

Expression profiling in hepatocellular carcinoma with intrahepatic metastasis: identification of high-mobility group I(Y) protein as a molecular marker of hepatocellular carcinoma metastasis

Makoto Chuma,^{1,3} Norihisa Saeki,^{2,5} Yoshiya Yamamoto,^{1,3} Tsutomu Ohta,² Masahiro Asaka,³ Setsuo Hirohashi¹ and Michiie Sakamoto^{1,4}

¹Pathology Division and ²Genomics Division, National Cancer Center Research Institute, Tokyo
³Department of Gastroenterology and Hematology, Hokkaido University, Sapporo, ⁴Department of Pathology School of Medicine, Keio University, Tokyo, Japan

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common and aggressive human malignancies. Its high mortality rate is mainly a result of intra-hepatic metastases. To investigate the detailed genetic mechanisms in cancer metastasis, we compared the expression profiles of 20 HCCs with intrahepatic metastasis and 10 HCCs without intrahepatic metastasis using an oligonucleotide array. Of the approximately 12600 genes that were analyzed, we identified 34 genes whose expression levels were significantly correlated with intrahepatic metastasis ($P < 0.05$). Of these genes, we further investigated the expression of high-mobility group I(Y) [HMG-I(Y)] protein. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed that HMG-I(Y) was up-regulated in HCC with intrahepatic metastasis, compared to its level in HCC without intrahepatic metastasis. Further immunohistochemical examination of HMG-I(Y) revealed a significant over-expression in HCC with intrahepatic metastasis, compared with that in HCC without intrahepatic metastasis ($P < 0.05$). These results indicate that the molecular signatures of HCC with intrahepatic metastasis and of HCC without intrahepatic metastasis are clearly different. HMG-I(Y) expression was associated with intrahepatic metastasis and may be a predictive marker of HCC intrahepatic metastasis. (Keio J Med 53 (2): 90–97, June 2004)

Key words: HCC, intrahepatic metastasis, HMG-I(Y), oligonucleotide array

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and one of the leading causes of death from cancer in Japan.¹ Recent advances in diagnostic modalities, such as imaging techniques and the measurement of serum tumor markers, have improved the rate of early detection of HCC, and therapeutic approaches to HCC have also progressed. However, the long-term survival of HCC patients continues to be poor because of the high incidence of recurrence within the liver after initial treat-

ment.² Pathological and genetic studies have identified two types of HCC recurrence: the multicentric development of new tumors and intrahepatic metastasis of the original HCC.^{3–5} Hematogenous intrahepatic metastasis of HCC is frequently observed in advanced cases and is thought to arise through tumor cell dispersal via the portal vein.⁶ Current studies have largely focused on individual candidate genes, an approach that may be insufficient to precisely define the genetic basis of metastatic HCC.^{7–9} A molecular understanding of intrahepatic metastasis is an important step toward the identification of predictive markers and more spe-

Present affiliation:⁵ *The Organization for Pharmaceutical Safety and Research, Tokyo*

Reprint requests to: Dr. Michiie Sakamoto, Department of Pathology, School of Medicine, Keio University, 35 Shinanomachi, Shinjyuku-ku, Tokyo, 160-8582, Japan, e-mail: msakamot@sc.itc.keio.ac.jp

cific targets for HCC recurrence. The establishment of microarray methods enabling the large-scale analysis of gene expression has made it possible to seek molecular markers for cancer classification and outcome prediction in addition to identifying molecules involved in carcinogenesis in a variety of tumor types.¹⁰⁻¹² Several studies have compared gene expression between non-tumorous liver tissue and HCC and shown that the gene expression patterns present in HCC are specific to that cell type.¹³⁻¹⁶ Here, we compared the expression profiles of 20 HCCs with intrahepatic metastasis and 10 HCCs without intrahepatic metastasis using an oligonucleotide array to identify genes generally involved in intrahepatic metastasis. Among the genes identified, we further investigated high-mobility group I(Y) (HMG-I(Y)). HMG-I(Y) is a family of low molecular mass, nonhistone nuclear DNA binding proteins. Over-expression of HMG-I(Y) has been described in several cancers, and a significant correlation between elevated HMG-I(Y) expression and a poor prognosis has been reported in patients with prostate and breast cancers. However, HMG-I(Y) expression has rarely been mentioned and its role is not clearly understood in HCC. Therefore we further investigated whether HMG-I(Y) expression is associated with intrahepatic metastasis using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry.

Materials and Methods

Patients and tissue samples

Thirty HCCs and corresponding noncancerous liver tissues were obtained from patients who had undergone surgical resections at the National Cancer Center Hospital, Japan. Specimens were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored until further use. The clinicopathological characteristics of the 20 HCCs with intrahepatic metastasis and the 10 HCCs without intrahepatic metastasis are shown in Table 1. There were no significant differences in factors between the two groups of HCCs. For the immunohistochemical analysis, 102 HCCs were analyzed. Sections were prepared from formalin-fixed, paraffin-embedded tissue samples that were resected surgically between 1990 and 2002. The main clinicopathological features of the specimens are presented in Table 2. The histopathological grade of tumor differentiation was assessed using a modification of the Edmondson Grading System.^{17,18} Macroscopically, HCC was subclassified into three types: single nodule (type 1), single nodule with extranodular growth (type 2), and confluent multinodule (type 3). Histological diagnosis of intrahepatic metastases was made according to the WHO criteria.¹⁹ Nodules apparently growing from portal vein tumor

Table 1 Patient Characteristics per Study Group

	vp(+)/im(+)	vp(-)/im(-)	<i>p</i> value
Number of cases	20	10	
Mean ages (years) ^a	59.5 ± 13.9	60.3 ± 7.5	0.87
Sex			0.77
Male	15	7	
Female	5	3	
Virus maker			0.69
HBs-Ag(+)	7	3	
HCV Ab(+)	9	6	
NBNC ^b	4	1	
Maximal size (mm)	63.9 ± 30.7	44.1 ± 20.4	0.08
Differentiation			0.1
Well	0	2	
Moderate	10	5	
Poor	10	3	
Macroscopic type			0.28
1	2	2	
2	8	6	
3	10	2	

^a Mean ± SE. ^b NBNC; patients without HBs-Ag(+) nor HCV-Ab(+).

Table 2 Immunohistochemical Examination of HMG-I(Y) in HCC

	HMGI expression		<i>p</i> value
	(-)	(+)	
Number of cases	60	42	
Mean ages (years) ^a	58.6 ± 9.54	60.3 ± 10.2	0.72
Sex			0.84
Male	38	25	
Female	22	17	
Virus maker			0.91
HBs-Ag(+)	16	12	
HCV Ab(+)	36	26	
NBNC ^b	8	4	
Maximal size (mm)	47.2 ± 22.4	52.5 ± 24.2	0.34
Differentiation			<i>p</i> < 0.05
Well	11	3	
Moderate	36	21	
Poor	13	18	
Macroscopic type			<i>p</i> = 0.182
1	13	5	
2	30	18	
3	17	19	
Intrahepatic metastasis ^c			<i>p</i> < 0.05
Present	24	26	
Absent	36	16	

^a Mean ± SE. ^b NBNC; patients without HBs-Ag(+) nor HCV-Ab(+)

^c included tumor thrombus in the portal vein.

thrombi or satellite nodules surrounding a large main tumor represent intrahepatic metastases, whereas other nodules can be considered multicentric HCCs if they satisfy any of the following three criteria (1) multiple, small early-stage HCCs or concurrent small early-stage

HCCs and classical HCCs; (2) presence of peripheral areas of well differentiated HCC in both lesions or in the smaller ones; and (3) multiple HCCs of obviously different histology. All the studies using these human materials had been approved by the Institutional Review Board.

RNA preparation and oligonucleotide array

Total RNA was extracted from bulk tissues using Trizol reagent (Invitrogen Corp., Carlsbad, CA). Biotin-labeled cRNA was synthesized from 10 µg of total RNA derived from each sample using a Super Script Choice System (Gibco-BRL, Rockville, MD) and a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), according to the manufacturers' instructions. The hybridization of each cRNA to the probe array, HG-U95Av2 (Affymetrix, Santa Clara, CA), and the detection of the signals were performed as instructed by the manufacturer. A previously described method was used for the data analysis,¹⁶ and the hybridization intensity data were normalized to the nearest 1000 of the total signal intensity in each array.

Real-time quantitative RT-PCR analysis

For the RT-PCR analysis, all RNA samples were treated with DNase I (Promega Corp., Madison, WI) to remove genomic DNA. The real-time quantitative RT-PCR analysis was then performed using a previously reported method.²⁰ The primer set 5'-ACTG-GAGTCTCCTGTGGTGTGT-3' (forward) and 5'-AGTGCTATTTCCCTCCCTTC-3' (reverse) was designed for the 3' untranslated region of HMGI. To standardize the amount of RNA, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was quantified using the primer set 5'-GAAGGTGAAGGTCGGAGTC-3' (forward) and 5'-CCCGAATCACATTCTCCAAGAA-3' (reverse). All PCR reactions were performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems, Foster City, CA) under the following conditions: 1 cycle at 50°C for 2 minutes; 1 cycle at 95°C for 10 minutes; and 40 cycles at 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Real-time detection of the emission intensity of SYBR Green was performed using an ABI prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems), using a previously reported method.²⁰ Quantitative RT-PCR was performed at least three times, including the use of a no-template run as a negative control. Statistical analyses were performed using unpaired *t*-tests.

Immunohistochemistry

Immunohistochemical staining was performed us-

ing formalin-fixed, paraffin-embedded tissue sections and previously described immunoperoxidase method.²¹ Briefly, each section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature and then washed in phosphate-buffered saline. Normal swine serum (DAKO, Glostrup, Denmark) was applied for 30 minutes and removed. The sections were then incubated with goat polyclonal antibodies for HMGI (SC-1564; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:250 overnight at 4°C, washed three times in phosphate-buffered saline, and incubated with secondary antibody for 30 minutes at room temperature. A Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used to detect the antibody signals.

Staining evaluation

Staining was evaluated by two independent observers. An equal or more intense nuclear staining, compared with that in lymphocytes, was considered a positive result. Correlations between the overexpression of HMGI protein and the clinicopathological features of the specimens were assessed, and the resulting data were analyzed using the χ^2 test and the Fisher's exact test. All statistical analyses were done using Stat View (Version 5.0) software (Abacus Concepts, Berkeley, CA). A value of $P < 0.05$ was considered significant.

Results

Identification of genes with different expression profiles in HCCs

First, we listed the upregulated and downregulated genes in each tumor and selected the genes with the following criteria: (1) presence (*i.e.*, expressed in the sample), (2) more than 1,000 times the average difference (mean of the difference in signal intensities between the match and mismatch probe set), and (3) a more than 2-fold increase or decrease in the average difference when compared to corresponding noncancerous liver tissues. Of the 142 genes that met the above criteria, 61 genes were up-regulated in 50% or more of the specimens and 81 genes were down-regulated in 50% or more of the specimens.

Two-way hierarchical clustering algorithm

To identify genes generally involved in intrahepatic metastasis, we compared the expression profiles of the 20 HCCs with intrahepatic metastasis and the 10 HCCs without intrahepatic metastasis using an oligonucleotide array and the 142 genes identified in the experiment described above. Next, we filtered the 142 genes

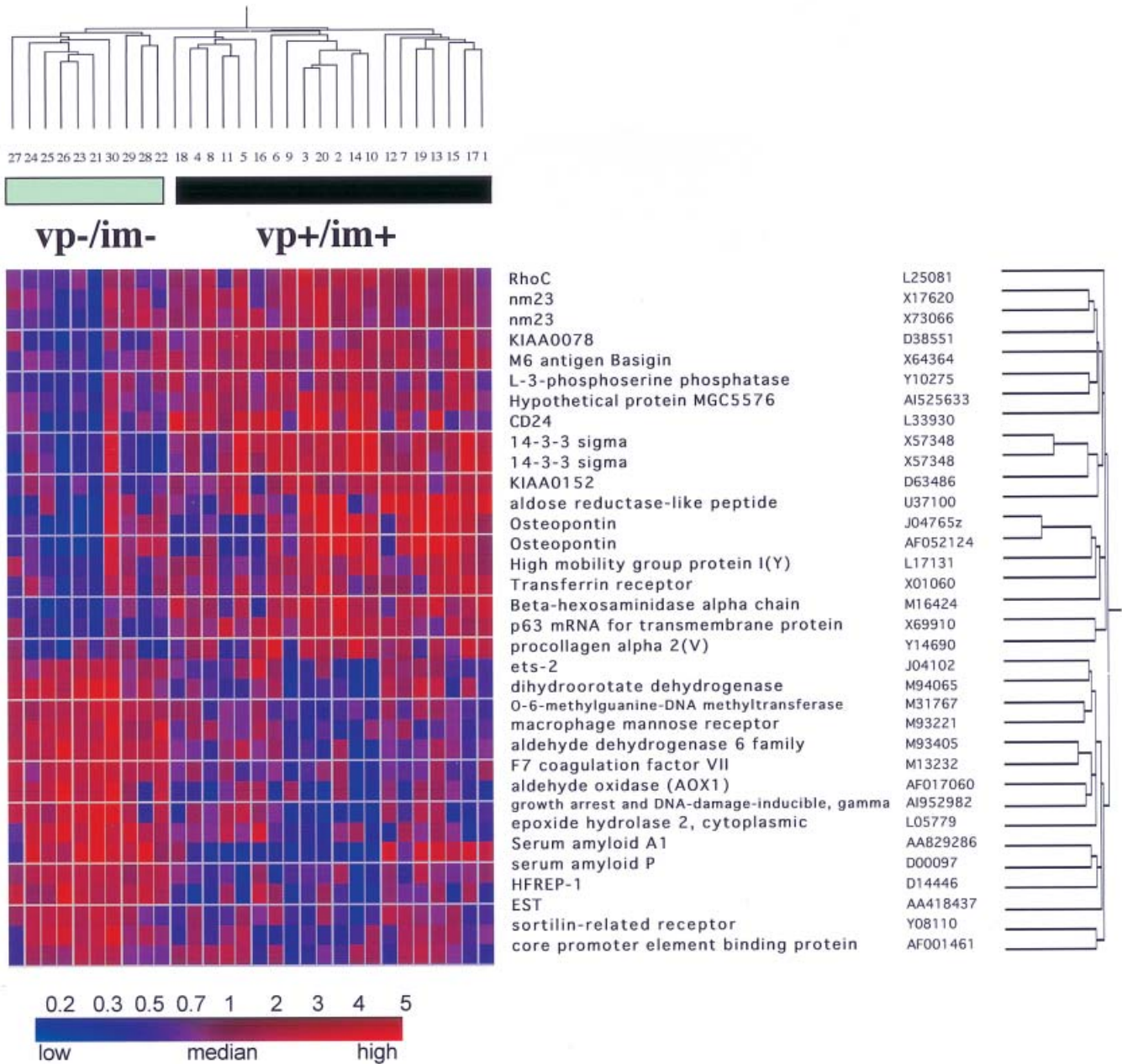


Fig. 1 A two-way hierarchical clustering algorithm. Cluster map and phylogenetic tree resulting from a two-way, pair-wise, average-linkage cluster analysis. Each color patch in the resulting visual map represents the expression level of the associated gene in that tissue sample, with a continuum of expression levels from blue (lowest) to bright red (highest). The two-way hierarchical clustering algorithm was able to successfully distinguish between HCC with intrahepatic metastasis and HCC without intrahepatic metastasis. The scale bar reflects the fold increase (red) or decrease (blue) for any given gene relative to the median level of expression across all samples.

using (4) a Mann-Whitney U-test with the significance set at $P < 0.05$ to identify genes expressed differently between the two groups. Finally, 34 genes were selected, and a two-way hierarchical clustering algorithm was used to successfully distinguished between HCC with intrahepatic metastasis and HCC without intrahepatic metastasis (Fig. 1). Nineteen of the 34 genes were up-regulated and 15 genes were down-

regulated in HCC with intrahepatic metastasis, compared to their levels in HCC without intrahepatic metastasis.

HMG-I(Y) mRNA expression in HCC

Of the listed 19 genes that are up-regulated in HCC with intrahepatic metastasis, we selected HMG-I(Y)

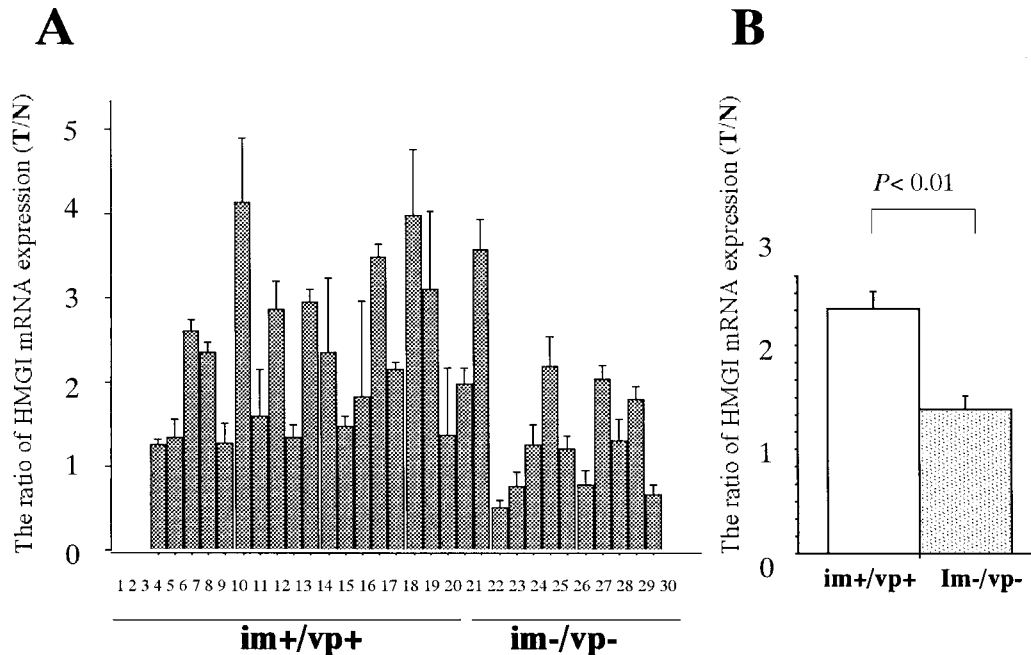


Fig. 2 Real-time quantitative RT-PCR analysis. (A) The ratio of relative HMG-I(Y) mRNA expression levels in 30 HCCs. The expression levels were normalized with GAPDH mRNA in each sample. The ratio of relative HMG-I(Y) mRNA expression (HMG-I/GAPDH) in HCCs (T) to that in corresponding noncancerous liver tissues (N) is indicated. (B) The average ratio in HCC with intrahepatic metastasis (□) was upregulated (2.37 ± 0.17) versus that in HCC without intrahepatic metastasis (▨) (1.40 ± 0.13), $P < 0.01$. Bars, SE.

and analyzed the relative expression level of HMG-I(Y) mRNA (HMG-I(Y)/GAPDH) using real-time quantitative RT-PCR. In 12 of the 20 cases of HCC with intrahepatic metastasis, the ratio of the relative HMG-I(Y) mRNA expression in the HCC specimens to the relative HMG-I(Y) mRNA expression in corresponding noncancerous liver tissues was more than two-fold; the same was true in 2 out of the 10 cases of HCCs without intrahepatic metastasis (Fig. 2A). The average ratio in HCCs with intrahepatic metastasis was upregulated (2.37 ± 0.17) versus that in HCCs without intrahepatic metastasis (1.40 ± 0.13), $P < 0.01$ (Fig. 2B).

Protein expression of HMG-I(Y)

To determine whether HMG-I(Y) was also overexpressed at the protein level and if an increase in expression was associated with intrahepatic metastasis, we performed an immunohistochemical study using an antibody against HMG-I(Y) (Fig. 3). Hepatocytes in noncancerous liver tissue with chronic hepatitis or cirrhosis showed no immunostaining or only focal or faint staining in the nucleus. However, the lymphocytes always stained strongly and thus served as an internal control for positive staining (Fig. 3B). As shown in Fig. 3, strong immunoreactivity was observed in the carcinoma cells, localized mainly in the nucleus. Some HCC cases without intrahepatic metastasis showed little HMG-I(Y) immunoreactivity, whereas HCC cases with intra-

hepatic metastasis showed strong HMG-I(Y) immunoreactivity (Fig. 3B). To evaluate the relation between HMG-I(Y) expression and prognostic factors, we divided these patients into two groups: an HMG-I(Y) positive expression (more than 20%) group and a low HMG-I(Y) expression (lower than 20%) group. The relations between HMG-I(Y) and seven prognostic factors were then examined. Table 1 shows that tumor histology grade and intrahepatic were significantly associated with HMG-I(Y) expression ($P < 0.05$).

Discussion

The recent development of cDNA microarray or oligonucleotide array technology, a high-throughput method of monitoring gene expression, has made it possible to analyze the expression of thousands of genes at once.^{22,23} Consequently, new classifications of cancers can now be proposed based on the altered expression profiles of multiple genes in tumor tissues.¹⁰⁻¹⁶ For example, molecularly distinct subtypes have been recognized on the basis of differences in gene expression patterns in multistage hepatocarcinogenesis.¹⁶ To investigate the detailed genetic mechanisms in cancer metastasis, we compared the expression profiles of 20 HCCs with intrahepatic metastasis and 10 HCCs without intrahepatic metastasis using an oligonucleotide array. We showed that a two-way hierarchical clustering algorithm successfully distinguished between HCC with

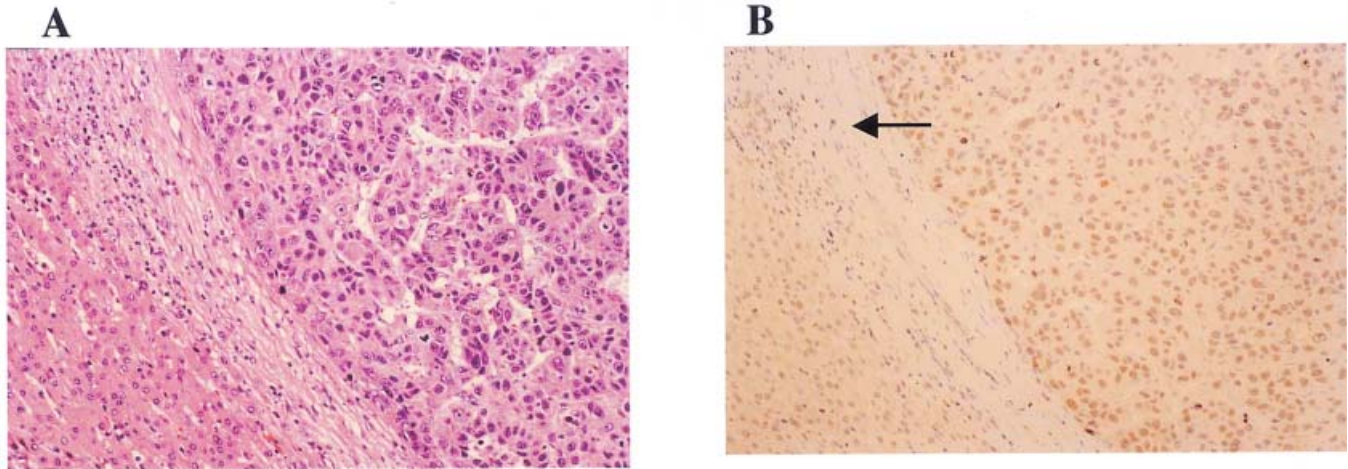


Fig. 3 Immunohistochemical localization of HMG-I(Y) in HCC. Representative histology of HCC with intrahepatic metastasis (A) (hematoxylin–eosin stain; original magnification, $\times 20$), and HMG-I(Y) immunostaining of the same section (B). Lymphocytes were used as a positive control (arrows). HCC cases with intrahepatic metastasis showed strong HMG-I(Y) immunoreactivity, mainly in the nucleus.

intrahepatic metastasis and HCC without intrahepatic metastasis. These results indicate that the molecular signatures of HCC with intrahepatic metastasis and of HCC without intrahepatic metastasis are distinctly different. We also identified 34 genes whose expression levels were significantly correlated with metastatic ability. We further showed that HMG-I(Y) was associated with intrahepatic metastasis and differentiation, although the reason why HMG-I(Y) expression increases in HCC with intrahepatic metastasis and poorly differentiated HCC is not clear at present. The mammalian HMG-I, HMG-Y, and HMG-C proteins are members of the HMG-I(Y) family of nonhistone chromatin proteins,²⁴ with the former two proteins being derived from the alternative processing of RNA transcripts from a single functional gene.²⁵ HMG-I(Y) has been implicated in both positive and negative regulation of gene transcription *in vivo*.²⁶ Numerous studies have demonstrated that HMG-I(Y) overexpression is frequently associated with both the neoplastic transformation of cells and metastatic tumor progression.^{27–29} Overexpression of HMG-I(Y) has been described in several cancers,^{29–33} and has been correlated with differentiation status.³³ Moreover, significant correlation between elevated HMG-I(Y) expression and a poor prognosis has been reported in patients with prostate³³ and breast cancers.³⁴ Recently, HMG-I(Y) has been shown to be a *c-myc* target gene involved in neoplastic transformation.³⁵ Several reports have concluded that the amplification and overexpression of *c-myc* is significantly correlated with the prognosis of HCC.³⁶ Thus, we speculated that these interactions exist in HCC and mediate key aspects of HCC progression.

In addition to HMG-I(Y), we were able to identify

several other candidate genes involved in intrahepatic metastasis. Some of the up-regulated genes identified in the present analysis have been previously correlated with tumor malignancy and metastasis in several types of cancers. For example, osteopontin is known to be a secreted adhesive glycoprotein that is associated with clinical outcome in prostate³⁷ and breast³⁸ cancer and is also involved in intrahepatic metastasis in HCC.¹⁴ CD24 is a small heavily glycosylated glycosylphosphatidylinositol-linked cell surface protein, which is expressed in a large variety of carcinomas, including HCC.³⁹ Aldose reductase, which efficiently reduces aliphatic and aromatic aldehydes, is also overexpressed in HCC.⁴⁰ RhoC, a ras-related GTP-binding protein of member C in the rho subfamily, regulates the reorganization of the actin cytoskeleton and is specifically expressed in invasive breast carcinomas capable of metastasis.⁴¹ RhoC is also associated with tumor progression in ovarian carcinoma⁴² and is involved in venous invasion of HCC.¹³ 14-3-3 sigma, a member of the 14-3-3 family, plays a role in the G2 checkpoint response to DNA damage, and the epigenetic inactivation of 14-3-3 sigma caused by aberrant hypermethylation has been reported in several cancers.^{43,44} However, Christine recently reported the overexpression of 14-3-3 sigma in pancreatic cancer and the hypomethylation of the 14-3-3 sigma promoter.⁴⁵ We also confirmed the overexpression of 14-3-3 sigma in HCC with intrahepatic metastasis using real-time quantitative RT-PCR and immunohistochemistry (unpublished observations). The overexpression of 14-3-3 sigma in HCC may be related to the anti-apoptotic role that has been described for this gene. It would be necessary to analyze the expression and methylation status of the 14-3-3 sigma in

larger HCC samples. Further analysis of these genes should improve our understanding of intrahepatic metastasis.

In conclusion, the molecular signatures of HCC with intrahepatic metastasis and HCC without intrahepatic metastasis were clearly different. HMG-I(Y) was associated with intrahepatic metastasis and may be a predictive marker for HCC with intrahepatic metastasis.

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