Methionine Adenosyltransferase 1: 간경화 환자에서 단백체학 분석을 통한 조기 간세포암 진단표지자

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Methionine Adenosyltransferase 1: A Proteomic Surrogate Marker of Early Hepatocellular Carcinoma in Cirrhotic Patients

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Background/Aims: Because there is a lack of effective biomarkers, we aimed to discover proteomic candidate markers for hepatocellular carcinoma (HCC) in cirrhotic patients at the highest-risk of HCC, and to validate the markers.

Methods: We collected tumor tissue from 5 cirrhotics with HCC, and from 5 cirrhotics without HCC, who underwent liver resection or transplantation. These tissue samples were analyzed by 2-dimensional difference gel electrophoresis coupled with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), and potential markers were validated at the transcriptional and translational levels. We also performed western blot assays using other blood samples from 10 cirrhotics with HCC and 10 without HCC.

Results: Among the 66 distinguishable spots on 2-D gel images, we identified 15 proteins over-expressed more than 1.5 fold in terms of volume ratio in the tumors. Ten of the over-expressed proteins were identified by MALDI-TOF MS; of those, only methionine adenosyltransferase 1 (MAT1), a protein specific for liver, and acyl-CoA dehydrogenase were significantly up-regulated in tumors in further immunoblotting analyses ($P < 0.05$). There was no between-pair difference in MAT1 mRNA measured by real-time polymerase chain reaction ($P = 0.96$). However, in western blots of serum samples, distinct MAT1 bands were observed in all 10 HCC patients, but in only 2 of the non-HCC patients.

Conclusions: MAT1 is a potential marker for surveillance in cirrhotic patients with and without prior HCC. (J Liver Cancer 2018;18:33-43)

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INTRODUCTION

Chronic liver disease caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, and the related liver cirrhosis are major risk factors for hepatocellular carcinoma (HCC) and thus established targets of periodic surveillance programs.^{1,2} Given the dismal prognosis of non-curable HCC, the detection of nodules at an early and thus treatable stage is a major aim of surveillance regimens.¹⁻³ However, ultrasonography, the only screening modality currently endorsed, faces challenges for detecting HCC at an early stage or on a coarse cirrhotic background, and also in terms of operator- and equipment-dependence.⁴ Serum alpha-fetoprotein (AFP) has been tested in many laboratory and clinical studies as an alternative to imaging approaches or even as an earlier surrogate. It is the most widely used marker in clinical practice and has no strong competition.^{4,5} However, due to its unacceptable sensitivity and specificity, especially for small tumors, the most recent global guidelines no longer recommend it as a complementary tool for monitoring high-risk populations.^{1,2} Recent advances in oncologic proteomic platforms have allowed researchers to identify and characterize molecular signatures specific for HCC, as has been done for other types of cancer.^{3,6,7} However, there is as yet no universal proteomic biomarker that has a clinical function and significance that justifies its use in actual patient care.

In this study, we identified candidate biomarkers of HCC in cirrhotic patients at highest risk, based on protein profiling using two-dimensional differential in-gel electrophoresis (2D-DIGE) followed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).⁸ We then examined the differential expression of the potential hepatocarcinogenic targets at the protein and RNA levels by immunoblotting and real-time polymerase chain reaction (PCR) analyses, respectively. To validate its potential clinical usefulness, one selected protein was further used in immunoblotting of the sera of an additional 20 cirrhotic patients with and without HCC.

METHODS

1. Origins of study samples

First, we prospectively collected fresh tumor tissues from 5 patients with cirrhosis and HCC who underwent liver resection, and fresh liver tissues from 5 cirrhotic patients without any neoplasms who underwent liver transplantation at Asan Medical Center, Korea, for proteomic analysis, and subsequent immunoblotting and real-time PCR assays. All the tissue samples were frozen at -80°C shortly after collection in the operating theatre. Second, we obtained serum samples from a separate prospective cohort of 10 cirrhotic patients with HCC and 10 without HCC just before liver resection or transplantation at the same hospital, and used them in immunoblotting assays to confirm the potential usefulness in clinical settings of a particular protein (i.e., methionine adenosyltransferase 1 [MAT1]) identified by the high-throughput proteomic assay. The centrifuged blood samples were stored at -80°C for further experiments. The study protocol and use of tissue samples were approved by the institutional review boards of Asan Medical Center (IRB No. 2012-0882).

2. Protein extraction and labeling with Cy-Dye

Each tissue sample was chopped and homogenized in 2-D lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate [CHAPS], pH 8.5). Extracted proteins were precipitated using ice-cold 50% trichloroacetic acid (TCA) solution and the pellets were dissolved in 2-D lysis buffer. The protein concentration was determined using a 2D Quant kit (GE Healthcare, Uppsala, Sweden). The procedure of two-dimensional difference gel electrophoresis (2D-DIGE) was performed in the dark condition. 50 µg of each sample and a pooled internal standard were cross-labeled with 400 pmol of Cy3-, Cy5- and Cy2-dyes (GE Healthcare, Uppsala, Sweden). Detailed methods are presented in a Supplementary Appendix.

3. 2D-DIGE and MALDI-TOF MS analysis

The Cy Dye-labeled gels were scanned on a Typhoon 9400 scanner (GE Healthcare, Chicago, IL, USA) at the manufacturer's recommended excitation and emission wavelengths. The 2D-DIGE spots of interest were excised manually from Coomassie Brilliant Blue (CBB)-stained preparative gels and processed for mass spectrometry (MS) analysis. The prepared samples were analyzed with a MALDI-TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster city, CA, USA). Data were acquired in positive MS reflector mode at a scan range of 800–4,000 *m/z* with 1000 shots per spectrum. A maximum of 15 precursors with minimum signal-to-noise ratio (S/N) of 50 were selected for MS/MS analysis. The MS/MS spectra were searched against the NCBI human database (NCBI 20110704) using the MASCOT algorithm (Matrix Science, Boston, MA, USA) for peptide and protein identification. Peptide mass fingerprinting (PMF) was carried out using the Mascot search engine included in the GPS Explorer software, and mass spectra used for manual *de novo* sequencing were annotated with the Data Explorer software (Applied Biosystems, Foster city). Detailed methods are presented in a Supplementary Appendix.

4. Western blot analysis

Western blotting was performed on the 5 tumors and 5 liver tissues used in the proteomic analysis using the following primary antibodies: rabbit anti- β -actin (1:1,000, Abcam, Cambridge, MA, USA), anti-carbamoylphosphate-synthetase 1 (1:1,000, Abcam, Cambridge), anti-MAT1 (1:1,000, Abcam, Cambridge), anti-galactokinase 1 (1:1,000, Abcam, Cambridge), anti-moesin (1:10,000, Abcam, Cambridge), anti-acetyl-CoA-dehydrogenase-short-branched chain (1:500, Abcam, Cambridge), anti-cytokeratin 9 (1:1,000, Abcam, Cambridge), anti-mitochondrial adenosine triphosphate (ATP) synthase (1:500, Abcam, Cambridge), and anti-glutamate dehydrogenase 1 (1:1,000, Abcam, Cambridge). In addition, western blots of the sera of 10 patients with HCC and 10 without HCC were examined using primary rabbit anti-MAT1 (1:2,000, Santa cruz biotechnology, Minneapolis, MN, USA) and anti-human

immunoglobulin G (IgG) (1:3,000, Santa cruz biotechnology, Minneapolis). Detailed methods are presented in the Supplementary Appendix.

5. Quantitative real-time PCR

To evaluate the expression of the *MAT1A* gene encoding MAT1 in the 5 tumors and 5 cirrhotic liver tissues used in the proteomic analysis, real-time quantitative PCR was performed using a Step-One-Plus RT PCR System (Applied Biosystems, Foster city, CA, USA). Relative mRNA expression levels were measured by TaqMan Assays (Applied Biosystems) for *MAT1A* (Hs01547962_mL) and GAPDH (Hs99999905_mL). The mRNA levels of *MAT1A* were normalized to those of GAPDH. Quantification was performed by the comparative CT method. Detailed methods are presented in the Supplementary Appendix.

6. Serological test for AFP

Serum AFP was determined with an immunoradiometric assay (IRMA: RIA-gnost® AFP, Cis-Bio International, Schering, Switzerland) based on the principle of the sandwich assay, using ¹²⁵I-labelled anti-AFP monoclonal antibody. Values of AFP <8 ng/mL were regarded as normal.

7. Statistical analysis

Student's *t*-test and the chi-square test were used to compare groups. For time-dependent recurrence analysis, we used the Kaplan-Meier method with log-rank tests and Cox proportional hazards models. A two-sided *P*-value <0.05 was considered as statistically significant.

RESULTS

1. Clinical characteristics of the subjects

The demographic and tumor characteristics of the first set of 10 patients who provided cancer or liver tissue for protein expression assays are presented in Table 1. One of the five cir-

Table 1. Clinical characteristics of the 5 HCC and 5 cirrhosis patients included in the proteomic analysis

Case No.	Age (years)	Gender	Etiology of liver disease	Serum AFP (ng/mL)	Child-Pugh class	Largest tumor size (cm)	Tumor number	Modified UICC stage	Edmonson grade (worst)
HCC_1	42	Male	HBV	2.3	A	3.0	1	II	III
HCC_2	54	Male	HCV	28.2	A	0.4	2	II	III
HCC_3	50	Male	HBV	12	A	3.5	2	III	III
HCC_4	42	Male	HBV	2,394.7	A	2.2	3	III	II
HCC_5	48	Male	HBV	8,735	A	3.2	1	II	IV
LC_1	53	Female	HBV	2.7	B	-	-	-	-
LC_2	51	Male	HBV	4.6	A	-	-	-	-
LC_3	56	Male	HBV	361	C	-	-	-	-
LC_4	47	Female	HBV	638.9	C	-	-	-	-
LC_5	44	Male	HBV	17.4	C	-	-	-	-

HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; Modified UICC, modified Union Internationale Contrele Cancer; HBV, hepatitis B virus; HCV, hepatitis C virus; LC, liver cirrhosis.

rhctic patients without HCC was infected with HCV, and the remainder with HBV. While all the HCC patients had Child-Pugh class A liver function, only one of the cirrhotic patients without HCC had class A liver function. With regard to the tumor-related parameters of the 5 HCC patients, mean tumor size and serum alpha-fetoprotein levels were 2.46 ± 1.25 cm, and 2,234.4 ng/mL, respectively, and 3 of the patients had multiple tumors. Gross vascular invasion was not observed and microscopic vascular invasion was observed in one patient. Four patients had Edmonson grade III or IV tumors.

2. Identification of proteomic differences between tumor and cirrhosis tissues

We have detected a total of 66 spots clearly differentially expressed in the two sets of tissues using 2D-DIGE technology; 26 spots were up-regulated in the tumors and 40 were down-regulated (Fig. 1A). A quantified volume ratio of 1.5 and a significant Student's *t*-test ($P < 0.05$) were used as cut-offs for the difference in average level. Fifteen protein spots, with more than 1.5 fold volume increases in the HCC tumor group compared to non-tumor cirrhotic group, were picked and subjected to in-gel MALDI-TOF MS analysis (Fig. 1B). Using MS/MS analysis and MASCOT software (www.matrixscience.com), the following 10 proteins were unambiguously identified as highly expressed in the tumors: carbamoylphosphate synthe-

tase I, cytokeratin 9, mitochondrial ATP synthase, human liver carboxylesterase 1, moesin, glutamate dehydrogenase 1, S-adenosylmethionine synthetase isoform type-1 (*i.e.*, MAT1), acyl-CoA dehydrogenase, galactokinase, and acetoacetyl-coenzyme A thiolase. Details are given in Table 2.

3. Validation of the selected proteomic candidates at the transcriptional and translational levels

To confirm the 2D-DIGE results, we performed western blot assays using the same samples as used for the proteomic analysis, and used Student's *t*-test to compare the ratios of the optical density (OD) measurements of the individual bands normalized to β -actin in the two sets. The ratios were clearly higher for the tumor set than the cirrhosis set for 5 out of the 10 proteins identified as up-regulated in the tumors in the main proteomic study, namely carbamoylphosphate synthetase I, moesin, MAT1, acyl-CoA dehydrogenase, and galactokinase, but the difference reached statistical significance only for MAT1 ($P = 0.046$) and acyl-CoA dehydrogenase ($P = 0.026$) (Table 3, Fig. 2). There were faint or no bands on western blot images for cytokeratin 9, mitochondrial ATP synthase, and glutamate dehydrogenase 1 (Table 3). We further examined, by real-time PCR analysis, the expression of *MAT1A*, which has received little attention in previous proteomic studies of HCC tissue and appeared potentially relevant to the tumors.⁹⁻¹³ The real-time PCR results failed

Figure 1. Representative 2D gel images. (A) Sixty-six spots were significantly differentially expressed in the hepatocellular carcinoma (HCC) and cirrhosis tissues; 26 spots were up-regulated in the tumors and 40 were down-regulated in the preparative 2-D gel image. (B) Fifteen protein spots >1.5 fold volume increases were picked and subjected to in-gel MALDI-TOF MS analysis using a significant Student's *t*-test ($P<0.05$) as a cut-off for the difference in average level. MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry.

to detect any significant difference in the respective amounts of *MAT1A* gene transcripts in the tumor samples compared with the non-tumor cirrhotic tissues; the relative expression values averaged over the five samples and normalized to GAPDH expression were 1.93 ± 0.82 for tumor tissue and 1.97 ± 0.45 for non-tumor cirrhotic tissue ($P=0.96$) (Fig. 3).

4. Validation of MAT1 in serum samples based on Western blot analysis

We used the sera of an independent cohort of 10 cirrhotic patients with HCC and 10 without HCC to validate the pro-

teomic behavior of MAT1 based on blood testing, which could be used more easily in practice. The parameters of these patients and tumors are summarized in Table 4. All the HCC and non-HCC patients were infected with HBV. In the HCC group, the median size of the tumors was 2.1 cm (range, 1.1–3.9 cm) and their median number was 2 (range, 1–4). The mean serum AFP levels by immunoradiometric assay were 53.8 ng/mL (standard deviation [SD], 56.4 ng/mL) and 24.9 ng/mL (SD, 47.2 ng/mL) in the HCC and non-HCC groups, respectively ($P=0.12$) (Table 5, Fig. 4), with one-half of HCC patients having less than 20 ng/mL of serum AFP, which was shown to be the optimal cut-off value as a diagnostic test for HCC.¹⁴ Moreover, serum AFP values were above 20 ng/mL in 2 of the 10 patients without HCC. In terms of the western blot results for the sera, the mean band intensity ratios of MAT1 normalized with control IgG were significantly higher in the HCC group than in the non-HCC group (267.1 ± 108.0 vs. 26.1 ± 12.9 ; $P<0.001$) (Table 5). Positive MAT1 signals were distinctly given by the sera of all 10 HCC subjects, as expected, but by only 2 of those from the 10 cirrhotics (serum No. C8 and C10; Fig. 4).

DISCUSSION

Recent advances in cancer proteomics make it possible to monitor many biomarkers simultaneously and generate proteomic-based signatures to construct meaningful protein networks and discover functional pathways. These approaches have been applied to HCC, and many protein indicators for early detection of HCC, which may subsequently lead to better prognoses, have been introduced.^{3,6,7} However, there is no invariant ready-to-use proteomic signature that can be directly applied in the clinical situation.^{3,15}

In the present tissue-based proteomic investigation, 15 protein spots that were more abundant in HCC tumors than in the cirrhotic tissue of patients with HBV or HCV infections were identified using the more sensitive 2D-DIGE approach, which has some advantages over traditional 2D-polyacrylamide gel electrophoresis (PAGE), including multiple pre-labeling co-detection and a wider dynamic range, as well as proven effectiveness for cancer biomarker discovery.^{7,16–18} Ten of

Table 2. Representative differentially expressed proteins in the HCC tumor tissues identified by 2D-DIGE and MALDI-TOF MS analysis

Spot No.	Accession No.	Protein name	Score [†]	P-value*	Average ratio*	Main action
265	gi 21361331	Carbamoylphosphate synthetase 1	139	0.002	3.21	Amino acid metabolism
275	gi 435476	Cytokeratin 9	39	0.0015	3.54	Cytoskeleton and extracellular matrix
276	gi 4757810	Mitochondrial ATP synthase	96	0.0035	3.39	Energy metabolism
368	gi 30749518	Human liver carboxylesterase 1	75	0.036	2.41	Detoxification, oxidoreduction
722	gi 4505257	Moesin	76	<0.001	2.48	Connecting cytoskeletal structures
1134	gi 4885281	Glutamate dehydrogenase 1	204	0.0094	2.09	Amino acid metabolism
1175	gi 4557737	Methionine adenosyltransferase isoform 1	138	0.046	1.53	Nucleic acid and methionine metabolism
1413	gi 4501859	Acyl-CoA dehydrogenase	64	0.019	1.62	Fatty acid metabolism
1441	gi 4503895	Galactokinase 1	131	0.0072	2.91	Carbohydrate metabolism
1461	gi 546901	Acetoacetyl-coenzyme A thiolase	74	0.011	1.57	Fatty acid metabolism

HCC, hepatocellular carcinoma; 2D-DIGE, two-dimensional difference gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; ATP, adenosine triphosphate; CoA, coenzyme A.

*P-values and average ratios were estimated using the biological variance analysis mode of the 2-dimensional DIGE analyzer; [†]MASCOT ion score using MASCOT algorithm (Matrix Science, Boston, MA, USA) for peptide sequences and protein identification (IPI_Human database). For protein identification, MASCOT ion score >30, was used as the criterion.

Table 3. Intensity ratios of the protein bands from western blots that were identified as highly expressed in HCC tumors using MS/MS analysis and MASCOT software

Spot No.	Protein name	Mean intensity ratio normalized with β -actin ($\times 100$)		P-value
		Non-tumor (cirrhosis)	Tumor (HCC)	
265	Carbamoylphosphate synthetase I	137.1	218.1	0.420
275	Cytokeratin 9	Weak signals	Weak signals	-
276	Mitochondrial ATP synthase	No bands	No bands	-
368	Human liver carboxylesterase 1	293.1	300.9	-
722	Moesin	57.9	77.8	0.281
1134	Glutamate dehydrogenase 1	No bands	No bands	-
1175	MAT1	161.8	351.9	0.046
1413	Acyl-CoA dehydrogenase	196.2	366.9	0.026
1441	Galactokinase 1	47.1	90.8	0.102
1461	Acetoacetyl-coenzyme A thiolase	120.6	85.2	-

HCC, hepatocellular carcinoma; MS, mass spectrometry; ATP, adenosine triphosphate; MAT1, methionine adenosyltransferase isoform 1; CoA, coenzyme A.

these identified spots were analyzed by MALDI-TOF MS followed by PMF or MS/MS analyses; this procedure has intrinsic advantages in terms of speed of analysis, low sample volume needed, high sensitivity, ease and cost of use, and wide range of mass coverage.^{3,6,17,19} These candidate proteins were found to have functions associated with cytoskeletal proteins (moesin and cytokeratin 9), amino acid metabolism (car-

bamoylphosphatesynthetase 1 and glutamate dehydrogenase 1), metabolic enzymes (mitochondrial ATP synthase), carbohydrate metabolism (galactokinase 1), fatty acid metabolism (acyl-CoA dehydrogenase and acetoacetyl-coenzyme A thiolase), nucleic acid and methionine metabolism (MAT1), and detoxication and oxidoreduction function (human liver carboxylesterase 1), as identified using a bioinformatics database

Figure 2. Western blotting of tissue specimens. (A) Western blotting was performed with the 5 tumor and 5 cirrhosis samples used for the high-throughput proteomic analysis. The data were generated from OD measurements of individual bands from Western blots and normalized to β -actin. The normalized ratios were higher for the tumor set than the cirrhosis set for only 5 (i.e., carbamoylphosphate synthetase I, moesin, methionine adenosyltransferase 1 [MAT1], acyl-CoA dehydrogenase, and galactokinase) of the 10 proteins identified as highly expressed in the tumors in the proteomic analyses. Three of the 5 tumor-enriched proteins, carbamoylphosphate synthetase I (B), moesin (C), and galactokinase (F), were not significantly different in terms of mean ratios in the two sets ($P=0.42$, 0.281 , and 0.102 , respectively by Student's *t*-test), but, another two tumor-enriched proteins, MAT1 (D) and acyl-CoA dehydrogenase (E), were significantly different in the two sets ($P_s=0.046$ and 0.026 , respectively by Student's *t*-test). M.W, molecular weight; HCC, hepatocellular carcinoma; CoA, coenzyme A; OD, optical density.

Figure 3. Results of real-time PCR of *methionine adenosyltransferase 1A* (*MAT1A*) in tissue specimens. The real-time PCR results did not show any significant difference in terms of the levels of *MAT1A* transcripts in the tumors compared with the cirrhotic tissues. The relative expression values averaged over the five samples and normalized to GAPDH expression were 1.93 ± 0.82 for tumors and 1.97 ± 0.45 for non-tumors ($P=0.96$). PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(<http://david.abcc.ncifcrf.gov/>; Table 2). In the next step of our experimental series, we performed immunoblot analyses to verify the differential expression of the characterized 10 proteins because of the moderate reproducibility of MALDI-TOF MS technology,^{7,19} and then only two of the proteins were confirmed by western blot.

Although the level of acyl-CoA dehydrogenase by the western blot assays was significantly higher in HCC patients than cirrhotic patients, little has been known about the tumorigenic role of the acyl-CoA dehydrogenase so far. MAT1, which is encoded by the *MAT1A* gene, has been found to be involved in the synthesis of S-adenosylmethionine (SAM) specifically in liver.^{9-13,20} In this context, we did focus on the MAT1 as a new biomarker for early detection of HCC. Reduced expression of *MAT1A* resulting in SAM deficiency was reported to predispose to HCC development, a result at least superficially not in line with our present findings.^{11-13,20} However, such changes in expression of proteins are substantially attenuated in slow-

Table 4. Clinical characteristics of the 10 HCC and 10 cirrhosis patients included in the validation set based on western blot analysis

Case No.	Age (years)	Gender	Etiology of liver disease	Child-Pugh class	Largest tumor size (cm)	Tumor number	Modified UICC stage	Edmonson grade (worst)
HCC_1	45	Female	HBV	A	2.7	1	II	III
HCC_2	56	Male	HBV	A	1.8	4	II	III
HCC_3	41	Male	HBV	B	2.7	2	III	IV
HCC_4	50	Male	HBV	C	3.4	1	II	III
HCC_5	53	Female	HBV	B	2.0	1	II	II
HCC_6	51	Male	HBV	A	3.9	2	III	III
HCC_7	26	Female	HBV	B	2.1	1	II	II
HCC_8	53	Female	HBV	A	2.6	2	III	IV
HCC_9	53	Male	HBV	B	1.1	2	II	III
HCC_10	48	Male	HBV	C	1.1	2	II	II
LC_1	44	Male	HBV	C	-	-	-	-
LC_2	55	Male	HBV	B	-	-	-	-
LC_3	48	Male	HBV	B	-	-	-	-
LC_4	68	Male	HBV	B	-	-	-	-
LC_5	45	Male	HBV	B	-	-	-	-
LC_6	51	Male	HBV	A	-	-	-	-
LC_7	49	Male	HBV	B	-	-	-	-
LC_8	54	Male	HBV	B	-	-	-	-
LC_9	59	Male	HBV	C	-	-	-	-
LC_10	46	Male	HBV	C	-	-	-	-

HCC, hepatocellular carcinoma; Modified UICC, modified Union Internationale Contrele Cancer; HBV, hepatitis B virus; LC, liver cirrhosis.

growing HCC models, which probably resemble the relatively early-stage tumors we used in our proteomic assay.²¹ On the other hand, a previous tissue-based proteomic study by Blanc et al. reported that MAT1 protein species were down-regulated in tumor tissue, compared to surrounding non-tumor tissue.²² However, it is important to note that the paired analyses comparing diseased and non-diseased tissues from the same patient that have been used in most proteomic protocols might mask alterations of protein expression patterns in malignant conditions, unlike blood serum-based models, because of con-

current changes in the underlying carcinogenic hepatic tissues.^{3,23} In this respect, our inter-individual approach comparing independent cirrhotic samples with and without HCC may provide more reliable markers specific for HCC arising from cirrhosis.

It is interesting that our quantitative real-time PCR data indicate that the overexpression of MAT1 protein in HCC tissue may not result from increased transcription but rather from post-translational mechanisms. In general, protein levels and their half-lives are not proportional to those of the corresponding mRNAs, and a multitude of post-translational modifications for controlling protein turnover and abundance have been described.^{24,25} Importantly, it is changes in protein concentrations that would be most useful for elucidating human disease processes.²⁶

In our further western blot analyses for evaluating blood-based levels of MAT1 protein, we found positive bands of MAT1 in all ten of the patients with even early HCC, compared to only 2 of the 10 patients without HCC. On the other hand, using the cut-off AFP level for HCC of 20 ng/mL that is currently recommended,^{1,14} we would have obtained false-negatives in 50% of the HCC group and false-positives in 20% of the non-HCC group. These observations indicate that MAT1 is a better proteomic surrogate than AFP if levels are measured in sera. Indeed, serum tumor markers may be especially useful in screening and monitoring programs because sera can be easily obtained from clinical subjects.

One limitation of this study is that our results were predominantly obtained from HBV-infected subjects, and thus might not be generalizable. Further studies using a diverse and large set of patients are necessary before clinically apply-

Table 5. Intensity ratios of the protein bands from western blots for serum MAT1 and results of immunoradiometric assays for serum AFP

Case No.	HCC patients		LC patients	
	MAT1	Serum AFP (ng/mL)	MAT1	Serum AFP (ng/mL)
1	203.7	3.7	46.4	45.6
2	386.2	7.0	24.7	15.3
3	220.7	8.5	39.9	6.8
4	198.9	22.6	25.5	1.2
5	516.7	137.7	11.6	3.6
6	234.3	79.8	36.7	4.1
7	298.6	11.0	25.8	3.1
8	155.2	110.1	31.4	3.7
9	237.3	19.8	7.9	154.0
10	219.3	137.8	11.3	11.3
Mean $\pm$ SD	267.1 $\pm$ 108.0*	53.8 $\pm$ 56.4 [†]	26.1 $\pm$ 12.9*	24.9 $\pm$ 47.2 [†]

Values are presented as mean $\pm$ SD unless otherwise indicated. MAT1, methionine adenosyltransferase isoform 1; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SD, standard deviation. *Mean band intensity ratios for MAT1 were significantly higher in the HCC group than in the LC group ($P < 0.001$ by Student's *t*-test); [†]Mean levels of serum AFP did not differ between the HCC and LC groups ($P = 0.12$ by Student's *t*-test).

Figure 4. Results of western blot analysis of serum specimens. Western blotting was performed on sera collected separately from 10 HCC patients (H1–H10) and 10 cirrhotic patients (C1–C10). There were MAT1 bands visible for all 10 HCC patients, but for only 2 non-HCC patients. Serum AFP levels were greater than 20 ng/mL in only 50% of the patients with HCC, and unexpectedly, in 20% of the patients without HCC. HCC, hepatocellular carcinoma; MAT1, methionine adenosyltransferase isoform 1; AFP, alpha-fetoprotein; IgG, immunoglobulin G.

ing MAT1 as a prognostic biomarker. In addition, future work should include serum-based clinical validation of MAT1 with a large cohort consisting of patients with and without HCC in order to test MAT1 as a real practical tool.

In conclusion, MAT1 protein is a promising histological or serological biomarker for HCC on a cirrhotic background. It may be useful for early detection of new or recurrent HCC in high-risk patients with cirrhosis.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this paper.

SUPPLEMENTARY MATERIAL

Supplementary data can be found with this article online <http://www.e-jlc.org/html/>.

Conflicts of Interest

The authors have no conflicts to disclose.

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