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Biochimica et Biophysica Acta 1690 (2004) 276–284

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Methylthioadenosine phosphorylase gene expression is impaired in human liver cirrhosis and hepatocarcinoma

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Received 27 April 2004; received in revised form 3 August 2004; accepted 10 August 2004
Available online 23 August 2004

Abstract

Methylthioadenosine phosphorylase (MTAP) is a key enzyme in the methionine and adenine salvage pathways. In mammals, the liver plays a central role in methionine metabolism, and this essential function is lost in the progression from liver cirrhosis to hepatocarcinoma. Deficient *MTAP* gene expression has been recognized in many transformed cell lines and tissues. In the present work, we have studied the expression of *MTAP* in human and experimental liver cirrhosis and hepatocarcinoma. We observe that *MTAP* gene expression is significantly reduced in human hepatocarcinoma tissues and cell lines. Interestingly, *MTAP* gene expression was also impaired in the liver of CCl_4-cirrhotic rats and cirrhotic patients. We provide evidence indicating that epigenetic mechanisms, involving DNA methylation and histone deacetylation, may play a role in the silencing of *MTAP* gene expression in hepatocarcinoma. Given the recently proposed tumor suppressor activity of MTAP, our observations can be relevant to the elucidation of the molecular mechanisms of multistep hepatocarcinogenesis.

Keywords: Liver; Methylthioadenosine phosphorylase; Cirrhosis; Hepatocarcinoma; Gene expression

1. Introduction

Methylthioadenosine phosphorylase (MTAP) (5′-deoxy-5′-methylthioadenosine: orthophosphate methylthio-ribosyltransferase, EC 2.4.2.28) is the rate-limiting enzyme in the methionine salvage pathway [1–3]. MTAP catalyzes the phosphorolytic cleavage of 5′-methylthioadenosine (MTA), a sulfur-containing adenosyl nucleoside that is formed from S-adenosylmethionine (AdoMet) in the polyamine biosynthetic pathway [4–6]. The reaction products, adenine and 5-methylthioribose 1-phosphate are reused: adenine is metabolized to adenine nucleotide pools via adenine phosphoribosyltransferase [7], and 5-methylthioribose 1-phosphate is metabolized to methionine through a series of reactions [8]. This methionine salvage pathway has been studied in detail in *Klebsiella pneumoniae* [9], but has also been demonstrated in mammalian tissues such as rat liver [8]. *MTAP* is expressed in all normal tissues and in non-transformed cell lines, however, many tumors and malignant cell lines, including breast, lung, colon and ovarian carcinomas, glioblastoma, leukemia and melanoma lack MTAP activity [1,10–15]. In most of these tumors and transformed cells, the complete absence of MTAP activity has been attributed to total or partial deletions of the *MTAP* gene, located on human chromosome 9p21 in a region frequently deleted in human malignancies [16,17]. Additionally, *MTAP* promoter hypermethylation has also been associated with reduced *MTAP* expression in human melanoma cell lines [18].

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doi:10.1016/j.bbadis.2004.08.002

Interestingly, it has been recently found that expression of MTAP in an MTAP-deficient breast adenocarcinoma cell line resulted in a dramatic inhibition of tumorigenicity, showing that *MTAP* can function as a tumor suppressor gene [19].

Consistent with its central metabolic role, the liver parenchyma is one of the normal human tissue types where *MTAP* gene expression is higher [17]. We have previously shown that the expression of genes involved in methionine metabolism is impaired in human liver cirrhosis and hepatocarcinoma (HCC) and in experimental models of chronic liver damage [20,21], and that these alterations may contribute to the progression to the transformed phenotype [22,23]. However, to our knowledge, there are no studies addressing *MTAP* status in human liver disease. The aim of this work was to characterize the expression of *MTAP* in human and experimental liver cirrhosis and hepatocarcinoma.

2. Materials and methods

2.1. Patients

We obtained specimens of liver tissue from three groups of subjects: (a) control individuals (n=19; 11 males; mean age 58, range 45–70). Control human liver tissue was obtained from patients in whom a cholecystectomy was performed for the treatment of a symptomatic cholelithiasis and who consented to be submitted to a liver biopsy during the surgical procedure. In the control group, both the liver function tests and liver histology were normal or showed minimal changes. (b) Patients with liver cirrhosis of different etiology (n=10; nine males; mean age 60, range 42–77): six patients with hepatitis C viral (HCV) cirrhosis, two with hepatitis B viral (HBV) cirrhosis and two alcoholic cirrhosis. Cirrhotic liver samples were obtained at the time of liver transplantation. (c) Patients with primary hepatocellular carcinoma (HCC) (n=9; all males; mean age 67, range 39–79). Cancerous liver tissues were obtained during surgical resection. All tissues were immediately frozen in liquid nitrogen for subsequent isolation of RNA. This study was approved by the human research review committee of the University of Navarra. Studies were conducted in compliance with the ethical standards formulated at the Helsinki Declaration of 1975 (revised in 1983).

2.2. Experimental model of cirrhosis

Male Wistar rats (Harlan, Barcelona, Spain) (150 g of weight at the onset of treatment), fed ad libitum a standard laboratory diet, were used. Cirrhosis was induced with CCl_4 injected intraperitoneally (0.15 ml/100 g body weight) twice a week for 9 weeks. Animals were sacrificed 24 h after the last injection. Liver samples were taken, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. The establishment of cirrhosis was monitored by histological examination of liver sections from treated animals. We performed all experimental procedures in conformity with our Institution's guidelines for the use of laboratory animals.

2.3. Cell culture

The human hepatocarcinoma cell lines HuH7, PLC, SK-Hep1 and Hep3B were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics (Gibco). HuH7 and PLC cells were treated with 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St. Louis, MO, USA) at 10 μM for 48 h. 5-Aza-dC was added directly to complete culture medium from a 10 mM stock solution in distilled water. Trichostatin A (TSA) (Sigma) was added at a final concentration of 100 nM.

2.4. RNA isolation, RT-PCR and real-time PCR

We extracted total RNA using TRI Reagent (Sigma). We treated RNA (2 μg) with DNaseI (Gibco-BRL, Paisley, UK) prior to reverse transcription with M-MLV Reverse Transcriptase (Gibco-BRL) in the presence of RNaseOUT (Gibco-BRL), to avoid amplification of *MTAP* pseudogene [24]. We designed all primers to distinguish between genomic and cDNA amplification and sequenced all PCR products to confirm the specificity. Real-time PCR was performed with 1/20 of the RT reaction using an iCycler (BioRad) and the iQ SYBR Green Supermix (BioRad). The primers used for *MTAP* amplification were: 5′-TGGAATAATTGGTGGAACAGGC-3′ and 5′-TGGCACACTCCTCTGGCAC-3′. *MTAP* mRNA levels were normalized according to β-actin quantitation in the same sample. The primers used for β-actin amplification were: human 5′-AGCCTCGCCTTTGCCGA-3′ and 5′-CTGGTGCCTGGGGCG-3′, rat 5′-CAACCTCCTTGCAGCTC-3′ and 5′-CTGGTGCCTAGGGCG-3′. To monitor the specificity, final PCR products were analyzed by melting curves and electrophoresis. The amount of *MTAP* transcript was calculated and expressed as the difference relative to the control gene β-actin ($2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycles between the target and control genes) essentially as described [25]. Total RNA extracted from human peripheral blood cells and the human lung adenocarcinoma cell line A549 [14] were used as positive and negative controls for the real-time PCR analyses of *MTAP* gene expression.

2.5. Genomic DNA isolation and analysis of MTAP promoter methylation

Genomic DNA was isolated from control and cirrhotic liver samples, HCC tissue and HCC cell lines as previously reported [20,21]. Where indicated, aliquots of genomic DNA (0.5 μg) were digested with the restriction

enzyme *Not*I (New England Biolabs, Beverly, MA, USA) (10 U/μg of DNA) at 37 °C for 18 h. Subsequently, a 606 bp region of *MTAP* promoter was amplified by PCR using the Immolase DNA polymerase from Bioline (Randolph, MA, USA) (32 cycles, 58 °C annealing temperature), and the following primers: 5′-CGTCCAGGCTAATTTGCAGC-3′ and 5′-CCTCAC-CAAGGCGGGTACTG-3′. This region of human *MTAP* promoter contains a CpG island (nucleotides −461 to −441) [18], harboring an internal *Not*I restriction site. *Not*I digestion is inhibited by cytosine methylation, consequently PCR amplification of this region will not occur in unmethylated DNA samples. *MTAP* gene promoter methylation was further examined by methylation-specific PCR (MSP) analysis. Genomic DNA samples (1 μg) were treated with sodium bisulfite and subsequently purified using the CpGenome DNA modification kit from Qbiogene, Inc. (Carlsbad, CA, USA) following the manufacturer's recommendations. Two sets of primers were used to amplify each region of interest (35 cycles, 58 °C annealing temperature), one pair recognized a sequence in which CpGs are unmethylated and the other recognized a sequence with methylated CpGs. The primers used were: unmethylated sense 5′-TTTGTTTTTGTTGTGGTGGTTGT-3′ antisense 5′-TTAACCCAATATTAATAACATCAAA-3′, methylated sense 5′-TTTGTTTTCGTCGCGGCGGTC-3′ antisense 5′-TTAACCCAATATTAATAACGTCGAA-3′, and included the *Not*I site in the *MTAP* promoter mentioned above.

2.6. Assessment of allelic status of MTAP

Allelic status (no deletion, mono-allelic deletion, bi-allelic deletion) of *MTAP* was studied in control (n=9) and cirrhotic (n=6) human liver samples, in human HCC samples (n=5), and in the HCC cell lines mentioned above, using a real-time quantitative PCR method previously described [26]. This method of gene dosage has been successfully applied for the assessment of the allelic status of *MTAP* in human acute lymphoblastic leukemia. A single-copy sequence *S9ribP* mapping at 19q13.4 [27], a region that is not deleted in HCC [28], is used as reference sequence. The relative copy number of *MTAP* was obtained by calculating the ratio of the value obtained for *MTAP* to the *S9ribP* value as described [26]. The normalized ratio of *MTAP* to *S9ribP* values is expected to be close to 1 if no deletion was present, around 0.5 in the case of mono-allelic deletion and 0 for bi-allelic deletion [25]. Real-time PCR was performed using an iCycler (BioRad) and the iQ SYBR Green Supermix (BioRad). The primers used were 5′-GATGAGAAGGACCCACGGCGTCGTTTCG-3′ and 5′-GCAAAGACTGATCCAGATACAC-3′ for *S9ribP*, and 5′-TGGAATAATTGGTGGAACAGGC-3′ and 5′-CACCTTCTCAGCTGATGGAAC-3′ for *MTAP*. The sense primer for *MTAP* was an exonic primer while the antisense *MTAP* primer was intronic, to avoid amplification of *MTAP* pseudogene [24].

2.7. Measurement of AdoMet and MTA in rat liver samples

AdoMet and MTA concentrations were determined by high-pressure liquid chromatography according to the procedure previously described [29]. Briefly, liver samples were homogenized in 1 ml of 0.4 M perchloric acid. Homogenates were centrifuged at 10,000×g and 4 °C for 15 min and 100 μl of the supernatant were analyzed on a Bio-Sil® ODS-5S column equilibrated in 0.01 M ammonium formate, 4 mM heptanesulfonic acid, pH 4.0. AdoMet and MTA were eluted with a two-step gradient of acetonitrile in the same buffer (0–25% in 31 min and 25–100% in 20 min). Chromatograms were analyzed with the Beckman System Gold software.

2.8. Measurement of MTAP activity

Liver specimens were homogenized in five volumes of 50 mM Tris/HCl, pH 7.4 with protease inhibitors. The cytosolic fraction was obtained by centrifugation at 100,000×g and 4 °C during 90 min. The activity assay was performed according to the conditions described before with minor modifications [5]. Briefly, the reaction mixture contained 50 mM potassium phosphate buffer, pH 7.4, 200 μM MTA and the amount of cytosol required for a protein concentration of 1 mg/ml in a final volume of 400 μl. The assay mixture was incubated at 37 °C and the reaction was stopped after 0 and 30 min by adding perchloric acid to a final concentration of 0.4 M. MTAP activity was calculated as the consumption of MTA during the activity assay. Consumption of MTA was estimated by subtracting the MTA remaining after 30 min incubation from the initial concentration measured at time 0. MTA was measured in 100 μl aliquots as described above.

2.9. Statistics

Data obtained in experiments performed with hepatoma cell lines are mean±S.E. of at least three independent experiments performed in triplicate. Statistical significance was estimated using the Mann–Whitney unpaired test. The statistical analysis was performed with the SPSS 6.0 program.

3. Results

3.1. Expression of MTAP in healthy and diseased human liver

We examined *MTAP* mRNA levels by real-time PCR in 19 normal liver samples, 9 HCC and 10 cirrhotic liver samples (Fig. 1). As mentioned before, the expression of *MTAP* is diminished or lost in many types of human malignancies, our present data indicate that *MTAP* gene expression is markedly reduced in human HCC with respect to control livers. It is known that a significant number of

cirrhotic patients develop HCC. In this regard, the cirrhotic liver can be considered as a pre-cancerous condition in which molecular alterations involved in the development of the malignant phenotype take place [21,30–32]. Interestingly, we observe that the mRNA levels of *MTAP* are already significantly reduced in cirrhotic liver tissue as compared to healthy controls, and that there are no differences between cirrhotic liver and HCC in terms of *MTAP* gene expression. *MTAP* gene expression was similarly reduced in all cirrhotic liver samples regardless of the etiology (data not shown). In concordance with the reduced mRNA levels of *MTAP* observed in the cirrhotic liver samples, MTAP specific activity was also decreased when compared to healthy controls (123±17 vs. 47±1 pmol/min/mg of protein. Values are mean±S.E. $P<0.05$).

The allelic status of *MTAP* was studied in randomly selected control, cirrhotic and HCC samples according to the recently described method of Bertin et al. [26]. This real-time PCR-based method determines the dosage of a target gene using a reference gene not deleted in the condition studied [25]. The theoretical target/reference ratios are 0, 0.5 and 1 for samples with bi-allelic deletion, mono-allelic deletion and no deletion, respectively. The mean values (±S.D.) obtained for *MTAP* gene in this assay were: 1.00±0.05 in control liver samples (n=9), 0.88±0.18 in cirrhotic liver samples (n=6) and 0.96±0.17 in HCC samples (n=5). The data obtained in cirrhotic and HCC samples were close to the theoretical value of 1, observed in the control samples, suggesting that no deletional events occurred in *MTAP* in our cirrhotic or tumoral liver samples.

3.2. Expression of MTAP in experimental liver cirrhosis

The impairment in *MTAP* gene expression observed in cirrhotic human liver was confirmed in the experimental model of CCl_4-induced liver cirrhosis in rats. As shown in Fig. 2, the mRNA levels of *MTAP* were markedly reduced in the liver of rats with established hepatic cirrhosis. Given the central role played by MTAP in MTA metabolism, we

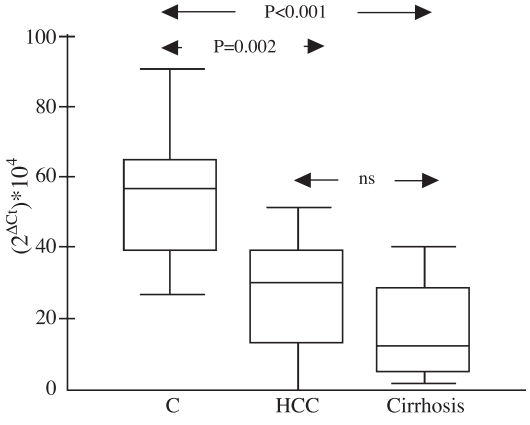

Fig. 1. Expression of *MTAP* in control, HCC and cirrhotic human liver. *MTAP* mRNA levels were determined in control (n=19), HCC (n=9) and cirrhotic (n=10) human liver samples by real-time PCR.

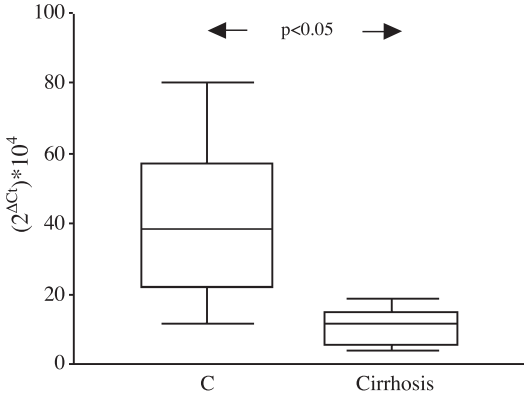

Fig. 2. Expression of *MTAP* in control and CCl_4-cirrhotic rat liver. *MTAP* mRNA levels were determined by real-time PCR in control (n=6) and cirrhotic rat liver (n=6). Values are mean±S.E. Differences between groups were statistically significant ($P<0.05$).

measured the hepatic contents of this metabolite in control and cirrhotic rat livers. We found that in chronically injured rat liver MTA levels were reduced by 47% when compared to control rats (6.65±0.5 vs. 3.53±0.2 pmol/mg of protein, n=6 animals per group. Values are mean±S.E. $P<0.05$). Thus, in spite of a compromised expression of MTAP, MTA is not accumulated in the cirrhotic liver. As indicated before, MTA is produced during polyamine biosynthesis from AdoMet, its metabolic precursor. We determined AdoMet contents in control and cirrhotic livers, and found that these were dramatically reduced in CCl_4-treated rats (80.3% decrease) (43.4±12.7 vs. 8.53±1.67 pmol/mg of protein, n=6 animals per group. Values are mean±S.E., $P<0.05$).

3.3. MTAP promoter is hypermethylated in the cirrhotic and transformed human liver. Involvement of epigenetic mechanisms in the downregulation of MTAP gene expression

Loss of *MTAP* gene expression in transformed cell lines and tissues has been mainly attributed to homozygous deletions and translocations at the chromosome 9p21 region [17]. We examined the expression of *MTAP* in four different human hepatocarcinoma cell lines by real-time PCR. As depicted in Fig. 3A, *MTAP* gene expression was detected in all of them, with the exception of SK-Hep1 cells. However, *MTAP* mRNA levels were markedly reduced, but not completely absent, when compared to levels found in healthy human liver. In addition, heterozygous deletions were not detected in these HCC cell lines, except in Sk-Hep1 cells in which *MTAP* was homozygously deleted. This is in agreement with the reduced expression of *MTAP* found in HCC tissues and our previous observation showing the absence of allelic losses of *MTAP* in HCC. Altogether, these data suggest the involvement of alternative mechanisms for the impaired expression of *MTAP*. Methylation at CpG dinucleotides in the promoter region of genes contributes to the silencing of many genes during cellular transformation [33–35]. It has been recently reported that hypermethylation of a CpG island (nucleotides −461 to−441) in *MTAP* gene

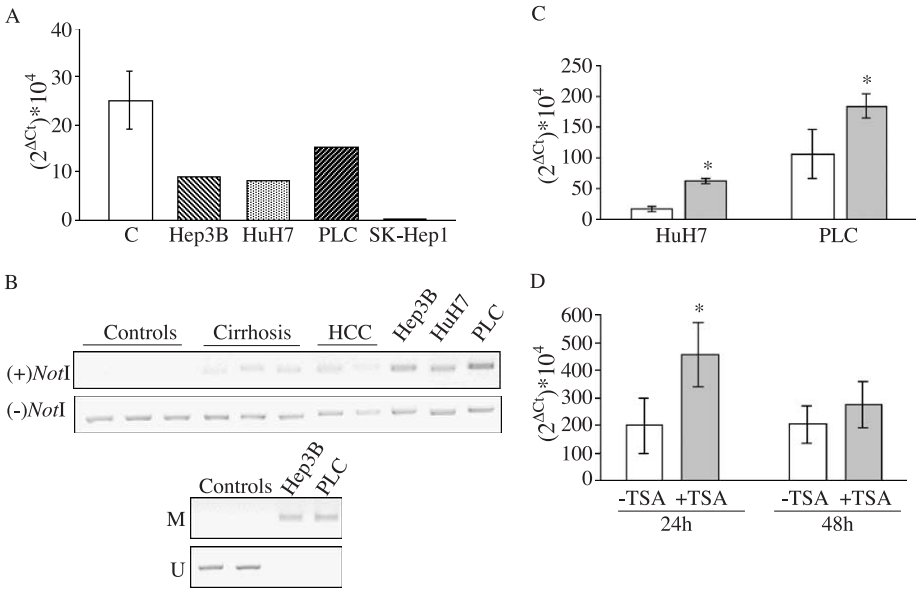

Fig. 3. (A) Expression of *MTAP* in human HCC cell lines. *MTAP* mRNA levels were determined in Hep3B, HuH7, PLC and SK-Hep1 cell lines by real-time PCR. Values found in control human liver (mean±S.E., n=19) are shown. (B) Upper panel: methylation analysis of human *MTAP* promoter in samples from control human liver, cirrhotic human liver, human HCC and the HCC cell lines Hep3B, HuH7 and PLC. Genomic DNA samples were treated or not with the methylation-sensitive *Not*I restriction enzyme and subsequently amplified by PCR using primers flanking the CpG island containing the *Not*I restriction site. PCR amplification in HCC cells indicates the hypermethylation of *MTAP* promoter. Lower panel: MSP analysis of *MTAP* promoter methylation in control human liver and the HCC cell lines Hep3B and PLC (M: methylated, U: unmethylated). (C) Effect of 5-aza-dC treatment (10 μM for 48 h, solid bars) on *MTAP* gene expression in HuH7 and PLC cells as determined by real-time PCR. Data are mean±S.E. of two experiments performed in triplicate. Differences between control and treated cultures were statistically significant (*P<0.05). (D) Effect of TSA (100 nM) on *MTAP* gene expression in PLC cells. Data are mean±S.E. of two experiments performed in triplicate. Differences between control and treated cultures were statistically significant (*P<0.05).

promoter occurs in human melanoma cells, and is responsible for the reduced expression of *MTAP* in this transformed cell type [18]. We examined the methylation status of *MTAP* gene promoter in three samples of cirrhotic human liver, two human HCC samples, three HCC cells lines and three samples of normal human liver. For this purpose, genomic DNA was incubated with the methylation-sensitive restriction enzyme *Not*I, and subsequently PCR amplified using primers flanking the CpG island containing the *Not*I site. As shown in Fig. 3B (upper panel), after *Not*I digestion the PCR product was detected in the cirrhotic and HCC samples as well as in HuH7, Hep3B and PLC cells, but not in control liver samples. However, in the absence of *Not*I treatment, genomic DNA amplification was observed in all samples (Fig. 3B, upper panel). The same results were obtained with the methylation-sensitive restriction enzyme *Sma*I and the appropriate primers interrogating a *Sma*I site located 409 nucleotides 3′ of the *Not*I site in *MTAP* promoter (data not shown). Further confirmation of *MTAP* promoter hypermethylation in transformed liver cells was obtained by MSP analysis of the HCC cell lines Hep3B and PLC and control liver samples (Fig. 3B, lower panel). Together, these observations prompted us to test if such epigenetic modification could play a role in the reduced expression of *MTAP* in HCC cell lines. For this purpose, we treated HuH7 and PLC cells with the demethylating agent 5-aza-dC (10 μM) for 48 h, and then measured *MTAP* gene expression by real-time PCR. As shown in Fig. 3C, *MTAP* expression was upregulated under these conditions. Epigenetic mechanisms involved in gene silencing involve chromatin changes that favor a close chromatin conformation preventing the normal transcription of genes [34]. Methylation of cytosine residues within CpG islands promote the recruitment of methylcytosine-binding proteins to the DNA methylated sites. Among the proteins recruited to methylated DNA regions are the histone deacetylases (HDACs). Deacetylated histones form tightly compacted and regularly spaced nucleosomes that exclude proteins that activate gene transcription [34,35]. The involvement of such epigenetic mechanisms in *MTAP* silencing in HCC was further confirmed when the expression of *MTAP* was induced in PLC cells upon treatment with the HDAC inhibitor TSA (100 nM) (Fig. 3D).

4. Discussion

In this work we have evaluated the expression of *MTAP* in human HCC tissues and cell lines by real-time PCR, and found it to be significantly reduced when compared to normal human liver. It has been extensively reported that *MTAP* gene expression is lost in a wide variety of human transformed cell lines and tumors [12–17]. The *MTAP* gene has been mapped to the chromosome 9p21 region [17,36,37]. This region also contains the cell cycle regulatory genes $p16^{INK4A}$ and $p14^{ARF}$, and is frequently

homozygously deleted in primary tumors [38]. These deletions are quite large and often involve multiple genes, as evidenced by the high incidence of co-deletion of *MTAP* and $p16^{INK4A}$ in malignant cells [39]. Our data revealed that although reduced, *MTAP* gene expression was detected in the human HCC tissues and cell lines examined, and that allelic deletions were not detected in human liver cirrhosis, HCC samples or cell lines. These facts, together with previous reports demonstrating that the $p16^{INK4A}$ locus is not frequently deleted in human HCC [40,41], suggested the involvement of alternative mechanisms in *MTAP* down-regulation. The hypermethylation of CpG islands in the promoter regions of genes is associated with the aberrant silencing of transcription observed in cancer. Methylated CpG dinucleotides are bound by methyl cytosine-binding proteins, such as MeCP2, that can in turn recruit protein complexes including HDACs. Histone deacetylation alters the configuration of nucleosomes, promoting chromatin condensation and impairing gene transcription [34,35,42]. Interestingly, de novo hypermethylation of $p16^{INK4A}$ promoter is the most frequent somatic alteration of this gene found in human HCC [41]. Our present data show that *MTAP* promoter is also hypermethylated in human HCC cell lines. We were able to upregulate *MTAP* gene transcription in HCC cells using 5-aza-dC, a demethylating agent, and TSA, an inhibitor of HDACs. Together, these observations suggest that epigenetic mechanisms are likely to participate in the downregulation of *MTAP* expression in HCC, as has been previously demonstrated in melanoma cells [18].

Chronic liver injury, inflammation, fibrosis and finally liver cirrhosis often precede the development of HCC. Liver cirrhosis is thus considered a preneoplastic stage in this multistep process, and certain genetic and epigenetic alterations found in HCC, such as $p16^{INK4A}$ hypermethylation, can be detected in this condition [20,21,30,31,43]. In this context we have previously shown that the hepatic mRNA levels of the main enzymes involved in methionine metabolism are reduced in the cirrhotic human liver and HCC [20]. In particular, the expression of methionine adenosyltransferase 1A gene (*MAT1A*), responsible for AdoMet synthesis in the liver, is reduced in human and experimental cirrhosis, and silenced in HCC, through mechanisms involving chromatin remodeling [20,21,23]. These findings are in line with our current observations showing that *MTAP* gene expression is already impaired in human and CCl_4-induced rat liver cirrhosis.

The expression of *MTAP* in human liver cirrhosis seemed lower than in HCC tissues. Although this difference did not reach statistical significance, there was indeed a tendency towards reduced expression in the first condition. It is worth mentioning that we have previously observed a similar situation for other genes involved in methionine metabolism. For example, the expression of betaine-homocysteine methyltransferase and methionine synthase were more compromised in a higher number of samples of liver cirrhosis than in HCC tissues [20]. It seems that the expression of methionine metabolism-related genes would be especially sensitive to the environmental conditions of liver cirrhosis.

It has been shown that re-expression of MTAP in cancer cell lines devoid of MTAP activity results in reduced cellular levels of MTA [44]. One could expect that the impairment in *MTAP* gene expression observed in our experimental model of liver cirrhosis could lead to the accumulation of this metabolite. However, the consequences of manipulating *MTAP* gene expression in a cancer cell line may be different from what occurs in chronic liver injury. In a similar model of CCl_4-induced cirrhosis, it was previously reported that hepatic MTA levels were diminished, and that MTA treatment reduced liver damage [45]. In agreement with this, we have observed a substantial decrease in MTA contents in the liver of cirrhotic rats. One possible explanation for this situation may lay in the reduced ability of the cirrhotic liver to efficiently convert methionine into AdoMet, which is the metabolic precursor of MTA [23]. This is supported by our present findings showing a dramatic reduction in AdoMet levels in our model of rats chronically treated with CCl_4.

Taken together, our findings suggest that in the chronically injured liver the metabolic flow through the methionine and adenine salvage pathways is impaired due to the combined deficiency in MTA availability and reduced *MTAP* gene expression and enzymatic activity. There are a number of potential pathogenic consequences for this situation. Considering that the endogenous production of adenine and adenine nucleotides is significantly dependent on MTAP activity [46], our present observations may contribute to explain the significant reduction in total adenine nucleotides found in the cirrhotic rat liver [47]. In addition, it has been recently reported that the reduction in downstream products of the methionine salvage pathway due to *MTAP* deletion may lead to ornithine decarboxylase (ODC) activation in human tumors [19]. ODC is the rate-limiting enzyme in the production of polyamines [4]. Elevated ODC activity has been reported in many human tumors and its overexpression is sufficient to cause transformation [4,48]. Re-expression of MTAP in a human mammary tumor cell line resulted in decreased ODC activity and tumorigenesis [19,49]. Consequently, our current observations may help to explain the elevation of ODC activity found not only in human HCC, but also in human and experimental chronically injured liver [50–52]. Hepatocarcinogenesis is a complex and multistep process and the precise genomic alterations that drive its development are not completely understood [28]. Aberrant promoter methylation and transcriptional inactivation of tumor suppressor genes is emerging as a major mechanism in the development of human cancer, and HCC is one of the tumor types with a high frequency of CpG island methylation [53–55]. Furthermore, CpG island hypermethylation is frequently observed in chronic liver disease and accumulates during the different stages of human hepatocarcinogenesis [43,55]. This situation has been described so far for a

number of genes that may be directly related to the control of cell growth [43,53–55]. The relevance of a persistent downregulation of *MTAP* gene expression for the development of cancer can be inferred from the recently observations showing the tumor suppressor activity of this gene [49]. However, in the case of hepatocarcinogenesis, the early loss of *MTAP* expression may have further significance. It is known that impairment of methionine metabolism in rodents by feeding a diet deficient in methionine and choline leads to the development of HCC [56], and we have recently shown that *MAT1A* knockout mice spontaneously develop HCC [57]. These facts support a strong link between a defective methionine metabolism in the liver and the development of neoplasia. Our present observations on the hypermethylation and impairment of *MTAP* gene expression in preneoplastic stages support this notion.

In summary, we have shown that *MTAP* gene expression is compromised in liver cirrhosis and HCC probably through epigenetic mechanisms. The reversible nature of these epigenetic modifications [33–35], make *MTAP* a candidate gene for the application of alternative therapeutic and preventive interventions in liver cancer [59]. Indeed, clinical trials using low doses of demethylating agents have yielded encouraging responses in hematopoietic malignancies (reviewed in Ref. [35]). Inhibitors of HDACs have been shown to reactivate tumor suppressor genes, and are currently being developed for their potential use in cancer therapy in combination with demethylating agents [35,58]. Finally, strategies aimed at the direct inhibition of the enzymatic activity of the different DNA methyltransferases, using compounds that do not have to be incorporated into DNA, are also being considered [35].

Acknowledgements

This work was supported by: the agreement between FIMA and the “UTE project CIMA”; grant Plan Nacional de I+D 2002-00168 from Ministerio de Educación y Ciencia to JMM; grant FIS PI020369 from Ministerio de Sanidad y Consumo to ERG; grant ROI AA-12677 from the National Institute on Alcohol Abuse and Alcoholism to MAA and JMM; grant R01 AT1576 from the National Center for Complementary and Alternative Medicine to MAA, FJC, and JMM; grants C03/02 and G03/015 from Instituto de Salud Carlos III to ERGT, JP, CB, JC, JMM, FJC and MAA; SAF 2004-03538 and SAF 2004-01855 to MAA and FJC. H. Hevia is a fellow of the Fundación Ramón Areces. The technical assistance of Eva Petri is acknowledged.

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