High mobility group A1 is expressed in metastatic adenocarcinoma to the liver and intrahepatic cholangiocarcinoma, but not in hepatocellular carcinoma: its potential use in the diagnosis of liver neoplasms

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Background. An increased level of high mobility group A (HMGA) gene/protein expression has been demonstrated to be associated with many human neoplasms originating from a variety of tissues. However, HMGA1 expression has not yet been studied in hepatic tumors. In this study, we analyzed HMGA1 expression in hepatic primary and metastatic tumors in order to verify whether determination of the HMGA1 expression level could provide any diagnostic advantages in the pathological diagnosis of hepatic tumors. ***Methods.*** Twenty samples of hepatocellular carcinoma, 5 samples of intrahepatic cholangiocarcinoma, and 21 samples of metastatic adenocarcinoma to the liver (15 metastatic tumors from colorectal carcinoma and 6 metastatic tumors from pancreatic carcinoma) were analyzed immunohistochemically using an HMGA1-specific antibody. ***Results.*** While no significant nuclear immunoreactivity was found in hepatocytes of non-neoplastic liver tissue, 40% (2/5) of intrahepatic cholangiocarcinomas, 53.3% (8/15) of metastatic lesions from colorectal carcinoma, and 100% (6/6) of metastatic lesions from pancreatic carcinoma showed positive immunoreactivity. In contrast, all 20 samples of hepatocellular carcinoma were negative for HMGA1 nuclear immunoreactivity. Thus, hepatocellular carcinoma represents the first case of malignant neoplasia in which HMGA1 expression is not induced, which presents a striking contrast to several previous studies demonstrating the significance of increased HMGA gene/protein levels in carcinogenesis and/or tumor progression. ***Conclusions.*** Based on these findings, we conclude that the HMGA1 protein level could serve as a potential diagnostic marker that may enable the differential diagnosis between hepatocellular carcinoma and intrahepatic cholangiocarcinoma or metastatic adenocarcinoma to the liver.

Introduction

The high mobility group A (HMGA) family of proteins in mammals is known to consist of four proteins: HMGA1a, HMGA1b, HMGA1c, and HMGA2. The first three of these proteins are generated from a single functional gene, i.e., *HMGA1* (formerly *HMGI(Y)*), while the last one is a product of a separate gene, i.e., *HMGA2* (formerly *HMGI-C*).[1,2] The HMGA proteins are nonhistone nuclear proteins, which bind to AT-rich regions in the minor groove of DNA via three AT-hook domains, and are thought to affect the transcription process by acting as architectural proteins.[3,4] Recent studies demonstrated an important role for the HMGA1 proteins (formerly HMGI(Y) proteins) in regulating gene expression,[5–10] although they have no transcriptional activity perse.[11] The HMGA1 proteins, however, participate in the assembly of protein complexes on the promoters of several inducible genes, and have thus been defined as architectural transcriptional factors.[5,9,12–14]

HMGA gene expression is negligible in normal adult tissues, being essentially restricted to embryonic development.[15,16] The HMGA1 gene/protein expression level, however, has been demonstrated to be elevated in many human neoplasms originating from a variety of tissues, including the thyroid, prostate, uterus, colorectum, and pancreas.[17–26] These findings indicate the critical role(s) of the HMGA1 proteins not only in normal cell proliferation and/or differentiation but also in tumorigenesis and/or tumor growth. Moreover, *HMGA1* has been reported to be an important c-Myc target gene involved in

neoplastic transformation.[27] Further investigations have actually shown that high levels of *HMGA1* mRNA expression are directly correlated with metastatic progression in several tumor cell lines.[28,29] We have also demonstrated a significant correlation between the levels of HMGA1 protein expression and factors closely associated with a poor prognosis in patients with colorectal cancer.[22] These data support the idea that HMGA1 proteins could be a potential target molecule for gene treatment, as well as a diagnostic marker for a wide variety of malignancies.

We previously demonstrated that colorectal carcinoma and pancreatic carcinoma express high levels of HMGA1.[21–23,25,26] However, HMGA1 expression has not yet been studied in hepatic tumors. In the present study, we analyzed HMGA1 expression in hepatic primary and metastatic tumors in order to investigate whether determination of the HMGA1 expression level could provide any diagnostic advantages in the pathological diagnosis of hepatic tumors. To this end, HMGA1 expression was determined at the protein level in hepatocellular carcinoma, intrahepatic cholangiocarcinoma, and metastatic adenocarcinoma to the liver, by immunohistochemical analysis.

Materials and methods

Tissue samples

Tissue samples were obtained at the time of surgery at the First Department of Surgery, Kyorin University Hospital, between January 1990 and March 2001. Written consent regarding inclusion of the removed tissues in the study was obtained from the patients. The tissues were fixed in 10% neutral buffered formalin within 4h after surgical removal, sectioned into blocks, and embedded in paraffin. The tissue samples obtained included 21 samples of metastatic adenocarcinoma to the liver (15 metastatic tumors from colorectal carcinoma and 6 metastatic tumors from pancreatic carcinoma), 5 samples of intrahepatic cholangiocarcinoma, and 20 samples of hepatocellular carcinoma (HCC), together with 32 samples of adjacent normal hepatic tissues.
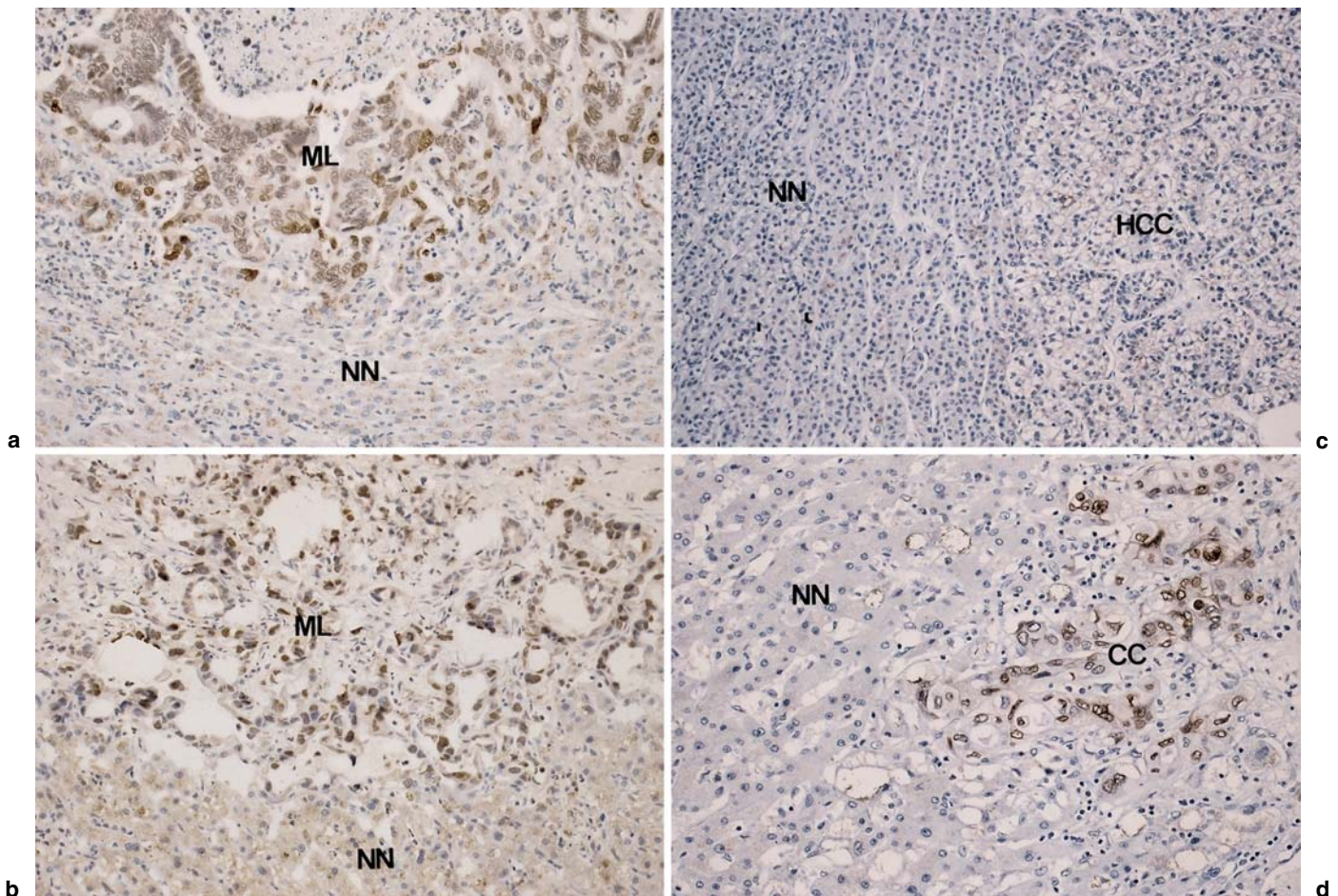
Immunohistochemical analysis

Immunohistochemical examinations were performed by the avidin-biotin complex immunoperoxidase technique, using an Avidin-Biotinylated Enzyme Complex kit (Vector Laboratories, CA, USA). A primary rabbit polyclonal antibody against an HMGA1-specific synthetic peptide, corresponding to the NH_2-terminal region of the molecule (Santa Cruz Biotechnology, CA, USA) was used in this study.[24,26] In brief, paraffin sec-

tions (4 m) were cut, transferred onto Matsunami Adhesive Silan (MAS)-coated slides, deparaffinized in xylene, and rehydrated through graded alcohol series. The sections were subjected to microwave antigen retrieval in citrate buffer in a calibrated microwave at high power seven times, each time for 3min, followed by quenching of the endogeneous peroxidase activity with 0.3% hydrogen peroxide in methanol. After a rinsing with phosphate-buffered saline (PBS), the sections were incubated with normal goat serum for 20min at room temperature to block nonspecific binding, and then incubated with the primary anti-HMGA1a antibody at a dilution of 1:100 for 14h at 4°C. After being washed in PBS with 0.2% Triton X-100, sections were further incubated with biotinylated anti-rabbit IgG for 30min at room temperature, followed by washes in PBS with 0.2% Triton X-100. Subsequently, sections were incubated with streptavidin-biotin-conjugated peroxidase for 30min at room temperature and washed in PBS with 0.2% Triton X-100, followed by visualization of the HMGA1 proteins by incubation of the sections with 3,3′-diaminobenzidine. The slides were then counterstained with Mayer's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and mounted. As the positive control, a pancreatic carcinoma tissue specimen overexpressing HMGA1 proteins was processed in a similar manner.[25,26] Negative control staining was carried out by replacing the primary antibody with normal rabbit serum under the same experimental conditions. The immunostained slides were evaluated microscopically by a single investigator (N.A.) according to the criteria previously published,[22,25] without prior knowledge of the clinical data for each case. The percentage of HMGA1-positive cells was scored by counting approximately 300–1000 tumor cells.[22,25,26] Immunnohistochemical evaluation was considered positive when HMGA1 nuclear immunoreactivity was detected in more than 20% of the cells according to the criteria previously published.[22,25]

Results

HMGA1 expression in metastatic lesions from colorectal and pancreatic carcinomas was analyzed first because these carcinoma species have been shown to express high levels of HMGA1 gene/protein at the primary sites.[22–26] Among the 15 samples of metastatic lesions from colorectal carcinoma, 8 showed positive HMGA1 immunoreactivity (53.3%). In these HMGA1-positive samples, the HMGA1 nuclear immunoreactivity tended to be distributed unevenly within the tumor lesion (Fig. 1a). In contrast, all the metastatic lesions from pancreatic carcinoma showed positive HMGA1 immunoreactivity characterized by intense nuclear

Fig. 1a–d. Immunohistochemical analysis of high mobility group A1 (HMGA1) protein expression in hepatic tumors. **a** Sections including both metastatic cancer cells from colon cancer and nonneoplastic hepatocytes (*NN*). *ML*, metastatic lesion. **b** Sections including both metastatic cancer cells from pancreatic cancer and nonneoplastic hepatocytes. **c** Sections including both hepatocellular carcinoma (*HCC*) and nonneoplastic hepatocytes. **d** Sections including both intrahepatic cholangiocarcinoma (*CC*) and nonneoplastic hepatocytes. Results of HMGA1 immunostaining demonstrate intense nuclear labeling (*brown staining*) in metastatic adenocarcinoma (**a**, **b**) and intrahepatic cholangiocarcinoma (**d**). In contrast, no significant HMGA1 labeling was observed either in hepatocellular carcinoma (**c**) or in nonneoplastic hepatocytes (**a–d**). **a–d** Sections were counterstained with Mayer's hematoxylin. **a**, **b**, **d**, ×200; **c** ×100

staining (100%), which was distributed homogeneously throughout the carcinoma lesion (Fig. 1b). In one sample, in which it was possible to compare the intensity as well as the distribution of HMGIA1 immunoreactivity between primary and metastatic pancreatic carcinoma obtained from a single patient, no significant difference was actually observed between the two lesions (data not shown). Thus, among the 21 samples of metastatic adenocarcinoma to the liver, 14 samples (66.7%) showed HMGA1 overexpression. Conversely, no significant nuclear immunoreactivity was identifiable in the 32 samples of nonneoplastic hepatic tissue. Such a marked difference in the HMGA1 immunoreactivity level between a metastatic lesion and nonneoplastic liver tissue was confirmed in a single section, in which

these two distinct components were intermingled with each other; that is, metastatic adenocarcinoma cells clearly showed very intense HMGA1 immunoreactivity, whereas neighboring nonneoplastic hepatocytes did not (Fig. 1a, b).

The HMGA1 expression in primary hepatic carcinomas was then examined. Results showed that the 20 samples of HCC were completely negative for HMGA1 nuclear immunoreactivity (Fig. 1c). In contrast, of the 5 intrahepatic cholangiocarcinoma samples examined, 2 lesions revealed multifocally distributed intense HMGA1 nuclear immunoreactivity (Fig. 1d). The percentages of the samples demonstrating positive HMGA1 immunoreactivity can thus be summarized as follows: 40% (2/5) for intrahepatic cholangiocarcinoma,

Table 1. High mobility group A1 (HMGA1) protein overexpression in hepatic tumors

	HMGA1 overexpression	
Histological type of lesion	Negative	Positive (positivity rates)
Hepatocellular carcinoma ($n = 20$)	20	0 (0%)
Cholangiocarcinoma ($n = 5$)	3	2 (40%)
Metastatic adenocarcinoma ($n = 21$)		
Pancreas ($n = 6$)	0	6 (100%)
Colorectum ($n = 15$)	7	8 (53.3%)
Nonneoplastic liver ($n = 32$)	32	0 (0%)

0% (0/20) for HCC, 100% (6/6) for metastatic lesions from pancreatic carcinoma, and 53.3% (8/15) for metastatic lesions from colorectal carcinoma (Table 1).

Discussion

The present study revealed that HMGA1 protein overexpression was observed in 66.7% of metastatic adenocarcinomas to the liver. Among the HMGA1-positive tumors, however, a significant difference in the HMGA1 staining pattern was found between metastatic tumors from pancreatic carcinoma and those from colorectal carcinoma. In metastatic adenocarcinomas of pancreatic origin, HMGA1-positive cells were characteristically distributed homogeneously throughout the lesion, which was similar to the pattern observed in their primary sites, as reported previously.[25,26] In contrast, HMGA1-positive cells in metastatic tumors from colorectal carcinoma were distributed heterogeneously, similar to those in their primary lesions.[22] Thus, characteristic patterns of HMGA1 immunoreactivity observed in the primary sites tended to be conserved even in the metastatic lesions, as far as pancreatic and colorectal carcinomas were concerned. These findings indicate that primary and metastatic pancreatic and colorectal carcinoma cells may have common biological characteristics, particularly those related to the expression/function of the HMGA1 gene/proteins. These findings, particularly those on colorectal carcinomas, may also provide an interesting insight into the relationship between the biological aggressiveness of carcinoma cells and their metastatic potential. There has been evidence indicating that biologically aggressive malignant cells tend to metastasize more frequently than those with less aggressiveness.[30,31] This would suggest that a metastatic tumor lesion is composed mainly of biologically aggressive tumor cells, or in this instance, strongly HMGA1-positive cells. This was, however, not the finding here; metastatic colorectal carcinomas from the liver were composed of cells with varied HMGA1 expression levels. The reason for this has to be determined.

The present study also revealed that HMGA1 protein overexpression was observed in 40% of intrahepatic cholangiocarcinoma samples. This is the first demonstration that a significantly increased expression level of HMGA1 protein could also be associated with malignant tumors originating from bile duct tissues. In contrast to intrahepatic cholangiocarcinoma or metastatic adenocarcinoma, neither HCC nor nonneoplastic hepatocytes expressed the HMGA1 proteins. The absence of the HMGA1 proteins in nonneoplastic liver tissue was consistent with a previous report that the *HMGA1* gene was expressed at very low levels in normal adult liver tissue, as determined by Northern blot analysis using poly-A RNA.[17] It is noteworthy that the aberrant expression of HMGA1 in metastatic carcinoma and intrahepatic cholangiocarcinoma did not occur in HCC. Thus, HCC represents the first case of malignant neoplasia in which HMGA1 expression is not induced, a finding that presents a striking contrast to several previous studies demonstrating the significance of increased HMGA1 gene/protein levels in carcinogenesis and/or tumor progression.[16–26] This suggests that HMGA1 proteins may not play an essential role(s) in the carcinogenesis of HCC. It is possible that genetic alterations leading to HCC could induce the expression of HMGA-related genes other than *HMGA1*, such as *HMGA2*, which is not detectable by the antibody we used. Also, we cannot exclude the possibility that the results for HCC obtained in the present study simply reflect that the amount of HMGA1 protein in HCC was too small to be detected by the immunohistochemical analysis.

Although there have been various histochemical and immunohistochemical attempts to distinguish among HCC, intrahepatic cholangiocarcinoma, and metastatic adenocarcinoma,[32–37] these studies have not been successful to date. In particular, in a minority of HCCs (high-grade tumors, undifferentiated tumors, and those with microglandular patterns), the appearance is quite variable, and confusion with intrahepatic cholangiocarcinoma and metastatic adenocarcinoma may arise on conventional histological examination.[37] Currently, the immunohistochemical panel used in distingui-

shing HCC from intrahepatic cholangiocarcinoma/adenocarcinoma includes: alpha-fetoprotein, keratin, Lewis antigen, and MOC31.[33–37] Despite this impressive array of antibodies, no combination has been proven reliable in distinguishing HCC from intrahepatic cholangiocarcinoma/adenocarcinoma. The present study disclosed that 40% of intrahepatic cholangiocarcinoma samples and 66.7% of metastatic adenocarcinoma samples overexpressed the HMGA1 proteins, while none of the HCC samples examined expressed the HMGA1 proteins. This suggests that the HMGA1 protein level could serve as a potential diagnostic marker that may enable the differential diagnosis between HCC and intrahepatic cholangiocarcinoma or metastatic adenocarcinoma, although it does not distinguish between intrahepatic cholangiocarcinoma and metastatic adenocarcinoma. Potential application of this assay could be in the evaluation of samples obtained by needle biopsy of a hepatic neoplasm.

This characteristic expression of HMGA1 gene/proteins indicates their potential role as a target of gene therapy for hepatic metastatic tumors. Metastatic disease in the liver is the primary cause of death in patients with colorectal and pancreatic carcinomas. Furthermore, as conventional systemic or regional chemotherapy has generally failed to improve the survival of patients,[38] novel therapeutic approaches are urgently required. A recent development in cancer gene therapy has enabled intratumoral gene expression, which allows more effective access to tumor cells, with less systemic toxicity.[39] Recently, an adenovirus carrying the *HMGA1* gene in an antisense orientation was introduced to tumors, which resulted in the induction of programmed cell death in several carcinoma cell lines,[40] suggesting that the fate of tumor cells could be altered by controlling *HMGA1* gene expression. Because the present study demonstrated that 66.7% of metastatic tumor samples, but not the adjacent normal hepatocytes, expressed the HMGA1 proteins, it may be possible to specifically suppress HMGA1 protein synthesis within metastatic hepatic tumors from colorectal and pancreatic carcinoma by the local administration of an HMGA1 antisense adenoviral vector.

In conclusion, the present study revealed that HMGA1 proteins were overexpressed in 40% of intrahepatic cholangiocarcinoma samples and 66.7% of metastatic adenocarcinoma samples, while none of the HCC samples examined expressed the HMGA1 proteins. Thus, HCC represents the first case of a malignant neoplasm in which HMGA1 expression is not induced. Based on these findings, we conclude that the HMGA1 protein level could serve as a potential diagnostic marker that may enable the differential diagnosis between hepatocellular carcinoma and cholangiocarcinoma or metastatic adenocarcinoma.

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