Differential Expression of Methionine Adenosyltransferase Genes Influences the Rate of Growth of Human Hepatocellular Carcinoma Cells

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ABSTRACT

Methionine adenosyltransferase (MAT) catalyzes the formation of S-adenosylmethionine (SAM), the principal methyl donor, and is essential to normal cell function. The two forms of MAT, liver specific and non-liver specific, are products of two genes, MAT1A and MAT2A, respectively. We have reported a switch from MAT1A to MAT2A gene expression in human liver cancer cells. In the current work, we examined whether the type of MAT expressed by the cell influences cell growth. HuH-7 cells were stably transfected with MAT1A and were subsequently treated with antisense oligonucleotides directed against MAT1A. MAT2A antisense treatment reduced the amount of MAT2A mRNA by 99% but had no effect on MAT1A mRNA. Cell growth and DNA synthesis rates were reduced by ~20–25% after transfection with MAT2A and by an additional 30–40% after MAT2A antisense treatment. SAM level and SAM:S-adenosylhomocysteine (SAH) ratio increased by 50–75% after MAT1A transfection and by an additional 60–80% after MAT2A antisense treatment. DNA methylation changed in parallel to changes in SAM level and SAM:SAH ratio. Supplemeting untransfected HuH-7 cells with SAM in the culture medium increased SAM level, SAM:SAH ratio, and DNA methylation and decreased cell growth and DNA synthesis. In conclusion, cell growth is influenced by the type of MAT expressed. The mechanism likely involves changes in SAM:SAH ratio and DNA methylation.

INTRODUCTION

MAT catalyzes the formation of SAM, the principal biological methyl donor, and is essential to normal cell function (1, 2). Mammalian MAT exists as three different isozymes, α (or MATI), β (or MATII), and γ (or MATIII; Refs. 3–5). MATI and MATIII are confined to the liver, composed of four and two identical subunits, respectively, produced by the same gene, MAT1A (see Ref. 5 for a consensus nomenclature of the mammalian MAT genes and gene products). MATII is a product of a different gene, MAT2A, and is widely distributed (3–5). MATII also predominates in the fetal liver and is progressively replaced by the liver-specific isoforms during development (6, 7).

Different isoforms of MAT differ in kinetic and regulatory properties (2, 8–12). The Km for methionine is lowest for MATII (~4–10 μM), intermediate for MATI (23 μM–1 mM), and highest for MATIII (215 μM–7 mM), with different studies reporting different absolute values depending on the purification procedure and purity of the enzyme (8–12). The activity of MAT is also modulated by SAM, the product of the reaction it catalyzes. SAM strongly inhibits MATII (IC50, 60 μM), whereas it minimally inhibits MATI (IC50, 400 μM) and stimulates MATIII (9).

We reported a switch in gene expression from MAT1A to MAT2A in various human-derived liver cancer cell lines and hepatoma tissues resected from patients (13). Consistent with the known differences in kinetic parameters, the MAT activity of cancerous liver cells was much higher at lower and physiologically relevant methionine concentrations (50–100 μM) than in cultured normal rat and human hepatocytes, which exhibited much higher MAT activity at high (5 mM) methionine concentration (13, 14). This led us to speculate that the altered gene expression from MAT1A to MAT2A might provide a growth advantage to cancerous liver cells. In the current work, we established a cell line model of HuH-7 cells that differ only in the type of MAT expressed, studied whether the type of MAT expressed influences cell growth and DNA synthesis, and examined the mechanisms involved.

MATERIALS AND METHODS

Materials. FBS, SAM, SAH, 5-methylcytosine, and cytosine were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM and G418 were purchased from Mediatech’s Cellgro (Tustin, CA). Methyl-L-[3H]methionine (214 mCi/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Total RNA isolation kits were obtained from Promega (Madison, WI). Normal human liver and kidney RNA were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). All other reagents were of analytical grade and were obtained from commercial sources.

Human Liver Cancer Cell Lines. HuH-7 and HepG2 cells were provided by the Cell Culture Core, USC Liver Disease Research Center. Both were cultured using DMEM containing 10% FBS. All cells were subcultured every 5–7 days at a cell density of 2 × 10⁶ cells per 75 cm² flask. Medium was changed every 3–4 days.

MAT Activity. MAT activity was measured using 50 μM or 5 mM methionine as described (13). Protein was determined by the method of Bradford (15). MAT activity is reported as nmol of SAM formed per mg protein per min.

Nucleic Acid Extraction. RNA was isolated from HuH-7 and HepG2 cells according to the method of Chomczynski and Sacchi (16). RNA concentration was determined spectrophotometrically before use, and the integrity was checked by electrophoresis.

RT-PCR. Total RNA (1 μg) was subjected to RT and subsequently amplified by PCR according to described methods (3, 13). Primers were designed to amplify human MAT1A (primer pair: nucleotide residues 24–43 and 171–190) and human MAT2A (primer pair: nucleotide residues 24–43 and 210–229) as described (3, 13). The sizes of the amplified fragments corresponding to human MAT1A and MAT2A were 167 and 206 bp, respectively.

Stable Transfection of HuH-7 and HepG2 Cells with MAT1A Expression Vector. The MAT1A expression vector pMAM-SSRL was constructed and kindly provided by Drs. Luis Alvarez and José Mato (Instituto de Investigaciones Biomédicas, Madrid, Spain). It contains the same MAT1A sequence as the vector pMEX-SSRL, which was used successfully in transfection experiments involving Chinese hamster ovary cells (17). The major difference is the substitution of enhancer/promoter from Moloney sarcoma virus long terminal repeat to mouse mammary tumor virus long terminal repeat, to allow further induction of expression by glucocortioids, which up-regulates the gene expression of MAT1A (18). The identity of the entire coding region was confirmed by sequencing using the dyeoxynucleotide chain-termination method (19).
Transfection experiments were done according to standard techniques (19). Huh-7 and HepG2 cells (6 × 10⁵) were plated on 100 × 15-mm plastic dishes and cultured in DMEM supplemented with 10% FBS. Duplicate plates were transfected with 30 μg of either pMAM-SSRL or control vector (pMAM-neo; Clontech) by the calcium phosphate precipitation method according to protocol from 5 Prime 3 Prime, Inc. (Boulder, Co). To obtain stably transfected cells for large-scale studies, transiently transfected cells (after 48 h of transfection) were selected for 4 days with 0.8 mg/ml of G418 neomycin analogue. The medium containing G418 was changed every 3 days. After 14 days, colonies of cells containing G418 were changed every 3 days. After 14 days, colonies of cells containing human MAT2A-specific sequence and the neutral DNA fragment were generated by the Molecular Biology Core Facility of the USC.

Construction Kit (Clontech) by the calcium phosphate precipitation method according to protocol described in the PCR MIMIC (130 bp) was obtained by amplifying the neutral DNA sequence were generated by the MacVector 4.1.4 software program, was used as control. Cell lysis was used as a way of rapid screening of the effectiveness of the antisense treatment, because cells that express only MAT2A would not be viable without the enzyme. Total RNA was extracted from these cells, and 1 μg of the RNA was subjected to RT.

In pilot experiments, we found that 0.5 μM nonsense phosphorothioated oligonucleotides were toxic to the cells. The best results were obtained with two treatments, 48 h apart, of 0.4 μM of antisense oligonucleotides. All reported results used this regimen.

The amount of 5-mC and cytosine in each sample was calculated by comparing the retention times for SAM and SAH were 12 and 18 min. respectively. SAM and SAH prepared at the same time as the samples. The identities of SAM and SAH could be easily distinguished by measuring absorbance at 280 nm at a sensitivity scale of 0.01. The amount of 5-mC and cytosine in each sample was calculated by comparing the area of the peaks with standard curves of 5-mC and cytosine. The identities of 5-mC and cytosine were also confirmed by adding known standards to the samples. The percentage of methylation was calculated by the ratio of mmol of 5-mC to mmol of cytosine multiplied by 100.

SAM and SAH Measurement. Cells were treated twice with vehicle control or 0.4 μM nonsense or antisense 1 and 2 at 0 and 48 h and processed for measurement of SAM and SAH levels as described with slight modifications (24). Cells (1 × 10⁵) from each condition were scraped off in 5 ml of PBS, and an aliquot was saved for protein determination by the method of Bradford (15). The cell suspension was pelleted at 500 × g for 15 min, and the aqueous layer was determined in the neutralized perchloric acid extracts by high-performance liquid chromatography (series 410 LC pump; Perkin-Elmer Corp.) with a LC-90 UV detector and a LC-100 integrator (Perkin-Elmer). Samples (75–100 μg) were injected into a Partisil SCX 10-μm column (25 × 0.44 cm inside diameter; Whatman, Clifton, NJ) at room temperature and a flow rate of 2.5 ml/min. Cytosine and 5-mC were eluted with 0.02 M ammonium phosphate/HCl buffer (pH 2.3). DNA bases were identified by measuring absorbance at 280 nm at a sensitivity scale of 0.01. The amount of 5-mC and cytosine in each sample was calculated by comparing the area of the peaks with standard curves of 5-mC and cytosine. The identities of 5-mC and cytosine were also confirmed by adding known standards to the samples. The percentage of methylation was calculated by the ratio of mmol of 5-mC to mmol of cytosine multiplied by 100.

Establishment of HuH-7 Cells That Express Different MAT.

Normal Huh-7 cells express only MAT2A (Fig. 1, Lanes 5 and 6). After stable transfection with pMAM-SSRL, the presence of MAT1A mRNA can be easily detected by RT-PCR (Fig. 1, Lanes 7 and 9). The gene products of MAT1A, MATI, and MATIII, exhibit much higher...
Fig. 1. RT-PCR of HuH-7 cells before and after transfection with pMAM-SSRL (MATIA). RNA (1 µg) from normal human liver and kidney and HuH-7 cells before and after transfection (different clones are represented here) were subjected to RT-PCR using MATIA-specific primers (product, 167 bp) or MAT2A-specific primers (product, 206 bp), and products were analyzed on a 2% agarose gel. The housekeeping gene, ß-titin, was equally expressed in all samples (not shown).

Table 1 MAT activity in MAT1A-transfected and MAT2A-antisense- or nonsense-treated HuH-7 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Methionine (50 µM)</th>
<th>Methionine (5 mM)</th>
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<tbody>
<tr>
<td>Untransfected</td>
<td>0.05 ± 0.003</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>MAT1A transfection</td>
<td>0.050 ± 0.005</td>
<td>2.68 ± 0.20</td>
</tr>
<tr>
<td>MAT2A transfection and treatment with antisense 1</td>
<td>0.052 ± 0.004*</td>
<td>2.51 ± 0.34*</td>
</tr>
<tr>
<td>MAT1A transfection and treatment with antisense 2</td>
<td>0.031 ± 0.002c</td>
<td>2.93 ± 0.30b</td>
</tr>
<tr>
<td>MAT2A transfection and treatment with nonsense</td>
<td>0.054 ± 0.001</td>
<td>2.60 ± 0.10b</td>
</tr>
</tbody>
</table>

Results represent means ± SE from three or four separate experiments. HuH-7 cells that normally express MAT2A were stably transfected with the MATIA expression vector pMAM-SSRL and subsequently treated with antisense oligonucleotides to MAT2A as described in “Materials and Methods.” MAT activity was determined at the end of the treatment. *P < 0.05 versus respective control and nonsense treated by ANOVA followed by Fisher’s test.

Kₘ's for methionine (mM range) than does the gene product of MAT2A, MAT1I (4–10 µM; Refs. 8–12). As expected, stable transfection with MATIA resulted in an increase in MAT activity at 5 mM methionine (Table 1).

We next treated untransfected and transfected cells with antisense oligonucleotides against MAT2A. Because untransfected cells express only MAT2A, elimination of this critical cellular enzyme would not be compatible with cell survival. Fig. 2 shows that as expected, antisense treatment (0.4 µM, treated twice, 48 h apart) selectively killed untransfected HuH-7 cells while sparing the transfected HuH-7 cells, which also express MAT1A.

We used competitive RT-PCR to measure the amount of reduction in MAT2A mRNA after antisense treatment. Fig. 3 shows a 10-fold dilution series (Lane 1, 720 attomol of MIMIC). MAT1A-transfected HuH-7 cells treated with either vehicle control or nonsense oligonucleotide (Fig. 4, top, represented by nonsense) express 3.6 attomol of MAT1A mRNA/µg of total examined the amount of MAT1A mRNA by competitive RT-PCR after these same treatments. Fig. 4 shows a 10-fold dilution series (Lane 1, 720 attomol of MIMIC). MAT1A-transfected HuH-7 cells treated with either vehicle control or nonsense oligonucleotide (Fig. 4, top, represented by nonsense) express 3.6 attomol of MAT1A mRNA/µg of total

Fig. 2. Effects of antisense and nonsense treatment against MAT2A on cell lysis in untransfected and MAT1A-transfected HuH-7 cells. Untransfected and MAT1A-transfected HuH-7 cells treated with vehicle control, nonsense, antisense 1, or antisense 2 against MAT2A had cell lysis measured, as described in “Materials and Methods.” Results represent means (bars, SE) from four experiments. *P < 0.05 versus respective control and nonsense treated by ANOVA followed by Fisher’s test.

Table 3 Competitive RT-PCR of MAT2A. RNA obtained from MAT1A-transfected HuH-7 cells after vehicle control, nonsense, antisense 1, or antisense 2 treatment against MAT2A was subjected to competitive RT-PCR using primers designed for MAT2A as described in “Materials and Methods.” Lanes represent a 10-fold dilution series; Lane 1, 45 attomol of MIMIC. Top, MAT1A-transfected HuH-7 cells treated with vehicle or nonsense (represented here by nonsense) contain 4.5 attomol of MAT2A mRNA/µg of total RNA (as the RT product represented 0.1 µg of total RNA); bottom, antisense 1- and 2-treated transfected HuH-7 cells contain only 1% of that amount (antisense 1 is shown here). In parallel, the same amount of cDNA products was subjected to PCR for 25 cycles using primers for ß-actin, and these treatments did not change the amount of ß-actin expression (not shown). A representative of four separate experiments is shown.

To ensure the specificity of the MAT2A antisense effect, we also
DNA synthesis is not limited to HuH-7 cells, we also examined HepG2 cells, another human liver cancer cell line that exhibited the switch in the gene expression from MATI\(^{1}\) to MAT2\(^{A}\) (13). HepG2 cells were stably transfected with MATI\(^{1}\), pMAM-SSRL, or the vector control pMAM-neo and had cell growth and DNA synthesis rates measured as described above. MATI\(^{1}\)-transfected HepG2 cells had lower rates of cell growth and DNA synthesis compared to untransfected or vector-transfected controls. The rate of cell growth in untransfected HepG2 cells was 1,815 ± 108/h, in vector-transfected cells it was 1,878 ± 101/h, and in MATI\(^{1}\)-transfected cells it was 1,132 ± 83/h. \(^{3}\)HThymidine incorporation into DNA in untransfected HepG2 cells was 16,451 ± 435 dpm/µg DNA, in vector-transfected cells it was 16,861 ± 298 dpm/µg DNA, and in MATI\(^{1}\)-transfected cells it was 10,931 ± 911 dpm/µg DNA. These results represent means ± SE from three separate experiments; \( P < 0.05 \) between MATI\(^{1}\)-transfected cells and untransfected or vector-transfected controls by ANOVA followed by Fisher’s test.

**Possible Mechanisms for the Influence of MAT Expression on Cell Growth**. Two major differences between the two forms of MAT, liver specific and non-liver specific, are in the kinetic parameters for methionine and ATP and in their response to SAM, which strongly inhibits MATII with IC\(50\) of 60 µM, minimally inhibits MATI with IC\(50\) of 400 µM, and strongly stimulates MATIII (8-12). Thus, changes in MAT gene expression are likely to result in changes in SAM level and the ratio of SAM:SAH. SAH, the reaction product of...
all transmethylation reactions, competitively inhibits all transmethylation reactions (25). Both an increased SAH level and a fall in the ratio of SAM:SAH are known to inhibit transmethylation reactions (25). Thus, we examined levels of SAM, SAH, and DNA methylation as a result of the changes in MAT expression.

Table 2 shows that SAM level increased by 52% after transfection with MATIA, and this was further increased by 50–65% after MAT2A antisense but not after nonsense treatment. The ratio of SAM:SAH increased in parallel with SAM level, whereas SAH level remained relatively unchanged. In accordance with changes in SAM level and SAM:SAH ratio, DNA methylation as estimated by the percentage of methylated cytosine decreased by 42% after MATIA transfection, and this was further increased by 88% after MAT2A antisense but not nonsense treatment.

To see whether the changes in SAM:SAH ratio were responsible for changes in DNA methylation and cell growth, untransfected HuH-7 cells were supplemented with SAM (1 mM) or vehicle control in the culture medium for 4 days, with a medium change at 48 h. SAM and SAH levels, DNA methylation, and DNA synthesis were measured at the end of the 4th day of SAM treatment. Cell growth rates were also measured as before during the 4 days of SAM treatment. SAM treatment increased significantly the intracellular SAM level and the ratio of SAM:SAH and DNA methylation and decreased significantly DNA synthesis and cell growth (Table 3). SAM treatment exerted no toxic effect on HuH-7 cells as measured by LDH release (not shown).

**DISCUSSION**

MAT is a critical cellular enzyme that catalyzes the formation of the predominant biological methyl donor, SAM. Inhibition of this enzyme leads to cell death. We previously reported a switch from antisense oligonucleotides to MATIA as described in “Materials and Methods.” SAM and SAH levels and percentage of total cytosine methylated were determined as described in “Materials and Methods.” Ps were determined by ANOVA followed by Fisher’s test.

<table>
<thead>
<tr>
<th>Table 2 Effect of MATIA transfection and MAT2A-antisense treatment on SAM and SAH levels and DNA methylation in HuH-7 cells</th>
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<tbody>
<tr>
<td>Condition</td>
</tr>
<tr>
<td>Untransfected</td>
</tr>
<tr>
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<tr>
<td>MATIA transfection and treatment with antisense 1</td>
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<tr>
<td>MATIA transfection and treatment with antisense 2</td>
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<tr>
<td>MATIA transfection and treatment with nonsense</td>
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<sup>a</sup> Results represent means ± SE from three or four separate experiments. HuH-7 cells that normally express MAT2A were stably transfected with MATIA expression vector and subsequently treated with antisense oligonucleotides to MAT2A as described in “Materials and Methods.” SAM and SAH levels and percentage of total cytosine methylated were determined as described in “Materials and Methods.” Ps were determined by ANOVA followed by Fisher’s test.

<sup>b</sup> nmol/mg protein.

<sup>c</sup> Percentage of cytosine methylated.

<sup>d</sup> P < 0.05 versus untransfected cells.

<sup>e</sup> P < 0.05 versus MATIA-transfected cells and MATIA-transfected cells treated with nonsense oligonucleotide.

What is the mechanism for the growth difference in cells expressing different MAT isoforms? We initially speculated that the advantage in cell growth was due to expression of an enzyme that was more active at low physiological methionine concentrations and more able to provide SAM. However, the steady-state SAM level was actually lower in cells expressing only MAT2A. Transfection of HuH-7 cells with MATIA increased SAM levels and SAM:SAH ratios. SAH levels were unchanged in the MATIA-transfected cells. Elimination of MAT2A by antisense treatment in transfected cells led to a further increase of SAM levels and SAM:SAH ratios. These findings can be largely explained by the known differences in the kinetic parameters and regulation by SAM of different MAT isoforms (2, 8–12). The gene product of MAT2A, MATII, is strongly inhibited by SAM, with an IC₅₀ of 60 μM (9), which is close to the normal intracellular SAM concentration (14, 26, 27). At the same SAM concentration, the gene products of MATIA are either unaffected (MATI) or stimulated (MATIII: Ref. 9). Thus, increased methionine availability to cells that express gene products of MATIA would offer liver cancer cells a growth advantage.

In tissues that express only the non-liver-specific MAT isoform or MATH, the rate of cell growth was due to expression of an enzyme that was more active largely explained by the known differences in the kinetic parameters and regulation by SAM of different MAT isoforms (2, 8–12). The gene product of MAT2A, MATII, is strongly inhibited by SAM, with an IC₅₀ of 60 μM (9), which is close to the normal intracellular SAM concentration (14, 26, 27). At the same SAM concentration, the gene products of MATIA are either unaffected (MATI) or stimulated (MATIII: Ref. 9). Thus, increased methionine availability to cells that express gene products of MATIA would offer liver cancer cells a growth advantage.

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In a rodent model of hepatocarcinogenesis, Feo and coworkers reported a 50% fall in SAM levels in preneoplastic lesions, as well as in frank hepatocellular carcinoma (28, 29). All of these studies assumed that hepatic SAM levels fell because of increased consumption by neoplastic cells (28, 29). However, the mechanism responsible for the fall in hepatic SAM levels and the ratios of SAM:SAH was not addressed. Our current work coupled with our previous observation of a switch in MAT gene expression from MATIA to MAT2A in various human liver cancer cells (13) strongly suggests that the switch in MAT gene expression can also contribute to the fall in SAM level during hepatocarcinogenesis.

In normal mammalian livers, SAM can be decarboxylated to be used as a precursor for the synthesis of polyamines, but most of it (≥99%) is used in transmethylation reactions, a process by which methyl groups are added to compounds and SAM is converted to SAH (30). In regenerating and preneoplastic livers, much more SAM is channeled into polyamine synthesis (29, 31). SAH is a potent competitive inhibitor of transmethylation reactions, and its level is kept low normally by conversion to homocysteine and adenosine, both of which are rapidly removed in vivo (25). Transmethylation reactions are modulated by SAH levels and SAM:SAH ratios. A fall in the ratio also leads to decreased activity of various methyltransferases (14, 25).

Consistent with this, in HuH-7 cells expressing different MAT isoforms, DNA methylation changed in parallel with changes in SAM and SAM:SAH ratios and appeared to be causally related, inasmuch as treatments of untransfected HuH-7 cells with exogenous SAM, which increased the intracellular SAM level and SAM:SAH ratio, also increased DNA methylation.

Can changes in DNA methylation explain changes in cell growth? Many studies have found DNA hypomethylation of certain oncogenes to correlate with development of carcinogenesis (23, 32–35). Activation of some genes has been ascribed to the demethylation of critical mCpG loci, and silencing of some genes may be related to the methylation of specific CpG loci (30). In this regard, several studies reported a fall in liver SAM level, SAM:SAH ratio (SAH levels remained unchanged), and overall DNA methylation and increased expression of proto-oncogenes such as c-myc, c-Ha-ras, and c-Ki-ras during the early stages of rat liver carcinogenesis (32, 35). All of these changes were prevented with exogenous SAM treatment (35). The protective effect of SAM appears to be related to DNA methylation, given that the protection was reversed by an inhibitor of DNA methylation, S-azacytidine (34). Thus, during hepatocarcinogenesis, a fall in the SAM level and the SAM:SAH ratio can lead to overall DNA hypomethylation. This change in DNA methylation can then affect the expression of genes critical for cell growth. The identity of these target genes is the subject of future studies. We cannot currently exclude mechanisms other than a change in DNA methylation to explain the difference in cell growth. Because a change in SAM:SAH ratio would affect all methylation reactions, protein, phospholipid, or RNA methylation may be affected as well. However, the reversal by 5-azacytidine on the protective effect of SAM against hepatocarcinogenesis would suggest that the predominant mechanism is DNA methylation (34).

In summary, we have created a convenient cell line model that expresses different MAT. We have shown that cell growth and DNA synthesis are strongly influenced by the type of MAT expressed, and the mechanism may be related to changes in the SAM:SAH ratio affecting DNA methylation. This cell line model will be useful in allowing additional studies to examine the expression of growth-related genes, the expression of which is controlled by methylation. Finally, our studies show that the switch in gene expression from MATIA to MAT2A in human liver cancer is pathogenetically important, as the cancer cell is expressing the MAT gene that facilitates cell growth through DNA hypomethylation.

ACKNOWLEDGMENTS

HuH-7 and HepG2 cells were provided by the Cell Culture Core, and oligonucleotide primers were generated by the Molecular Biology Core of the USC Center for Liver Disease Research. We thank Drs. Luis Alvarez and José Mato for providing the human MATIA expression vector pAM-MSSRI and Rosemarie Nino for technical assistance with transfection.

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