Characterization of Methylthioadenosin Phosphorylase (MTAP) Expression in Malignant Melanoma

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Homozygous deletions of human chromosomal region 9p21 occur frequently in malignant melanoma and are associated with the loss of the tumor suppressor genes p16(INK4a) and p15(INK4b). In the same chromosomal region the methylthioadenosine phosphorylase (MTAP) gene is localized and therefore may also serve as a tumor suppressor gene. The aim of this study was to analyze MTAP mutations and expression patterns in malignant melanomas. To examine the MTAP gene and expression of MTAP protein we screened 9 human melanoma cell lines and primary human melanocytes by reverse transcriptase-polymerase chain reaction, sequencing, and immunoblotting. Analyzing the melanoma cell lines we found significant down-regulation of MTAP mRNA expression. In only one cell line, HTZ19d, this was due to homozygous deletion of exon 2 to 8 whereas in the other cell lines promoter hypermethylation was detected. MTAP expression was further analyzed *in vivo* by immunohistochemical staining of 38 tissue samples of benign melanocytic nevi, melanomas, and melanoma metastases. In summary, we demonstrate significant inverse correlation between MTAP protein expression and progression of melanocytic tumors as the amount of MTAP protein staining decreases from benign melanocytic nevi to metastatic melanomas. Our results suggest an important role of MTAP inactivation in the development of melanomas. This finding may be of great clinical significance because recently an association between MTAP activity and interferon sensitivity has been suggested. (*Am J Pathol* 2003, 163:683–690)

Methylthioadenosin phosphorylase (MTAP, EC 24.2.28) plays a major role in polyamine metabolism and is important for the salvage of both adenine and methionine.

MTAP catalyzes the phosphorylation of methylthioadenosine (MTA), a by-product of the synthesis of polyamines, which acts as a potent inhibitor of polyamine aminopropyltransferase and methyltransferases. MTAP is expressed abundantly in normal cells and tissues. The MTAP gene consists of 8 exons and 7 introns and is located on human chromosome 9p21. Starting from the centromeric end, the gene order on human chromosome 9p21 was mapped as p15—p16—MTAP—IFNA—IFNB.

Many malignant cells lack MTAP activity^{2–5} and cultured MTAP-deficient cells were shown to secrete MTA instead of metabolizing it.⁶ The reason for frequent loss of MTAP activity became clear after determining the chromosomal location of MTAP on human chromosome 9p21 where many tumors reveal selective deletions. Recently, MTAP gene deletions were described in endometrial cancer, osteosarcoma, and in hematological neoplasias like lymphoblastic leukemia or non-Hodgkin's lymphomas.^{2–5} Especially for melanoma, loss of this chromosome region is commonly found. Mainly, p16 was speculated to be the tumor suppressor gene on chromosome 9p21; however, recent results showing that p16 deletions do not frequently occur in sporadic melanomas led to the search of further gene deletions in this chromosomal region.⁷

The aim of this study was to analyze MTAP deletion or deregulation of expression in malignant melanoma. This is of particular significance as an association between MTAP activity and interferon sensitivity via the transcription factor STAT1 has been suggested recently.¹

Materials and Methods

Cell Lines and Culture Conditions

The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, Mel Ju, SK-Mel-28, HMB2, and HTZ19d have been described in detail previously.^{8,9} The cell lines Mel Ei, Mel Wei, Mel Ho, and Mel Juso were derived from a primary cutaneous melanoma and Mel Im, Mel Ju, SK-

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Mel-28, HMB2, and HTZ19d were derived from metastases of malignant melanomas. For tissue culture the cells were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (400 U/ml), streptomycin (50 μ g/ml), L-glutamine (300 μ g/ml), and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and split 1:5 every 3 days.

Human primary melanocytes derived from normal skin were cultivated in melanocyte medium MGM-3 (Gibco, Eggenstein, Germany) under a humidified atmosphere of 5% CO₂ at 37°C. Cells were used in passages 6 to 10 and not later than 3 days after trypsinization. Cells were detached for subcultivation or assay with 0.05% trypsin, 0.04% ethylenediaminetetraacetate in PBS.

For demethylation assays the cells were treated for 24 or 48 hours with 5-azacytidine (Sigma) at a final concentration of 20 μ mol/L.¹⁰

Stable Transfection of Melanoma Cells with MTAP Expression Plasmid

A panel of Mel 1m cell clones showing re-expression of MTAP were established by stable transfection with MTAP-sense expression plasmid (MTAP full-length cDNA cloned into pcDNA3; Invitrogen NV Leek, Holland). Controls received pcDNA3 alone. Transfections were performed using the lipofectamin plus method (Gibco). One day after transfection, cells were placed into selection medium containing 50 μ g/ml G418 (Sigma). After 25 days of selection, individual G418-resistant colonies were subcloned. The amount of MTAP expression in these clones was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis.

Assays for Cell Function

Proliferation, migration, invasion, and the ability to grow in soft agar of the stably transfected melanoma cell clones were measured as previously described.⁸

Preparation of Genomic DNA and Analysis for MTAP Deletions

Genomic DNA specimens were prepared from melanoma cell lines using the QIAamp blood kit following the manufacturer's instructions (Qiagen, Hilden, Germany). PCR was performed under standard conditions (35 cycles; annealing temperature, 56°C) in a total reaction volume of 50 μ l containing 2 μ l of diluted genomic DNA, using the pairs of primers published by Garcia-Castellano et al³ (Exon 2 to 7) and Wong et al⁴ (exon 1 and 8). PCR for genomic β -actin was used as a control.³ PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

RNA Isolation and Reverse Transcription

For RT-PCR total cellular RNA was isolated from cultured cells using the RNeasy kit (QIAGEN, Hilden, Germany).

The integrity of the RNA preparations was controlled on a 1% agarose/formaldehyde gel and subsequently cDNAs were generated by reverse-transcriptase reaction. The RT-reaction was performed in 20 μ l reaction volume containing 2 μ g of total cellular RNA, 4 μ l of 5X first strand buffer (Gibco), 2 μ l of 0.1 mol/L DTT, 1 μ l of dN₆-primer (10 mmol/L), 1 μ l of dNTPs (10 mmol/L) and diethyl pyrocarbonate (DEPC)-water. The reaction mix was incubated for 10 minutes at 70°C. Then 1 μ l of Superscript II reverse transcriptase (Gibco) was added and RNAs were transcribed for 1 hour at 37°C. Subsequently, reverse transcriptase was inactivated at 70°C for 10 minutes and RNA was degraded by digestion with 1 μ l RNase A (10 mg/ml) at 37°C for 30 minutes. cDNAs were controlled by PCR amplification of β -actin.

MTAP-RNA Mutational Analysis

The complete coding region of MTAP was amplified by RT-PCR from cDNA using specific primers: MTAP 84 forward GCC CAC TGC AGA TTC CTT TC and MTAP 983 reverse CTG GGC AGC CAT GCT ACT TT, resulting in a 899 bp fragment. The PCR reaction was performed in 50 μ l reaction volume containing 5 μ l 10X *Taq*-buffer, 1 μ l of cDNA, 1 μ l of each primer, 0.5 μ l of dNTPs, 0.5 μ l of *Taq* polymerase, and 41 μ l of water. The amplification reactions were performed by 33 repetitive cycles of denaturing for 1 minute at 94°C, annealing for 1 minute at 62°C and a final extension step at 72°C for 1.5 minutes. The PCR products were resolved on 1.0% agarose gels. For sequencing, the products were purified through polyethylene glycol precipitation to remove unincorporated primers and dNTPs. The sequencing reaction containing 4 μ l of terminator-ready reaction mix, 2 μ l of PCR product, 1 μ l of sequencing primer (MTAP 84 forward, MTAP 983 reverse, MTAP for2: GCG AAC ATC TGG GCT TTG or MTAP rev2: GCA CCG GAG TCC TAG CTT C, respectively), and 20 μ l of DEPC-water was performed for 10 seconds at 95°C, 5 seconds at 64°C, 4 minutes at 60°C, 25 cycles. Sequencing products were precipitated with 80 μ l of high-pressure liquid chromatography-water, 10 μ l of 3 mol/L sodium citrate solution, and 250 μ l of 100% ethanol to remove unincorporated terminators, and resuspended in 25 μ l of template suppression reagent. Products were run on an automatic sequencer from Applied Biosystems (Foster City, CA). Both strands were sequenced for each PCR product from at least two independent PCR reactions. Sequences were compared with the GenBank by means of BLAST search (National Center of Biotechnology Information).

Analysis of MTAP Expression by Quantitative PCR

Quantitative real-time PCR was performed on a Lightcycler (Roche, Mannheim, Germany). 2 μ l cDNA template, 2 μ l 25 mmol/L MgCl₂, 0.5 μ mol/L of forward and reverse primers (MTAP for2, MTAP rev2) and 2 μ l of SybrGreen LightCycler Mix in a total of 20 μ l were applied to the following PCR program: 95°C for 30 seconds (initial de-

nuration); a temperature transition rate of 20°C per second, up to 95°C for 15 seconds, 58°C for 3 seconds, 72°C for 5 seconds, and 81°C acquisition mode single, repeated 40 times (amplification). The PCR reaction was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 1.8% agarose gels.

Analysis of the Methylation Status of the MTAP Promoter

Genomic DNA was isolated from all melanoma cell lines and from normal human epidermal keratinocytes using the QIAamp blood kit (Qiagen). Sequence analysis revealed one CpG island at -461 to -441 with an internal *NotI* restriction site. PCR amplification of this region was performed under standard conditions (45 cycles; annealing temperature, 56°C) in a total reaction volume of 50 μ l containing 2 μ l of diluted genomic DNA, using the following primer (MTAP Prom-550for: GTC TTG GGT CAA GTC CAT CC, MTAP-Prom-10rev: GCC TTG ACT CCT CCT CTT C) and resulting in a 540-bp PCR product. To detect methylation, genomic DNA was incubated with *NotI*, resulting in a failure of PCR amplification in the case of an unmethylated CpG islet.

Reporter Gene Assays

1230 bp of the promoter region of the MTAP gene were amplified by PCR using the following primers: MTAP-Prom-1119SacI GCG AGC TCG CAT CCA GGC TAA CAT CAC, MTAP Prom + 111HindIII GCA AGC TTC ACG GGA AAG GAA TCT GC and subcloned into pGL3-basic (Promega, Mannheim, Germany). Activity of the construct was measured in the melanoma cell lines. For transient transfections 2×10^5 cells per well were seeded into 6-well plates and transiently transfected with 0.5 μ g of MTAP promoter reporter plasmid using the lipofectamine plus method (Gibco) according to the manufacturer's instructions. Twenty-four hours after transfection the cells were lysed and the luciferase activity in the lysate was measured. To normalize transfection efficiency, 0.2 μ g of a pRL-TK plasmid (Promega) was co-transfected and renilla luciferase activity was measured by a luminometric assay (Promega). All transfection experiments were repeated 3 times.

Protein Analysis in Vitro (Western Blotting)

For protein isolation, 2×10^6 cells were washed in 1X PBS and lysed in 200 μ l radioimmunoprecipitation assay buffer (Roche, Mannheim, Germany). The protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Balanced amounts of cell proteins (20 μ g) were denatured at 94°C for 10 minutes after addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and subsequently separated on Nu-Page-SDS gels (Invitrogen, Groningen, The Netherlands). After transferring the proteins onto polyvinylidene

difluoride membranes (Bio-Rad, Richmond, CA), the membranes were blocked in 3% BSA/PBS for 1 hour and incubated with a 1:1000 dilution of primary polyclonal chicken anti-MTAP antibody (a gift from Dr. D. Carson, University of California) overnight at 4°C. A 1:20,000 dilution of rabbit anti-IgY-AP (Sigma) was used as secondary antibody. Staining was performed using BCIP/NBT-tablets (Sigma).

Immunohistochemistry

Paraffin-embedded preparations of tissues from patients with benign melanocytic nevi, malignant melanomas, and melanoma metastases were screened for MTAP protein expression by immunohistochemistry. The tissues were deparaffinized, rehydrated, and subsequently incubated with primary polyclonal chicken MTAP-antibody (1:1500) overnight at 4°C. The secondary antibody (biotin-labeled anti-chicken, 1:1000; Jackson ImmunoResearch, West Grove, PA) was incubated for 30 minutes at room temperature, followed by incubation with streptavidin-POD (DAKO) for 30 minutes. Antibody binding was visualized using amino ethyl carbazole-solution (DAKO). Finally, the tissues were counterstained by hemalaun. Semi-quantitative evaluation of the staining intensity was performed in analogy to hormone receptor expression measurements in breast carcinomas by the Remmele score.¹¹ The intensity and percentage of staining were graded as follows: negative, 0; weak, 1; medium, 2; strong, 4; 0% to 10%, 1; 10% to 50%, 2; 50% to 80%, 3; 80% to 100%, 4; the score was calculated as the product of the values from intensity and percentage. Statistical analysis (Mann-Whitney test) was performed on a personal computer using the software GraphPad Prism. The mean and SE were determined.

Results

Analysis for MTAP Deletion

Exons 1 to 8 of the MTAP gene were amplified by PCR to detect homozygous genomic losses. Part of the genomic locus of β -actin was co-amplified to reveal integrity of genomic DNA and successful PCR amplification. Homozygous deletion of the MTAP gene was detected only in HTZ19d cells. Here, deletion of exon 2 to 8 was found. In all other 8 melanoma cell lines no changes were seen compared to the human primary melanocytes. Previous CGH analysis revealed loss of heterozygosity of the 9p21 region in the melanoma cells analyzed¹² (data not shown) but further analysis confirmed the genomic regions of p16 and p14ARF to be retained.⁷

Analysis for Mutations in MTAP RNA

All 9 human melanoma cell lines were evaluated for mutations in the MTAP mRNA and compared to primary human melanocytes (PHM). The complete coding region was specifically amplified by RT-PCR. With the exception of HTZ19d cells, where homozygous deletion occurred,

Figure 1. Amplification of the complete coding region of MTAP mRNA by PCR. The complete coding region was specifically amplified by RT-PCR. All melanoma cells except of HTZ19d were shown to express full-length MTAP mRNA. Strength of expression cannot be estimated by this experiment as the PCR was carried out to saturation to illustrate full-length mRNA expression and to obtain enough product for sequencing.

all melanoma cell lines were shown to express MTAP mRNA (Figure 1).

The PCR products were purified and subsequently sequenced using 4 different primers. The mutational analysis of all sequenced PCR products did not reveal any sequence variation with the exception of a known polymorphism (G166A) present in 7 of the 9 melanoma cell lines but not in SK Mel 28 and HTZ19d (GenBank accession numbers: XM 027613 and NM 002451). The polymorphism leads to the conservative amino acid exchange V56I.

Expression of MTAP in Melanoma Cell Lines

The entire panel of 9 human melanoma cell lines were further evaluated for levels of MTAP mRNA expression using quantitative PCR and compared to human primary melanocytes (NHEM). Strong reduction of expression was found in all melanoma cell lines compared to melanocytes (Figure 2, black bars).

As this could not be explained by loss of the MTAP gene in all melanoma cell lines with the exception of HTZ19d, we hypothesized promoter hypermethylation as a reason. All cell lines were exposed to 5-azacytidine for demethylation and MTAP expression was quantified. Our results clearly show significant up-regulation of MTAP expression after demethylation reaching the level of expression of normal melanocytes (Figure 2A, gray bars). Sequence analysis of the MTAP promoter led to the detection of a CpG island proximal to the transcription start (at -461 to -441). This region was shown to be methylated in melanoma cell lines but not in human keratinocytes used as a control for strong MTAP expression (Figure 2B). To further prove that promoter methylation is responsible for loss of MTAP expression we subcloned 1230 bp of the MTAP promoter containing the CpG island into pGL3-basic and revealed strong activity of the constructs compared to pGL3-basic in all melanoma cell lines (mean: 84.4-fold activity). This result clearly indicates that an unmethylated MTAP promoter construct is able to confer high reporter gene expression in melanoma cell lines.

MTAP Protein Expression in Melanoma Cell Lines

The 9 different melanoma cell lines were next screened for MTAP protein expression by Western blotting using an

Figure 2. MTAP expression in melanoma cell lines compared to primary melanocytes. **A:** By real-time PCR the amount of MTAP mRNA expression was carefully quantified. All melanoma cell lines showed significant reduction in MTAP expression compared to human primary melanocytes set as 1 (black bars). In HTZ19d this was due to loss of the genomic region. By treatment with the demethylating agent 5-azacytidine, re-expression of MTAP was found in all melanoma cell lines except of HTZ19d (gray bars). **B:** By PCR amplification the methylation status of the MTAP promoter was analyzed. Genomic DNA was incubated with *NotI* which cuts in the CpG island of the unmethylated MTAP promoter. Methylation interferes with *NotI* activity (gDNA->*NotI*->PCR). As a control for integrity of the genomic DNA, DNA without *NotI* digestion was analyzed (gDNA->PCR). PCR amplification was successfully obtained with genomic DNA of all melanoma cell lines after *NotI* digestion but not in normal human epidermal keratinocytes (NHEK). Keratinocytes were used as control for strongly MTAP-expressing cells (see Figure 4, immunohistochemistry).

anti-MTAP-antibody (Figure 3). Consistent with the reduced amount of mRNA expression, only weak levels of MTAP protein were detected in all of the melanoma cell lines compared to human primary melanocytes. In summary, both results from quantitative RT-PCR and Western blots indicated reduced MTAP expression in melanoma cells as compared to human primary melanocytes.

Analysis of MTAP Protein Expression in Vivo

To examine MTAP expression *in vivo*, 38 tissue samples from patients with benign melanocytic nevi, primary ma-

Figure 3. Western blot analysis of MTAP protein expression in melanoma cell lines. Expression of MTAP protein was detected in primary human melanocytes. In melanoma cell lines the expression was reduced. As loading control the blot was counterstained with a β -actin antibody.

Figure 4. Immunostaining of MTAP in human melanocytic tumors. **A and B:** Strong cytoplasmic immunosignals in benign melanocytic nevi (**blue arrows**). **C and D:** Focally positive- (**blue arrows**) and negative-staining results (**black arrow in D**) in primary cutaneous melanomas. Completely unstained melanoma cells in **F** are marked by **black arrows**. Small round cells underneath the metastatic infiltrate represent resident lymphocytes of the lymph node. Immunosignals were developed in red (AEC) and are distinct from the brown melanin pigment. Slides were counterstained with hemalaun and magnified $\times 250$.

lignant melanomas, and metastatic malignant melanomas were immunostained. Representative stainings are presented in Figure 4. The pattern of MTAP expression is summarized in Figure 5. The intensity and the percentage of MTAP positive cells was significantly reduced in primary malignant melanomas (scoring: 3.87 ± 0.74) compared to nevi (scoring: 11.33 ± 0.44 ; $P < 0.001$). In metastasis further reduction of expression was seen (scoring: 2.21 ± 0.884) and with the exception of very few cases semi-quantitative immunoscore discriminated benign from malignant melanocytic tumors ($P = 0.0435$). All nevi generally revealed strong staining with a high percentage of positive cells. In primary malignant melanomas we never found more than 80% positive cells. Most frequently 0 to 50% of the cells showed staining with weak to medium intensity and 3 of 15 tissues (20%)

showed no staining at all. In metastatic melanomas 8 of 14 tumors (57%) revealed no staining for MTAP, the rest displayed mostly weak staining with a low percentage of positive cells.

In summary, these data show an inverse correlation between protein expression and tumor progression as the amount of MTAP protein staining decreased from benign melanocytic nevi to primary melanomas and even further from primary melanomas to metastatic melanomas *in vivo*.

Functional Relevance of Loss of MTAP Expression

To analyze the functional role of MTAP in malignant melanomas we re-induced expression of MTAP by stable

alyzed the melanoma cell lines used for loss of p16/p14ARF expression and were able to show that loss is due to promoter methylation but not to genomic deletion.⁷ The *MTAP* locus is located telomeric to the *INK4* locus and deletion of the *MTAP* gene has been observed in several different neoplasias.^{2-5,13,14} However, no previous studies on *MTAP* gene deletion or regulation in malignant melanomas have been reported so far.

Our study reveals loss of the *MTAP* genomic region in only 1 of 9 analyzed melanoma cell lines, HTZ19d. In the remaining 8 cell lines all 8 exons were amplified successfully by PCR. This finding was supported by the fact that in these 8 cell lines full-length cDNA was generated by RT-PCR. Sequencing revealed no functional mutations but determined a known polymorphism in the amino acid valin 56 which was exchanged to isoleucin in 7 of the cell lines. This exchange is known as a conservative polymorphism replacing an uncharged amino acid by another uncharged amino acid which is only slightly larger in size.

Quantitation of mRNA expression by real-time PCR showed a marked down-regulation of MTAP expression in all analyzed melanoma cell lines. Further experiments revealed that this was due to promoter hypermethylation and could be reversed after demethylation by treatment with 5-azacytidine. Hypermethylation of the genomic 9p21 region was implicated in different cancer types including malignant melanoma¹⁵⁻¹⁸ leading to down-regulation of p16 and p15 expression. However, MTAP was not analyzed in this context before.

By semi-quantitative immunostaining of melanocytic tumors, significant down-regulation of MTAP expression correlated with malignant tumor progression. In nevi almost all samples showed strong MTAP expression, whereas in primary malignant melanoma and in metastasis a decrease in or complete loss of expression was found.

Loss of MTAP expression has been reported previously for several tumor types. In melanoma, as shown in this study, this is mainly due to hypermethylation of the promoter region.

Analyzing the functional relevance we can show that MTAP has no influence on melanoma cell proliferation. The same results were gained by Christopher et al² in a breast cancer cell line. In contrast to the results in the breast cancer cell line we were not able to see inhibition of growth in soft agar. We additionally tested the effect of MTAP re-expression on tumor cell migration and invasion. Here, we can clearly show that melanoma cells re-expressing MTAP revealed strong reduction in their invasive potential.

MTAP expression has a significant impact on STAT1 activity as illustrated by the recent study of Mowen et al.¹ Reduced activity of MTAP in the cell leads to accumulation of MTA, which acts as an inhibitor of methyltransferases. Mowen et al¹ determined the methylation of arginine 31 in STAT1 by PRMT1 as an important modification. Loss of this modification leads to enhanced binding of protein inhibitor of activated STAT (PIAS1) to STAT1 and therefore inhibition of STAT1 DNA binding activity. As STAT1 is the main player in the interferon- α and - β signaling pathway, loss of MTAP is therefore ex-

pected to cause reduced response of the cells to interferon treatment.

The role of adjuvant treatment in the therapy of malignant melanoma is an area of intense investigation. Therapy with high-dose interferon^{19,20} was shown to have beneficial effects and was approved by the FDA in 1995. However, the results of more recent studies have questioned this interpretation and lowered the initial enthusiasm.^{21,22} Our data suggest that loss of MTAP expression in malignant melanomas may have an impact on therapeutic success by compromising tumor response to interferon treatment. On the other hand, analysis of MTAP in tumor tissue may potentially be used as a prognostic marker for successful interferon therapy. Clearly, this important question needs to be addressed further by quantifying MTAP expression in malignant melanomas during treatment with interferons.

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