 protocols, which have been reported previously. The specimens were obtained in accordance with a protocol approved by the Memorial Hospital Insti-
MTAP metabolic pathway. a, de novo AMP biosynthesis is shown on the left side of the diagram. This pathway is a target for several chemotherapeutic agents, including L-alanosine. The AMP salvage pathway is shown on the right side of the diagram. MTA is cleaved into adenine and MTR-1-P by MTAP. Adenine is recycled to AMP by the enzyme adenosine phosphoribosyl transferase. MTR-1-P is converted into methionine by MTAP metabolic pathway.

Cell Lines. Human osteosarcoma cell lines HOS, U2OS, and SaOS-2 and the fibroblast cell line COS 7 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in MEM-α medium containing 10% FCS in a 37°C humidified, 5% CO₂ environment.

Analysis for MTAP Deletions. Genomic DNA was isolated from osteosarcoma patient samples and from cell lines using a genomic DNA Isolation kit (DNAzol; Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s instructions. PCR was performed for each of the samples and cell lines for exons 2–7 of the MTAP gene. Each PCR reaction was performed in triplicate using a Taq DNA Polymerase kit (Life Technologies, Inc.) in a Perkin-Elmer 9700 thermal cycler. Briefly, PCR was performed with 50 ng of genomic DNA in a total volume of 32 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 0.5 μM of each sense and antisense primers (exons 2–7; Table 1), and 2.5 units of Taq DNA polymerase. The PCR conditions were 94°C for 2 min, 35 cycles at 94°C, 56°C, 72°C, each for 1 min, and a final extension at 72°C for 5 min. Primers for β-actin were used as a positive control for the presence of DNA. Electrophoresis of PCR products was performed on a 1.4% agarose gel subsequently stained with ethidium bromide and photographed.

MTAP Template Analysis. The PCR reactions for SSCP were performed as described previously with the addition of 0.1 Ci/mmol of [α-32P]dATP. To perform SSCP, the PCR products were diluted 1:10 in formamide-dye loading buffer (95% formamide, 10 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) and incubated for 3 min at 95°C and cooled on ice for at least 2 min. For electrophoresis, 3 μl of each solution was loaded onto a 6% polyacrylamide/10% glycerol gel for overnight-room temperature electrophoresis and onto a 6% polyacrylamide gel for electrophoresis for 6–9 h at 4°C. Gels were dried and exposed to X-ray film for autoradiography.

Analysis of MTAP RNA Expression. From the total patient samples analyzed, a subgroup of 20 cases with identified MTAP gene deletions and 20 cases without gene deletion were selected. In these cases, MTAP mRNA expression was evaluated by semiquantitative RT-PCR. RNA was isolated using a
total RNA Isolation kit (TRIzol; Life Technologies, Inc.) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed on RNA using 2.5 μM random primer in 20-μl reactions containing 1 unit/μl murine leukemia virus reverse transcriptase, and buffer supplied by the manufacturer (Life Technologies, Inc.) at 42°C for 1 h. The cDNA was amplified by PCR as described previously using the primers shown in Table 1. Radioactive dATP (0.1 Ci/mmol of [α-32P]dATP) was included in the reaction. Electrophoresis of the products was performed on an 8% polyacrylamide gel. After electrophoresis, the gels were vacuum dried and exposed to X-ray film for 24–48 h at −80°C. MTAP:β-actin ratios were calculated by determining the linear range for each sample, plotting the best fit line and determining the MTAP:β-actin ratio at the X intercept. The cell lines COS 7 and HOS were used for standardization and as controls for each run.

**Immunohistochemistry.** In the same subgroup of patients analyzed for mRNA expression and in the three cell lines, protein expression was studied by immunohistochemistry using an avidin-biotin immunoperoxidase assay on 5-μm-thick OCT embedded frozen blocks. Sections were fixed with cold methanol:acetone (1:1 dilution). After blocking endogenous peroxidase, sections were incubated for 15 min with 10% normal horse serum, followed by a 2-h incubation with primary antibody against MTAP (1:500 dilution). The antihuman MTAP chicken antibody was a kind gift of Dr. Dennis Carson (University of California at San Diego Cancer Center). After the sections were washed extensively, they were incubated for 30 min with biotinylated rabbit antichicken IgG antibodies (1:1000 dilution) and then incubated for 30 min with avidin-biotin-peroxidase complex (1:25 dilution). Diaminobenzidine (0.06%) was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. The intensity of immunoreaction was scored as − (negative) when <10% of tumor cells exhibited cytoplasmic immunostaining; + (weak) when 10–20% of cells displayed cytoplasmic immunostaining; ++ (moderate) when 20–50% of cells showed reactivity for MTAP; and +++ (strong) when >50% demonstrated positive immunostaining. Histological specimens were evaluated by a pathologist (C. C-C.) blinded to patient identity, clinical information, and prior studies of MTAP status.

**Statistical Analysis.** To determine the association between MTAP gene status and clinical data, the χ² test was used.

**RESULTS**

**Clinical Data.** The clinical data are summarized in Table 2. Of 96 cases, 44 cases were males and 52 females (ratio M:F, 1:1.2). The osteosarcomas were subclassified as osteoblastic (n = 52), chondroblastic (n = 27), fibroblastic (n = 5), giant cell-rich (n = 3), telangiectatic (n = 2), or mixed (n = 7). In 51 patients, the specimens were obtained from the primary site, in 30 samples from the recurrence, and in 15 patients from the metastatic site. The location of the tumors were distal femur (n = 44), proximal tibia (n = 18), proximal humerus (n = 14), pelvis (n = 12), foot (n = 3), spine (n = 2), and head (n = 3). At the time of diagnosis, distinct metastases were present in 24 patients (25.0%) and absent in 72 patients (75.0%). The chemotherapy responses were classified according to the Huvos grading system as: grade I (n = 12; 12.7%), grade II (n = 23; 23.6%), grade III (n = 23; 23.6%), and grade IV (n = 10; 10.9%).

**Analysis for MTAP Deletions.** In 36 of 96 (37.5%) osteosarcoma samples, deletion of at least one exon of MTAP was observed (summarized in Table 3). The majority of deletions were only partial deletions. The most frequently deleted exon was exon 7. A representative gel for exon 6 is shown in Fig. 2. In one of the three cell lines (HOS), an MTAP deletion was observed (Fig. 3).

**Clinical Correlation.** No significant correlations were observed between genetic alterations of MTAP and clinical data including sex, age, histological subtype, site of primary tumor, type of specimen, the presence or absence of metastasis at diagnosis, and Huvos grade. However, in 4 of 20 paired cases (20%) when the MTAP gene status was compared in the same patient at biopsy and at a later time point (definitive surgery or recurrence), a difference was identified. In all of these cases, the MTAP gene was present at diagnosis but deleted at the later time point.

**Sequence Analysis.** SSCP analysis of tumors and osteosarcoma cell lines with intact MTAP revealed no suggestion of point mutation.

**Analysis of MTAP RNA Expression.** In all 20 osteosarcoma samples analyzed with an intact MTAP gene, expression of MTAP mRNA was observed by semiquantitative RT-PCR. In the other subset of 20 samples that demonstrated an
MTAP deletion, no MTAP mRNA expression was observed. This included both samples with partial and full gene deletions. No detectable MTAP mRNA was observed in the MTAP-deleted HOS cell line. These results are summarized in Table 3.

**DISCUSSION**

A number of human neoplasias have been shown to have structural defects in the chromosome 9p21 region (24). In the
short arm of chromosome 9, the cyclin-dependent kinase inhibitors p15INK4B and p16INK4A have been mapped. These tumor suppressor genes encode a M, 15,000 protein (p15INK4B) and a M, 16,000 protein (p16INK4A), which arrest the cell cycle through inhibiting retinoblastoma protein phosphorylation (25). The MTAP gene is telomeric to the INK4 locus. Codeletion of the MTAP gene with these tumor suppressor genes has been observed in different neoplasias (10, 12, 14, 22, 24). In osteosarcoma cell lines, five of eight (62.5%) demonstrate deletions observed in different neoplasias (10, 12, 14, 22, 24). In osteosarcoma patient samples, a p16INK4A gene alteration has been observed in 9–38% of cases (27–30). A p15INK4B gene alteration has been observed in 14% of the cases (28). No previous studies of MTAP gene deletion have been reported in patients with osteosarcoma.

MTAP deletions were observed in 37.5% of the osteosarcoma samples and in one of three cell lines. This proportion of deletions is similar to those found in certain other malignancies, such as T-cell acute lymphocytic leukemia and non-small cell lung cancer. The studies of MTAP deletion were confirmed at the mRNA and protein levels in a subset of the samples.

In four cases when the MTAP gene status was compared in the same patient at biopsy and at a later time point (definitive surgery or recurrence), a difference was identified. In all of these cases, the MTAP gene was present at diagnosis but deleted at the later time point. It is difficult to assess because of the limited number of cases, but this suggests that a relationship between MTAP deletion and disease progression may exist. These results are in agreement with Dreyling et al. (10) and Hori et al. (12), who found an association between changes in MTAP gene status and disease progression in leukemia and lymphoma, respectively.

Cancer cells lacking the MTAP gene are not able to salvage adenine from MTA and, therefore, are more dependent on the de novo synthesis of purines (11). This absence of MTAP function therefore makes the cells more susceptible to inhibitors of de novo purine biosynthesis including methotrexate (3, 11). Of interest, the more MTX-responsive malignancies, such as T-cell acute lymphocytic leukemias, appear to have high incidences of 9p21 deletions, including the MTAP locus (31, 32). Furthermore, methotrexate is more efficacious in MTAP-negative pancreatic carcinoma cell lines than in MTAP-positive normal epithelial cells in which the MTAP-dependent adenine salvage pathway is effective (3). The high rate of MTAP deletion in osteosarcoma is consistent with the observed activity of methotrexate in this disease and may partly explain its efficacy.

In this report, we have demonstrated that a significant proportion of osteosarcoma tumor samples have deletions in the MTAP gene. Several drugs that specifically inhibit de novo purine biosynthesis have been developed or are in development. These include drugs such as L-alanosine (Triangle Pharmaceuticals), lometrexol (DDATHF; Tularik, Inc.), and AG2037 (Aguon Pharmaceuticals). The high incidence of MTAP deletions in osteosarcoma and the limited number of agents effective in the treatment of this disease suggest Phase II trials of these agents should be considered in MTAP-deficient patients. At Memorial Hospital a Phase II clinical trial of L-alanosine in tumors proven to be MTAP negative, including osteosarcoma, is currently under development.

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