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Promoter-hypermethylation is causing functional relevant downregulation of methylthioadenosine phosphorylase (*MTAP*) expression in hepatocellular carcinoma

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The methylthioadenosine phosphorylase (*MTAP*) gene is localized in the chromosomal region 9p21. Here, frequently homozygous deletions occur in several kinds of cancer associated with the loss of tumour suppressor genes as *p16* and *p15*. The aim of this study was to analyse *MTAP* expression in hepatocellular carcinoma (HCC) and to get an insight into the regulation and functional role of *MTAP* in hepatocancerogenesis. Compared with primary human hepatocytes *MTAP* expression was markedly downregulated in three different HCC cell lines as determined by real-time PCR and western blotting. This was not due to genomic losses or mutations but to promoter-hypermethylation. Reduced *MTAP*-expression was confirmed *in vivo* in HCC compared with non-cancerous liver tissue on both mRNA and protein levels. To study the functional relevance of the downregulated *MTAP* expression in HCC, *MTAP* expression was re-induced in HCC cell lines by stable transfection. In these *MTAP* re-expressing cell clones the invasive potential was strongly reduced, whereas no effects on cell proliferation were observed in comparison with mock transfected cell clones. Furthermore, in *MTAP* re-expressing cells interferon (IFN)-α and IFN-γ induced a significantly stronger inhibition of cell proliferation than in mock transfected cells. In conclusion, our results suggest a functional role of *MTAP* inactivation in HCC development and invasiveness. Furthermore, in the light of a recent report revealing an association between *MTAP* activity and IFN sensitivity, our findings may have clinical significance for therapeutic strategies.

Introduction

Hepatocellular carcinoma (HCC) is one of the world's most common human malignancies and accounts for >90% of all primary liver cancers. Although the associated aetiological factors are recognized, the interactions between individual factors and the molecular mechanisms by which they lead to cancer remain largely unclear (1,2).

Methylthioadenosine phosphorylase (*MTAP*, EC 24.2.28) plays a major role in polyamine metabolism and is important for salvaging both adenine and methionine. *MTAP* catalyses 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 (3).

In contrast, many malignant cells lack *MTAP* activity (4–7). Tumorous cells lacking this enzyme, unlike enzyme-positive cells, excrete MTA instead of metabolizing it (8). The reason for the frequent loss of *MTAP* activity became evident after determining the chromosomal location of *MTAP*. Starting from the centromeric end, the gene order on human chromosome 9p21 was mapped as *p15—p16—MTAP—IFN*-α—*IFN*-β (3). In this region many tumours reveal selective deletions. Recently, *MTAP* gene deletions were described in endometrial cancer, osteosarcoma and in haematological neoplasias like lymphoblastic leukaemia or non-Hodgkin's lymphomas (4–7).

In HCC, mainly *p16* was speculated to be the tumour suppressor gene on chromosome 9p21. However, recent studies showing that *p16* deletions do not frequently occur in HCC (9–11) led to the search for further gene deletions in this chromosomal region.

In contrast, overexpression of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) is frequently observed in tumours and has been shown to be tumorigenic *in vitro* and *in vivo* (12–14). Also in HCC, ODC activity was shown to be correlated with the degree of malignancy (15,16). Interestingly, a recent study showed that products of the methionine salvage pathway regulate polyamine biosynthesis suggesting that *MTAP* deletions may lead to ODC activation in humans (17).

In addition to its role in polyamine metabolism, *MTAP* expression has recently been shown to have significant impact on the activity of STAT1 (signal transducer and activator of transcription 1) (18). STAT1 is essential for interferon (IFN) signalling pathways (19) and plays an important role in the pathophysiology of hepatocarcinogenesis (20).

Recently, we detected a CpG island proximal to the transcription start of the *MTAP* promoter (at −461 to −441) (21). In the present study, we aimed to assess whether promoter methylation impaires *MTAP* expression in HCC, and analysed *MTAP* expression in HCC on both mRNA and protein levels. Besides epigenetic modifications we searched for genomic losses and mutations of the *MTAP* gene in HCC cell lines and HCC tissue, respectively. In addition, to study the functional relevance of the downregulated *MTAP* expression in HCC, *MTAP* expression was re-induced in HCC cell lines by stable transfection.

Abbreviations: HCC, hepatocellular carcinoma; IFN, interferon; ODC, ornithine decarboxylase; MTA, methylthioadenosine; MTAP, methylthioadenosine phosphorylase; PHH, primary human hepatocytes; STAT1, signal transducer and activator of transcription 1.

Materials and methods

Patients and patient materials

Human liver tissue. HCC tissue and non-tumorous liver tissue of the same individual were obtained from 15 HCC patients undergoing partial hepatectomy. The underlying liver disease was chronic hepatitis B ($n = 2$) or C infection ($n = 1$), haemochromatosis ($n = 1$), or chronic alcohol abuse ($n = 6$). In five patients no underlying liver disease was known. Histological examination of surrounding non-tumorous liver tissue revealed the presence of cirrhosis in nine cases, whereas no liver fibrosis or only less advanced liver fibrosis was found in six specimens.

Liver tissue samples were immediately frozen in liquid nitrogen and stored at −80°C for RNA isolation or formalin-fixed for immunohistochemistry. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee.

Primary human hepatocytes (PHH). Tissue samples from human liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumours of colorectal cancer. Experimental procedures were performed according to the guidelines of the local Ethics Committee, University Hospital of Regensburg, with informed patient's consent. Only liver tissues judged microscopically as non-cancerous by an anatomical pathologist were used for cell preparation. Further exclusion criteria were known liver disease or histological evidence for liver fibrosis or inflammation in surrounding non-tumorous liver tissue. Hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously (22).

Cell lines and culture conditions

The cell lines PLC (ATCC CRL-8024; American Type Culture Collection, Manassas, VA), Hep B3 (ATCC HB-8064) and Hep G2 (ATCC HB-8065) were used for *in vitro* experiments. For tissue culture the cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), L-glutamine (300 μg/ml) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and splitted 1, 5 at confluence. Cells were detached for subcultivation or assay with 0.05% trypsin and 0.04% EDTA in PBS.

For demethylation assays the cells were treated for 48 h with 5-azacytidine (Sigma) at a final concentration of 10 μM (23).

Cell proliferation was measured using the XTT assay (Roche, Mannheim, Germany) following the manufacturer's description.

Preparation of genomic DNA and analysis for MTAP deletions

Genomic DNA specimens were prepared from 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 μl containing 2 μl of diluted genomic DNA, using the pairs of primers published by Garcia-Castellano *et al*. (5) (exons 2–7) and Wong *et al*. (24) (exons 1 and 8). PCR for genomic β-actin was used as a control (5). 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 or tissue using the RNeasy kit (Qiagen). 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 μl reaction volume containing 2 μg of total cellular RNA, 4 μl of 5× first strand buffer (Gibco, Karlsruhe, Germany), 2 μl of 0.1 M DTT, 1 μl of dN_6-primer (10 mM), 1 μl of dNTPs (10 mM) and DEPC-water. The reaction mix was incubated for 10 min at 70°C. Then 1 μl of Superscript II reverse transcriptase (Gibco) was added and RNAs were transcribed for 1 h at 37°C. Subsequently, reverse transcriptase was inactivated at 70°C for 10 min and RNA was degraded by digestion with 1 μl RNase A (10 mg/ml) at 37°C for 30 min. 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 for, GCC CAC TGC AGA TTC CTT TC, *MTAP* 983 rev, CTG GGC AGC CAT GCT ACT TT resulting in a 899 bp fragment. The PCR reaction was performed in 50 μl reaction volume containing 5 μl 10× *Taq*-buffer, 1 μl of cDNA, 1 μl of each primer, 0.5 μl of dNTPs, 0.5 μl of *Taq* polymerase and 41 μl of water. The amplification reactions were performed by 33 repetitive cycles of denaturing for 1 min at 94°C, annealing for 1 min at 62°C and a final extension step at 72°C for 1.5 min. The PCR products were resolved on 1.0% agarose gels.

For sequencing, the products were purified through PEG precipitation to remove unincorporated primers and dNTPs. The sequencing reaction containing 4 μl of terminator ready reaction mix, 2 μl of PCR product, 1 μl of sequencing primer (*MTAP* 84 for, *MTAP* 983 rev, *MTAP* for 2, GCG AAC ATC TGG GCT TTG or *MTAP* rev2, GCA CCG GAG TCC TAG CTT C) and 20 μl of DEPC-water was performed for 10 s at 95°C, 5 s at 64°C, 4 min at 60°C, 25 cycles. Sequencing products were precipitated with 80 μl of HPLC-water, 10 μl of 3 M sodium citrate solution, 250 μl of 100% ethanol to remove unincorporated terminators and resuspended in 25 μl 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 gene data bank by means of BLAST search (National Center of Biotechnology Information, NCBI, Bethesda, MD).

Analysis of MTAP expression by quantitative PCR

To quantify the expression of *MTAP* the real-time PCR LightCycler system (Roche) was used as described previously (21). Briefly, 2 μl cDNA template, 2 μl 25 mM $MgCl_2$, 0.5 μM of forward and reverse primers and 2 μl of SybrGreen LightCycler Mix in a total of 20 μl were applied. The following sets of primers were used, β-actin forward 5′-CTA CGT CGC CCT GGA CTT CGA GC-3′, β-actin reverse 5′-GAT GGA GCC GCC GAT CCA CAC G-3′, *MTAP* forward 5′-GAA GGA GCA CAC CAT CA-3′ and *MTAP* reverse 5′-CTG GCA CAA GAA TGA CTT CC-3′. The following PCR program was performed, 30 s 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 15 s, 3 s 58°C, 5 s 72°C, 81°C acquisition mode single, repeated for 40× (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.

Protein analysis in vitro (western blotting)

For protein isolation, 2×10^6 cells were washed in 1× PBS and lysed in 200 μl RIPA-buffer (Roche). The protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL). Balanced amounts of cell proteins (20 μg) were denatured at 94°C for 10 min after addition of Roti-load-buffer (Roth, Karlsruhe, Germany) and subsequently separated on NuPage–SDS–PAGE (Invitrogen, Groningen, The Netherlands). After transferring the proteins onto PVDF-membranes (Bio-Rad, Richmond, CA), the membranes were blocked in 3% BSA/PBS for 1 h and incubated with a 1:1000 dilution of primary polyclonal chicken anti-MTAP antibody (generous gift from Dr D. Carson, University of California) overnight at 4°C. A 1:20 000 dilution of rabbit anti-Ig-AP (Sigma) was used as secondary antibody. Staining was performed using BCIP/NBT-tablets (Sigma).

Analysis of the methylation status of the MTAP promoter

Genomic DNA was isolated from all HCC cell lines and from normal human epidermal keratinocytes [used as a control for unmethylated promoter region (25)] using the QIAamp blood kit (Qiagen). Sequence analysis revealed one CpG island at −461 to −441 with an internal NotI restriction site. PCR amplifying this region was performed under standard conditions (45 cycles, annealing temperature, 56°C) in a total reaction volume of 50 μl containing 2 μl of diluted genomic DNA, using the following primer (*MTAP*_Prom-550 for, GTC TTG GGT CAA GTC CAT CC, *MTAP*-Prom-10rev, GCC TTG ACT CCT CCT CTT C) and resulted 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 island.

Reporter gene assays

The promoter region of 1230 bp of the *MTAP* gene was 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 HCC cell lines. For transient transfections 2×10^5 cells per well were seeded into 6-well plates and transiently transfected with 0.5 μg of *MTAP* promoter reporter plasmids using the lipofectamine plus method (Gibco) according to the manufacturer's instructions. The cells were lysed 24 h after transfection, and the luciferase activity in the lysate was measured. To normalize transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega) was cotransfected and renilla luciferase activity was measured by a luminometric assay (Promega). All transfection experiments were repeated three times.

Stable transfection of HCC cell lines with MTAP expression plasmid

A panel of PLC cell clones showing re-expression of *MTAP* was established by stable transfection with *MTAP*-sense expression plasmid (*MTAP* full-length cDNA cloned into pCDNA3/Invitrogen NV Leek, The Netherlands). Controls received pCDNA3 alone. Transfections were performed using the lipofectamine plus method (Gibco). One day after transfection, cells were placed into selection medium containing 50 μg/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 RT–PCR and western blot analysis.

Immunohistochemistry

Paraffin-embedded preparations of tissues from patients with HCC were screened for MTAP protein expression by immunohistochemistry. The tissues were deparaffinated, rehydrated and subsequently incubated with primary polyclonal chicken MTAP-antibody (1:1500) overnight at 4°C. The secondary antibody (biotin-labelled anti-chicken, 1:1000, Immuno Research, West Grove, PA) was incubated for 30 min at room temperature, followed by incubation with streptavidin–POD (DAKO, Hamburg, Germany) for 30 min. Antibody binding was visualized using AEC-solution (DAKO). Finally, the tissues were counterstained by hemalaun.

Invasion assay

Invasion assays were performed in Boyden Chambers containing polycarbonate filters with 8 μm pore size (Costar, Bodenheim, Germany), as described previously (26). Briefly, filters were coated with a commercially available reconstituted basement membrane (Matrigel, diluted 1:3 in H_2O; Becton Dickinson, Heidelberg, Germany). The lower compartment was filled with fibroblast-conditioned medium used as a chemo-attractant. HCC cells were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of 2×10^5 cells/ml and placed in the upper compartment of the chamber. After incubation at 37°C for 4 h filters were removed. Cells adhering to the lower surface were fixed, stained and counted.

Statistical analysis

Results are expressed as mean ± SD (range) or percentage. Comparison between groups was made using the Student's unpaired *t*-test. Welch's correction was performed when required. A *P*-value of <0.05 was considered statistically significant. All calculations were performed using the SPSS-10 for Windows statistical computer package (SSPS, Chicago, IL).

Results

Search for genomic losses and mutations in the MTAP gene

Since *MTAP* gene deletions were described in several types of cancer, exons 1–8 of the *MTAP* gene were amplified by PCR to detect homozygous genomic losses using genomic DNA of the HCC cell lines PLC, Hep3B and HepG2. Furthermore, genomic DNA isolated from three HCC tissue samples was analysed. Part of the genomic locus of β-actin was co-amplified to assess integrity of genomic DNA and successful PCR amplification. In agreement with a recent study by Berasain *et al*. (27) homozygous deletion of the *MTAP* gene was not detected in any of the three analysed HCC cell lines or in the HCC tissues (data not shown).

To search for mutations in the *MTAP* mRNA, the complete coding region was specifically amplified by RT–PCR. All three HCC cell lines were shown to express full length *MTAP* mRNA. Subsequently, the PCR products were purified and sequenced using four different primers. The mutational analysis of all sequenced PCR products did not reveal any sequence variation (data not shown).

MTAP expression in HCC cell lines and PHH

Next, *MTAP* expression was determined in HCC cells in comparison with PHH. Analysis of *MTAP* mRNA expression by quantitative PCR revealed a significant reduction in all three HCC cell lines compared with PHH (0.17 ± 0.01 in HepG2 and 0.07 ± 0.03 in Hep3B, respectively) (Figure 1A). PLC cells almost completely lacked *MTAP* mRNA expression.

To assess MTAP protein expression in HCC cell lines, western blotting was performed using an anti-MTAP-antibody (Figure 1B). Consistent with the reduced amount of mRNA expression, only weak levels of MTAP protein were detected in HepG2 and Hep3B cells compared with PHH. Also PLC cells revealed only a very faint band.

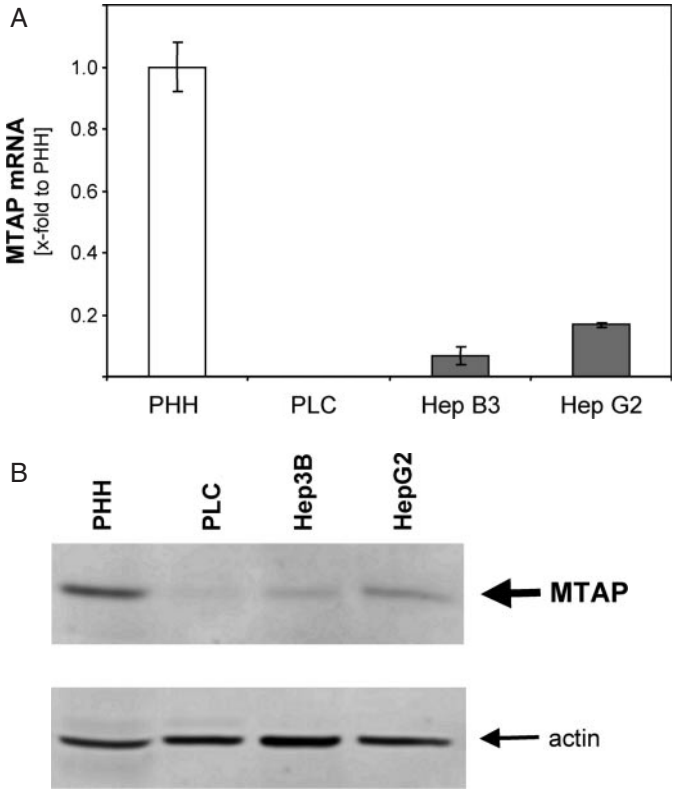

Fig. 1. *MTAP* expression in HCC cell lines compared with PHH. (**A**) *MTAP* mRNA expression was quantified by real-time PCR. All three HCC cell lines showed a strong reduction of *MTAP* expression compared with human primary hepatocytes (set as 1). Data represent the mean (±SD) of three independent analyses. (**B**) Western blot analysis of MTAP protein expression in HCC cell lines. Strong expression of MTAP protein was detected in PHH. In contrast, in HCC cell lines the expression was reduced or almost not detectable (in case of PLC cells). As loading control the blot was counterstained with a β-actin antibody.

MTAP expression in HCC

To further address *MTAP* expression in HCC *in vivo*, we analysed a panel of 15 paired specimens obtained from patients with HCC. From each HCC patient RNA was isolated from cancerous tissue and surrounding non-tumorous liver tissue, and *MTAP* mRNA expression was measured by quantitative RT–PCR (Figure 2). In 12 HCC specimens *MTAP* mRNA expression was significantly reduced compared with matched non-tumorous tissue (healthy liver (set as 1.0) versus HCC, mean of difference 0.65 ± 0.13%; $P < 0.0001$), including two HCC tissue samples (nos 3 and 6), where no *MTAP* mRNA expression was detectable at all. In one patient (no. 13) *MTAP* mRNA expression was not significantly different compared with non-tumorous tissue, and only two HCC specimens (nos 14 and 15) revealed increased *MTAP* mRNA expression levels compared with matched non-tumorous tissue specimens (both 1.7-fold). Hereby, relative *MTAP* expression levels showed no association with the stage of liver fibrosis or the underlying liver disease.

In order to assess *MTAP* expression in HCC *in situ*, we performed immunohistochemical staining for *MTAP* in tumorous tissue of five HCC patients. Furthermore, five tissue samples obtained from healthy livers were immunostained for comparison. Representative stainings are presented in Figure 3. Immunohistochemistry revealed strong cytoplasmatic immunosignals in all hepatic cells, particularly

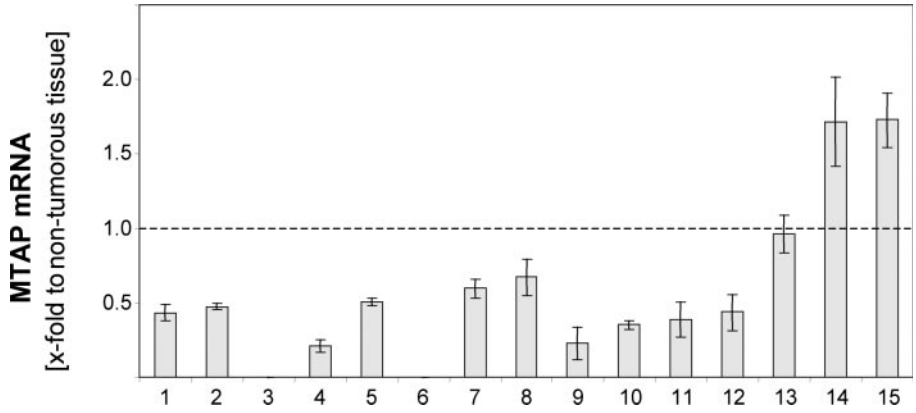

Fig. 2. *MTAP* expression in tissue of healthy liver compared with HCC *MTAP* mRNA expression was quantified in HCC tissue and in matching non-tumorous surrounding liver tissue of the same patients by real-time PCR. In 12 HCC-specimens *MTAP* mRNA expression was undetectable or significantly reduced compared with matched non-tumorous tissue (set as 1.0). In one patient *MTAP* mRNA expression was not significantly different, and only two HCC specimens revealed increased *MTAP* mRNA expression levels. Data represent the mean (±SD) of three measurements.

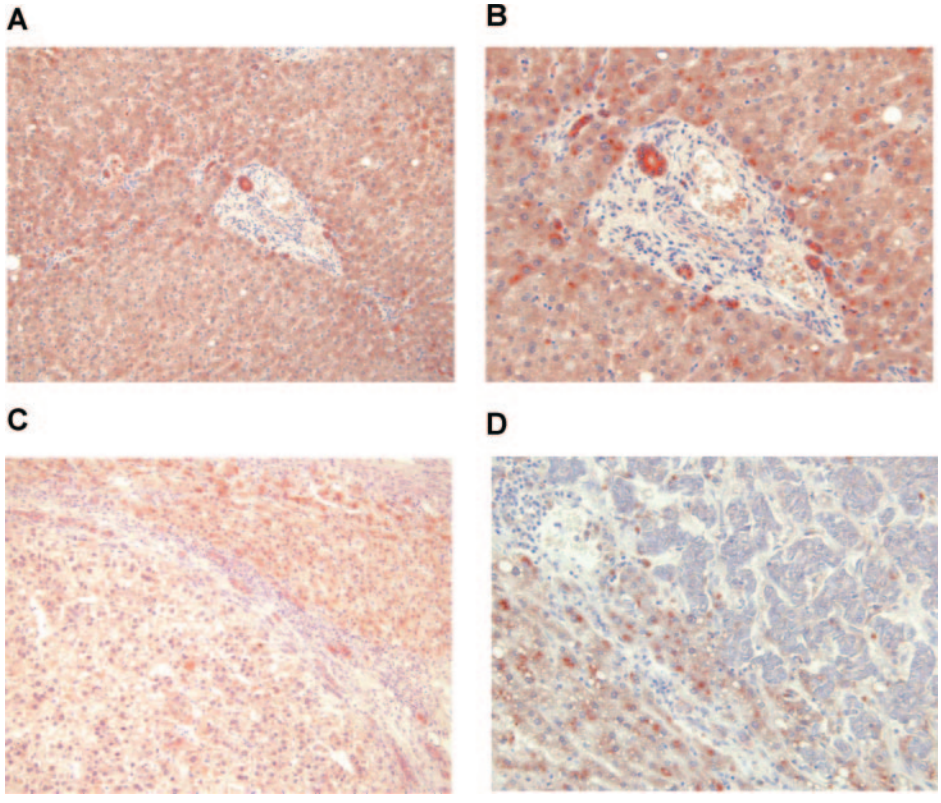

Fig. 3. Immunostaining of *MTAP* in HCC immunohistochemistry revealed strong cytoplasmatic immunosignals in all hepatic cells, particularly hepatocytes of normal, non-tumorous human liver (**A** and **B**). Similarly, non-tumorous hepatocytes in HCC surrounding cirrhotic liver tissue showed clear cytoplasmatic staining, although slightly fainter than hepatocytes in non-cirrhotic liver tissue (**C** and **D**). In contrast, *MTAP* staining signal was reduced or even undetectable in cancerous cells (C and D). Immunosignals were developed in red (AEC) and are distinct from brown pigment. Counterstaining was performed with haemalaun. (A, 100×; B–D, 200×).

hepatocytes of normal, non-tumorous human liver (Figure 3A and B). Similarly, non-tumorous hepatocytes in HCC surrounding cirrhotic liver tissue showed clear cytoplasmatic staining, although slightly fainter than hepatocytes in non-cirrhotic liver tissue (Figure 3C and D). In contrast, *MTAP* staining signal was reduced or even undetectable in cancerous cells (Figure 3C and D).

Regulation of MTAP expression in HCC cell lines

Recently, we detected a CpG island proximal to the transcription start (at −461 to −441) of the *MTAP* promoter (21), and Berasain *et al*. (27) provided evidence that DNA methylation plays a role in silencing *MTAP* expression in HCC. In accordance with the study by Berasain *et al*. (27), this promotor region was shown to be methylated in Hep3B, HepG2 and PLC cells (Figure 4A).

Furthermore, we confirmed the functional relevance of promoter hypermethylation in all three HCC cell lines. Exposure to 5-azacytidine for demethylation induced a strong upregulation of *MTAP* expression (Figure 4B). In contrast, in PHH 5-azacytidine treatment did not change *MTAP* mRNA expression levels (data not shown). To demonstrate

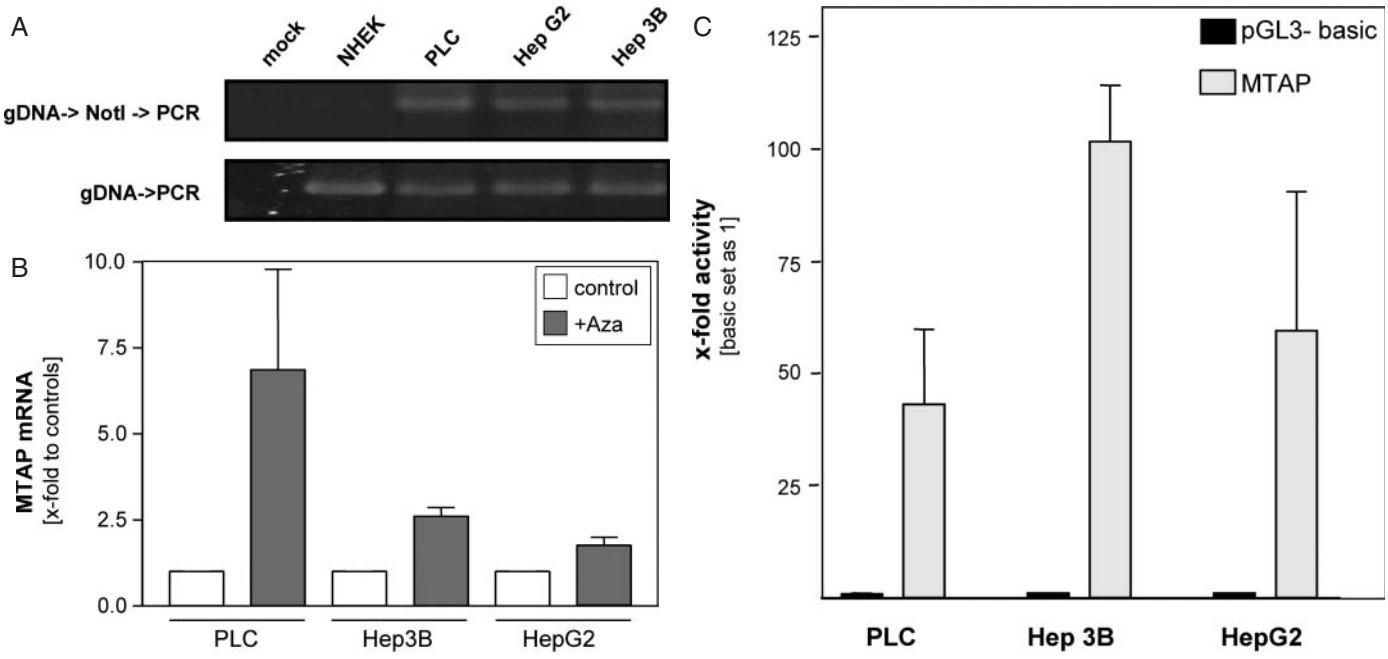

Fig. 4. Regulation of *MTAP* expression in HCC cell lines. (**A**) The methylation status of the *MTAP* promoter was analysed by PCR amplification. Genomic DNA was incubated with NotI, which cuts in the CpG island of the unmethylated *MTAP* promoter. Methylation interferes with NotI activity (gDNA→NotI→PCR). As a control for integrity of the genomic DNA, DNA without NotI digestion was analysed (gDNA→PCR). PCR amplification was successfully obtained with genomic DNA of all HCC cell lines after NotI digestion but not in normal human epidermal keratinocytes (NHEK). Keratinocytes were used as control for strongly *MTAP* expressing cells as shown previously (26). (**B**) By treatment with the demethylating agent 5-azacytidine (Aza), re-expression of *MTAP* was induced in all three HCC cell lines. Data represent the mean (±SD) of three independent analyses. (**C**) A reporter construct containing the luciferase gene under the control of the human *MTAP* promoter was transiently transfected into HCC cell lines. The unmethylated *MTAP* promoter revealed strong activity in all three HCC cell lines investigated. Data represent the mean (±SD) of three independent analyses.

that 5-azacytidine does not unspecifically affect *MTAP* mRNA expression, as an additional control, we analysed *IL-8* mRNA expression in 5-azacytidine treated and untreated cells. Despite the presence of four CpG sites located within the vicinity of the *IL-8* promoter no differences in *IL-8* expression levels were observed (data not shown), indicating differences in the methylation status between the *MTAP* and *IL-8* gene.

To further prove that promoter methylation is responsible for the reduced *MTAP* expression in HCC cell lines, we subcloned 1230 bp of the *MTAP* promoter containing the CpG island into the expression plasmid pGL3-basic. Transient transfection experiments revealed strong activity of the constructs compared with pGL3-basic in all three HCC cell lines investigated (mean, 84.4-fold increased compared with controls) (Figure 4C). These results clearly indicated that an unmethylated *MTAP* promoter construct is able to confer high reporter gene expression in HCC cell lines.

Functional relevance of loss of MTAP expression

To analyse the functional role of *MTAP* in hepatocarcinoma, we re-induced expression of *MTAP* by stable transfection of the HCC cell line PLC with an *MTAP* expression construct. By quantitative RT–PCR (data not shown) and western blotting re-expression of *MTAP* in the cell clones (PLC *MTAP*1 and 2) was shown, whereas no changes of *MTAP* expression was seen in mock transfected cell clones (PLC mock1 and 2) (Figure 5A).

Proliferation assays revealed no changes in proliferation comparing the *MTAP* expressing cell clones with the mock controls (doubling time: PLC *MTAP*1: 1.78 ± 0.07 days, PLC *MTAP*2: 1.50 ± 0.07 days, PLC mock1: 2.46 ± 0.05 days, and PLC mock2: 1.48 ± 0.09 days).

Interestingly, invasion assays using the Boyden Chamber system showed a strong reduction of invasive potential in the *MTAP* re-expressing cell clones (Figure 5B) [reduction to 57.3 ($P = 0.0007$) and 69.2% ($P < 0.0001$), compared with untransfected cells]. In contrast, the invasive potential of mock transfected cell clones did not differ significantly from untransfected PLC cells (PLC mock1: 104.2% and PLC mock2: 121.8%).

Reaction to IFN treatment

Since a recent report has suggested an association between *MTAP* activity and IFN sensitivity (28), we performed proliferation assays with the PLC cell clones, either *MTAP* expressing or mock, with and without stimulation with IFN-α at different concentrations (Figure 6). The assay revealed that the *MTAP* re-expressing PLC cell clones (PLC–*MTAP*) showed a significantly stronger inhibition of proliferation by IFN-α compared with the mock transfected (PLC-mock) or untransfected PLC cells. Already at a concentration of 10 U/ml IFN-α reduced cell growth in PLC–*MTAP* significantly to 75.0% as compared with untransfected PLC, whereas proliferation was not significantly affected in PLC–mock. At higher IFN-α concentrations (100 U/ml), also PLC and PLC–mock proliferation was inhibited, but the anti-proliferative effect was significantly stronger in the *MTAP* re-expressing PLC cell clones.

Similarly, IFN-γ revealed a stronger anti-proliferative effect on *MTAP* re-expressing PLC cell clones as compared with the mock transfected or untransfected PLC cells. At a concentration of 100 U/ml, IFN-γ reduced cell growth in PLC–*MTAP* to 59.6% compared with untransfected PLC whereas proliferation was not affected in PLC–mock. At higher IFN-γ

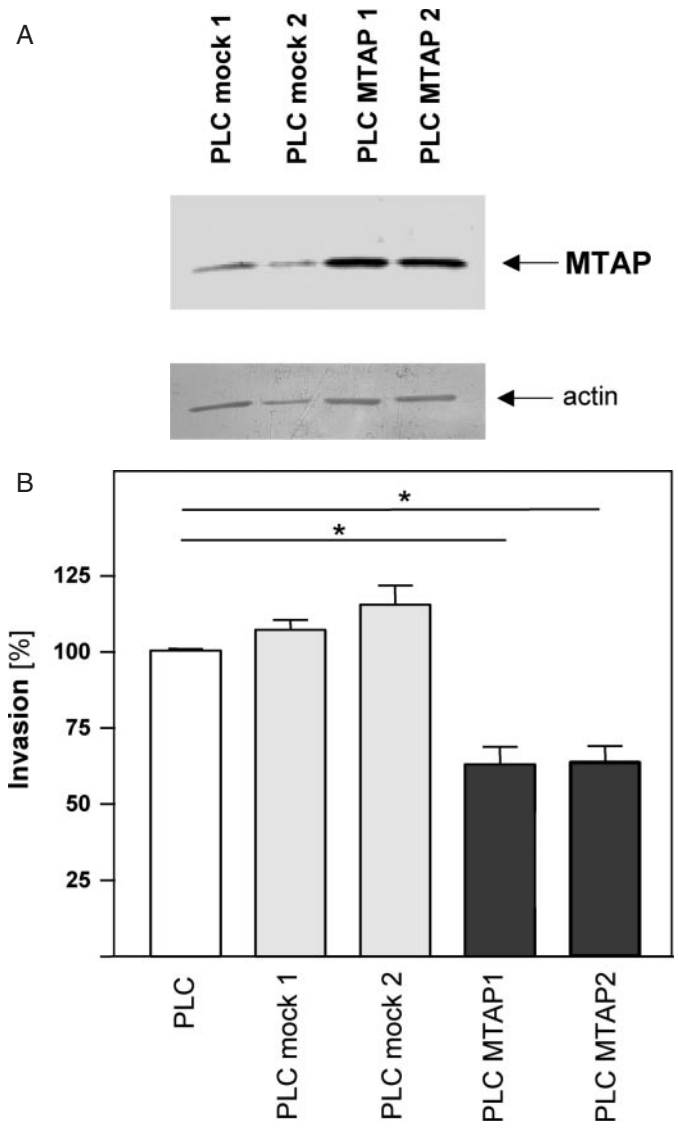

Fig. 5. Functional relevance of *MTAP* expression in HCC cell lines. (**A**) Western blot analysis of the PLC cell clones stably transfected with an *MTAP* expression plasmid (PLC–*MTAP*1 and 2). Both cell clones showed increased expression of *MTAP* whereas *MTAP* expression in the mock transfected cell clone (PLC–mock1 and 2) remained unchanged. As loading control the blot was counterstained with an anti-β-actin antibody. (**B**) Analysis of the cell clones re-expressing *MTAP* (PLC–*MTAP*1 and 2) revealed strong reduction of their invasiveness as shown by Boyden Chamber assays. Data represent the mean (±SD) of four independent analyses ($^{*}P < 0.05$).

concentrations (1000 U/ml), also PLC and PLC–mock proliferation was inhibited to ∼20%. At this high concentration, proliferation of *MTAP* re-expressing PLC cell clones was already almost completely inhibited (1.7% compared with unstimulated cells).

Discussion

The aim of this study was to analyse *MTAP* expression in HCC and to get insight into regulation and functional role of *MTAP* in hepatocancerogenesis.

In accordance with a recent publication of Berasain *et al*. (27), quantification of mRNA expression by real-time PCR showed a marked downregulation of *MTAP* mRNA expression in HCC cell lines as well as HCC tissues. Noteworthy, here, we compared *MTAP* mRNA expression in HCC-specimens with matched non-tumorous tissue samples of the same patient, whereas Berasain *et al*. (27) based their findings on the comparison of cancerous tissues with unrelated healthy liver tissue samples of different patients. This difference is of importance, since *MTAP* mRNA expression was also found to be downregulated in cirrhotic livers (27) and in the majority of cases HCC develops in a cirrhotic liver (29,30). Therefore, in general, liver cirrhosis can be considered a pre-cancerous condition in which molecular alterations involved in the development of the malignant phenotype take place (29,30). In the present study, in the majority of cases *MTAP* mRNA was downregulated or even lost in HCC as compared with surrounding non-tumorous tissue regardless of the degree of liver fibrosis. Moreover, these findings were confirmed on the protein level by immunohistochemical staining. Since MTAP is expressed ubiquitously in all normal cells, it cannot be excluded that changes found in MTAP expression analysing whole tissue samples are actually caused by stroma or inflammatory cells rather than differences between cancerous and non-cancerous parenchymal cells, respectively. However, immunohistochemical staining allowed a semi-quantitative comparison of MTAP staining intensity in normal hepatocytes and malignant cells. Here, in accordance with our mRNA expression data in PHH and three HCC cell-lines, MTAP protein expression was reduced or even undetectable in cancerous cells, whereas strong cytoplasmatic immunosignals were detectable in surrounding non-tumorous hepatocytes. Taken together these findings clearly indicate downregulation or loss of *MTAP* expression in hepatocytes during malignant transformation.

The *MTAP* gene is located on human chromosome 9p21. Loss of this chromosomal region has been shown in several human neoplasias including HCC (9,31). Previously, the cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p16^{INK4A}$ and p14ARF have been mapped to this region. These proteins are of high interest in cancer research and, consequently, have been studied by many groups. P16 and p14ARF were also implicated in HCC development and progression, but were found to be affected by deletions in only a small subgroup of HCC patients (9–11).

Despite the fact that deletion of the *MTAP* gene has been observed in several different neoplasias (4–7), our study revealed no loss of the *MTAP* genomic region in three analysed HCC cell lines and three HCC tissue samples. Our data are in line with the previous report of Berasain *et al*. (27) who could not find genomic deletions in HCC samples and cell lines with exception of the HCC cell line SK-Kep1. These findings are further supported by the fact that in all three HCC cell lines investigated full length cDNA was generated by RT–PCR. Moreover, sequencing revealed no mutations in the *MTAP* mRNA.

Hypermethylation of the genomic 9p21 region was implicated in different cancer types including HCC (32–36) leading to downregulation of *p16* and *p15* expression. Recently, we detected a CpG island proximal to the transcription start of the *MTAP* promoter (at –461 to –441) (21), and Berasain *et al*. (27) provided evidence that DNA methylation plays a role in silencing *MTAP* expression in HCC. This finding was confirmed in the present study. Furthermore, we could show strong *MTAP* promoter activity in all three HCC cell lines using an ummethylated promoter.

To study the functional relevance of *MTAP* promotor methylation and subsequent downregulation of *MTAP*

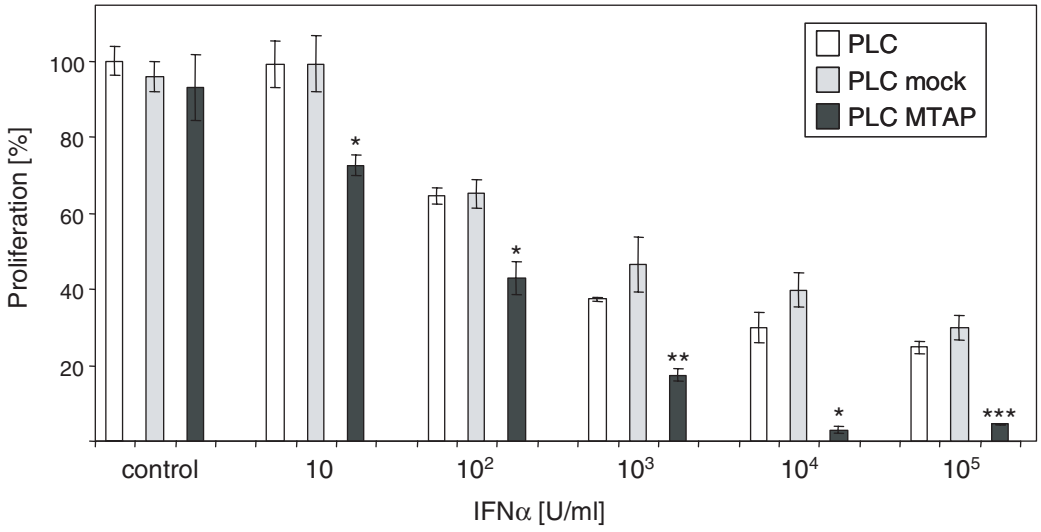

Fig. 6. *MTAP* re-expression leads to higher sensitivity to IFN proliferation assays of PLC cell clones, either *MTAP* re-expressing (PLC–*MTAP*; black bars) or mock transfected (PLC–mock; grey bars), and untransfected PLC cells (white bars). Cells were stimulated with IFN-α (IFNα) at different concentrations (10–10^5 U/ml). PLC–*MTAP* revealed a significantly stronger inhibition of proliferation by IFN-α compared with PLC–mock or untransfected PLC cells ($^*P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$).

expression in HCC, *MTAP* expression was re-induced in HCC cell lines by stable transfection. Interestingly, *MTAP* re-expression had no effect on cell proliferation. Similar results were gained by Christopher *et al*. (37) in a breast cancer cell line and our group in malignant melanoma (25) analysing anchorage-dependent growth. However, it can be speculated that *MTAP* may affect anchorage-independent growth of HCC, as shown for breast cancer cell lines (37).

Here, we additionally investigated the effect of *MTAP* re-expression on tumour cell invasion. This analysis clearly revealed that re-expression of *MTAP* in HCC cell lines causes a strong reduction of their invasive potential. A recent publication by Subhi *et al*. (17) demonstrating a regulatory link between the methionine salvage pathway and the polyamine pathway may provide a potential mechanism for this finding. This study suggests that reduced *MTAP* expression may lead to activation of the polyamine biosynthetic enzyme ODC (17). Overexpression of ODC is frequently observed in several tumours including HCC, and has been shown to be tumorigenic and to be correlated with the degree of malignancy (12–16).

Increased ODC activity was also detected in a previous study investigating HCC tissue from an animal model, but different than in our study analysing human HCC specimens and in contrast to the findings of Subhi *et al*. (17), high *MTAP* activity was found (38). In this rat model hyperplastic nodules and neoplasia in the liver were induced by combining partial hepatectomy and treatment with 2-acetylaminofluorene and phenobarbital, respectively. These findings indicate that in different species or in chemically induced hepatocancerogenesis the pathophysiological role of *MTAP* may vary.

In addition to its role in polyamine metabolism, *MTAP* expression has significant impact on STAT1 activity as illustrated by a recent study of Mowen *et al*. (18). STAT1 is essential for IFN signalling pathways (19) and plays an important role in the pathophysiology of hepatocancerogenesis (20). Mowen *et al*. (18) demonstrated that reduced activity of *MTAP* in the cell leads to accumulation of MTA, which acts as an inhibitor of methyltransferases. The methylation of arginine 31 in STAT1 by PRMT1 was found to be an important modification. Loss of this modification leads to enhanced binding of PIAS1 (protein inhibitor of activated STAT) to STAT1, and, therefore, inhibition of STAT1 DNA binding activity. As STAT1 is the main player in the IFN signalling pathway, loss of *MTAP* is, therefore, expected to cause reduced response of the cells to IFN treatment.

We analysed this hypothesis by treating the *MTAP* re-expressing cell clones with IFN-α or IFN-γ, and found a strong reduction of proliferation of the *MTAP* re-expressing cell clones but not of the mock transfected controls or untransfected HCC cells.

The role of adjuvant treatment in the therapy of HCC is an area of intense investigation. IFN-α is proven to be effective in treating various types of cancers, e.g. hairy cell leukaemia, Kaposi's sarcoma or melanoma (39–41). It also has a powerful antiproliferative effect on human HCC cell lines *in vitro* and has been shown to prevent and to delay hepatocancerogenesis in an animal model *in vivo* (42). However, systemic treatment of HCC patients with IFNs, either alone or in combination with other therapeutics revealed therapeutic success in only a small fraction of patients (43–46).

Our data suggest that loss of *MTAP* expression in HCC may have an impact on therapeutical success by compromising tumour response to IFN treatment. On the other hand, analysis of *MTAP* in tumour tissue may potentially be used as a prognostic marker for successful IFN therapy. Clearly, this important question needs to be addressed further in clinical studies by quantifying *MTAP* expression in HCC during treatment with IFN and correlating the results with the clinical response.

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