Pathol. Oncol. Res. (2012) 18:983–987
DOI 10.1007/s12253-012-9514-z

RESEARCH

Expression of High Mobility GroupA2 is Associated with Poor Survival in Hepatocellular Carcinoma

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Received: 3 November 2011 / Accepted: 1 March 2012 / Published online: 30 March 2012

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common solid malignancies [1]. Despite recent improvements in surveillance, diagnosis and clinical treatment strategies, HCC prognosis remains dismal. Patients often experience high metastasis or recurrence rates, and postoperative 5-year survival ranges from 17 % to 53 % [2]. To date, many studies have reported aberrant gene expression in HCC, but molecular factors identified as potential therapeutic targets remain limited. Therefore, it is a challenging task to identify relevant biomarkers or potential pharmacological targets in clinical practice.

The high mobility group A2 (HMGA2; also called HMGI-C) protein, a member of the high mobility group AT-hook (HMGA) family, is a small non-histone chromosomal protein that has no intrinsic transcriptional activity, but can modulate transcription by binding to DNA and altering chromatin architecture [3–5]. By interacting with many different transcription factors, HMGA2 is an important regulator of cell growth, differentiation, apoptosis, and transformation [5]. HMGA2 protein is expressed at a high level during embryogenesis, but is hardly detectable in adult human tissues [6, 7]. HMGA2 is frequently involved in chromosomal translocations that occur in benign human tumors, such as lipomas [8], uterine leiomyomas [9], and lung hamartomas [10]. Furthermore, increased HMGA2 expression has been detected in various human epithelial-type tumors, including breast cancers [11], lung cancers [12, 13], pancreatic carcinomas [14], thyroid carcinomas [15], and gastric cancers [16]. These studies suggest that HMGA2 protein overexpression may lead to neoplastic transformation. However, there have been no reports of HMGA2 expression in hepatocellular carcinoma. In this study, we investigated HMGA2 expression in HCC to determine its clinicopathologic and prognostic value.

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Materials and Methods

Tissue Specimens

Twenty three primary hepatocellular carcinoma specimens and matched adjacent nontumor tissues (taken>2 cm from the tumor margin) were collected from patients at the Department of General Surgeon, Xiangya Hospital, Central South University, China in 2011, and used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Patients were histopathologically diagnosed as primary hepatocellular carcinoma, newly diagnosed and untreated, or no history of other tumor. All samples were obtained immediately following surgical removal, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Informed consent was obtained from all patients in accordance with the guidelines of Central South University, and the study protocols were approved by the Ethics Committee of Central South University.

For immunohistochemical assay, a total of 107 paraffin-embedded HCC samples and the adjacent nontumor liver tissues were obtained from patients who underwent curative

liver resection between January 2006 and January 2007 at the Department of General Surgery in Xiangya Hospital of Central South University, China. The criteria for study enrollment were: histopathological diagnosis of primary hepatocellular carcinoma, no other therapy was received before the operation, and no history of previous tumor. The patients included 73 males and 34 females from 18 years to 64 years (mean age, 49 years). The clinicopathologic variables, such as alpha fetoprotein (AFP) concentration, cirrhosis, Edmondson grading, tumor size, and microscopic venous invasion were recorded. The follow-up period was defined as the interval between the date of the operation and the date of either the patient's death or the final follow-up. Deaths from non-HCC related causes were treated as censored cases. The follow-up time ranged from 4 months to 48 months. Follow-ups were performed by telephone.

Quantitative Real-time RT-PCR for HMGA2

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. RNA was reverse transcripted into cDNAs with RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). Briefly, 200 ng of each RNA sample was mixed with reaction mixture consisting of 4 μl 5× reaction buffer, 1 μl Oligo(dT)18 primer, 2 μl dNTP mix, 0.5 μl Ribonuclease Inhibitor, 1 μl RevertAidTM M-MulV Reverse Transcriptase, and 13.5 μl DEPC-treated water. They were incubated at 42 °C for 60 min and then 70 °C for 10 min. Quantitative PCR was carried out using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, CA,USA) and SYBR Green PCR Master Mix (Applied Biosystems). The sample mixture of a total 25 μl reaction volume contained complementary DNA, 2× SYBR Green PCR Master mix, primers and nuclease-free water. The reaction conditions was first 95 °C for 5 min, then 40 cycles at 94 °C for 20 s, 59 °C for 20 s and followed by 72 °C for 20 s. The primer sequence was designed using Prime3.0 (ProMab, Changsha, China). The primer for HMGA2 was forward: 5′-CAAGAGTCCCTCTAAAGCAG -3′ and reverse: 5′-CTAGTCCTCTTCGGCAGAC -3′. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ProMab) was used as an internal control, with primer sequences: forward: 5′- CAATGACCCCTTCATTGACC -3′ and reverse: 5′- GACAAGCTTCCCGTTCTCAG -3′.

Each sample was analyzed in triplicate, the *Ct* value for each sample was calculated using the $\Delta\Delta Ct$ method and the results were expressed as $2^{-\Delta\Delta Ct}$ [17].

Immunohistochemistry

The paraffin-embedded tissues sections were analyzed for the presence of HMGA2 using labeled streptavidin-biotin. After deparaffinization and antigen retrieval using the autoclave oven technique, the sections were incubated at 4 °C overnight with rabbit polyclonal anti-HMGA2 antibody (1:500; Abcom Inc., Cambridge, MA). Biotinylated anti-rabbit antibody was used as the secondary antibody. The antigen-antibody complexes were detected using the cobalt-3, 3′-diaminobenzidine (Co-DAB) reaction. Negative controls included incubation without the primary antibody. Nuclear staining was considered as positive staining for HMGA2. A total of 500 to 1000 cells for each sample were counted and the percentage of HMGA2-stained tumor cells was divided as negative staining <10 %, positive staining ≥10 %.

Statistical Analysis

Possible differences between groups were analyzed using the Mann–Whitney *U*-test. The significance of the relationships between HMGA2 protein expression and clinicopathological parameters was evaluated using $\chi 2$ tests. Survival curves were obtained using the Kaplan–Meier method; comparisons between curves were made using the log-rank test. Prognostic factors were examined using univariate and multivariate analyses (Cox proportional hazards model). SPSS 13.0 software was used for statistical analysis. A value of $P < 0.05$ was considered statistically significant.

Results

HMGA2 mRNA Expression in Liver Tissue Specimens

To determine if HMGA2 is overexpressed in HCC, we measured HMGA2 mRNA expression levels in fresh clinical samples using quantitative real-time RT-PCR. The mean (± SD) HMGA2 mRNA expression level was higher in tumor tissues (38.70±10.41) than in nontumor tissues (8.41±4.06; $p<0.01$) after normalization to GAPDH (Fig. 1).

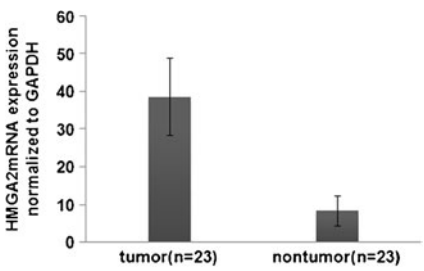

Fig. 1 HMGA2 mRNA expression in 23 fresh tissue specimens. The HMGA2 mRNA levels were measured by quantitative RT-PCR and normalized to human GAPDH as a loading control

HMGA2 Protein Expression in Paraffin-embedded HCC Samples and its Relationship to HCC Clinical Variables

Given the significantly high level of HMGA2 mRNA expression in clinical specimens, we sought to determine if HMGA2 proteins are overexpressed in HCC cases when compared to normal tissues. In this study, we analyzed HMGA2 protein levels using immunohistochemical methods in 107 HCC cases. As shown in Fig. 2, HMGA2 protein expression was localized in the nucleus of cancer cells. HMGA2 positivity in HCC tissues was 47.66 % (51/107), compared to 17.76 % (19/107) in adjacent nontumor liver tissues ($p<0.01$). As shown in Table 1, HMGA2 protein expression was significantly correlated with tumor size, capsule invasion and vascular invasion ($p<0.05$). However, HMGA2 protein expression was not associated with other clinicopathological features such as age, sex, hepatitis B, serum AFP elevation, Edmondson grading, or tumor number.

Prognostic Significance of HMGA2 Expression in HCC

On the basis of immunohistochemical results, the 107 paraffin-embedded cases were divided into two groups: positive HMGA2 expression ($n=51$) and negative HMGA2 expression ($n=56$). HCC patients in the positive HMGA2 expression group had a poorer overall survival rate than patients in the negative HMGA2 expression group (Fig. 3). The results from univariate and multivariate analyses of overall HCC patient survival are shown in Table 2. Univariate analysis showed that the following factors were significantly related to postoperative survival: tumor size, capsule invasion, vascular invasion, and HMGA2 expression. Multivariate analysis, using Cox proportional hazards model, indicated that HMGA2 expression was found to be a significantly independent prognostic factor in HCC.

Discussion

HMGA2 is expressed primarily in the developing mesenchyme of various organs, but is absent in the adult tissues. Overexpression of HMGA2 has been detected in many kinds of benign tumors and cancers, but is also highly expressed in various human epithelial-type neoplasias, such as breast cancers [11], pancreatic carcinomas [14], thyroid carcinomas [15], gastric cancers [16] and oral squamous cell carcinomas [18]. In this study, we showed that HMGA2 was highly expressed in HCC when compared with corresponding normal tissues. The mean HMGA2 mRNA expression level was higher in tumor tissues (38.70±10.41) than in nontumor tissues (8.41±4.06; $p<0.01$). The HMGA2 protein positivity rate in HCC tissues was 47.66 % (51/107), compared with 17.76 % (19/107) in adjacent nontumor liver tissues ($p<0.01$). We correlated HMGA2 protein expression with respect to various clinicopathologic factors in 107 HCC patients. Our findings show

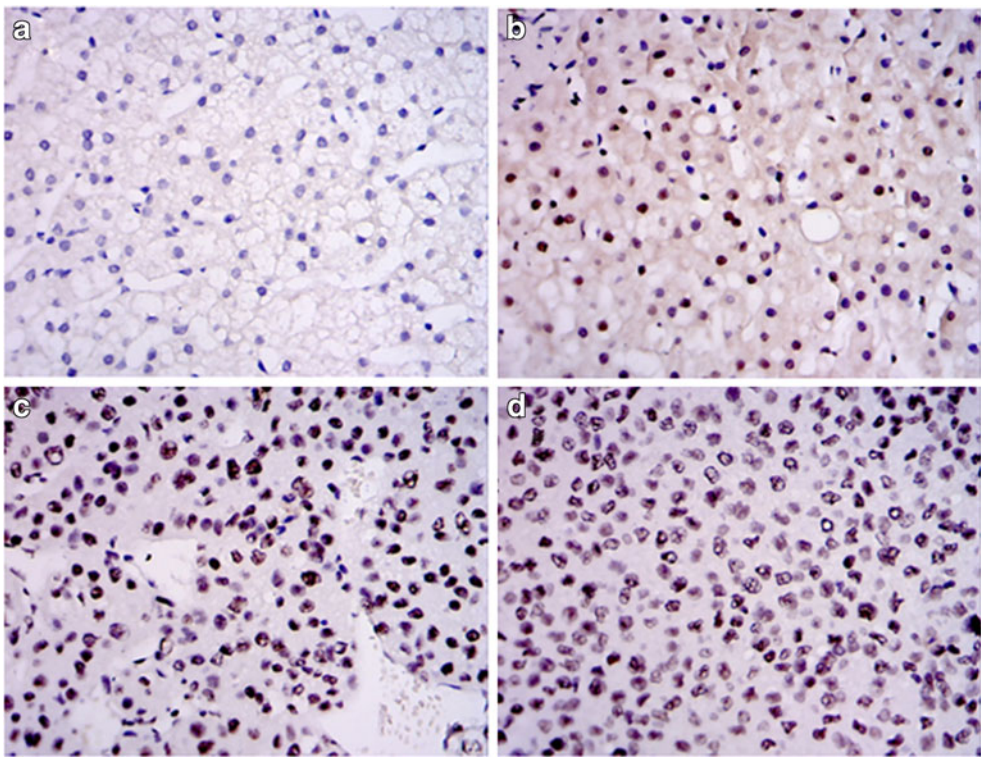

Fig. 2 Representative immunohistochemical staining of HMGA2 protein in HCC tissue and adjacent nontumor liver tissue (original magnification, ×400). **a** Negative HMGA2 staining in adjacent nontumor liver tissue. **b** Positive HMGA2 staining in adjacent nontumor liver tissue. **c** Positive HMGA2 staining in the well differentiated HCC tissue. **d** Positive HMGA2 staining in poor differentiated HCC

Table 1 Correlation between HMGA2 protein expression and clinicopathologic features of HCC patients

Parameters	*n*	HMGA2		*P*
		Positive	Negative	
Gender				
Male	73	31	42	0.115
Female	34	20	14	
Age, years				
≥40	66	29	37	0.328
<40	41	22	19	
HBsAg infection				
Positive	93	43	50	0.446
Negative	14	8	6	
Cirrhosis				
Yes	69	31	38	0.445
No	38	20	18	
AFP, ng/ml				
≥20	84	42	42	0.355
<20	23	9	14	
Tumor number				
Single	67	28	39	0.115
Multiple	40	23	17	
Tumor size				
≤5 cm	41	14	27	0.027
>5 cm	66	37	29	
Capsule invasion				
Yes	34	22	12	0.016
No	73	29	44	
Vascular invasion				
Yes	31	20	11	0.026
No	76	31	45	
Edmondson grading				
I–II	81	36	45	0.239
III–IV	26	15	11	

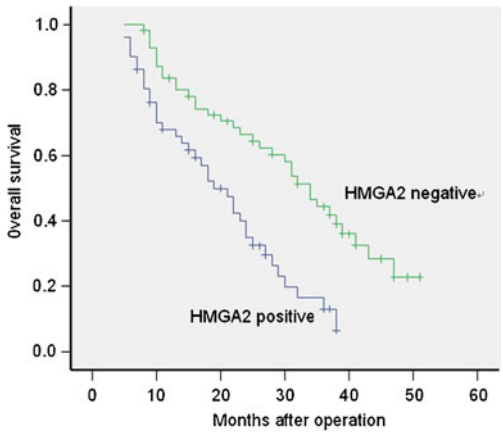

Fig. 3 Overall survival in 107 HCC patients based on the expression of HMGA2. The graph summarizes Kaplan–Meier survival analysis for patients with positive or negative HMGA2 expression (p=0.01)

Table 2 Univariate and multivariate analysis of overall survival in HCC

Parameters	Univariate analysis		Multivariate analysis	
	RR(95 % CI)	P	RR(95 % CI)	P
Tumor size				
≤5 cm >5 cm	0.647(0.398–1.049)	0.077	–	–
Tumor number				
single multiple	0.628(0.388–1.108)	0.059	–	–
Vascular invasion				
Yes No	2.230(1.334–3.726)	0.002	1.564(0.908–2.694)	0.107
Edmondson grading				
I–II III–IV	1.800(1.066–3.042)	0.028	1.479(0.867–2.524)	0.151
Capsule invasion				
Yes No	1.981(1.172–3.347)	0.011	1.406(0.814–2.428)	0.222
HMGA2 expression				
Negative Positive	2.476(1.152–4.054)	0.000	1.975(1.170–3.335)	0.011

that increased HMGA2 protein expression was significantly associated with tumor size, capsule invasion and vascular invasion, which implies that HMGA2 proteins may play a crucial role in the development and progression of HCC. HMGA2 protein overexpression is a constant feature of malignant human neoplasias, and recent studies implicated HMGA2 in the development and progression of human carcinomas. For example, Sun-Mi Park et al. [19] found that HMGA2 expression represents an early event during ovarian cancer progression. Fabjani et al. [11] reported that in invasive ductal breast tumors, HMGA2 expression was noted predominantly in tumors with a high histological grade. Hristov et al. [20] reported that HMGA2 nuclear immunoreactivity correlated positively with lymph node metastases and high tumor grade in pancreatic ductal adenocarcinoma. Motoyama et al. [16] reported that high HMGA2 expression in gastric cancer correlated with tumor invasiveness and was a independent prognostic factor. HMGA2 protein exerts their oncogenic activity from its ability to down- or up-regulate genes that have a critical role in controlling cell proliferation and invasion, such as induction of E2F1 and AP1 activity and regulation of epithelial-mesenchymal transition (EMT). For example, HMGA2 protein has been reported to increase the AP-1 complex activity that is responsible for neoplastic transformation [21]. Fedele et al. [22] reported that HMGA2-mediated E2F1 activation was a crucial event during tumorigenesis in transgenic mice. Thuault et al. [23] reported that

transforming growth factor β (TGFβ) mediated EMT by inducing HMGA2 through the Smad pathway. HMGA2, in turn, regulated EMT-associated transcription factors such as Snail, Slug, Twist, and inhibitor of differentiation 2, resulting in repression of E-cadherin expression. These observations supported our findings that HMGA2 overexpression is related to tumor development and progression in HCC.

HMGA2 protein represents a poor prognostic index as its overexpression often correlates with metastasis and with reduced survival. In this study, we found that increased HMGA2 protein expression in HCC was significantly associated with poor prognosis and low overall survival. Furthermore, a multivariate analysis showed that HMGA2 protein expression was an independent prognostic factor associated with overall survival rate. A previous study of primary lung adenocarcinomas also found that high levels of HMGA2 nuclear staining correlated with poor survival [12]. In oral squamous cell carcinomas, HMGA2 expression was an independent prognostic value for disease-specific overall survival [18]. HMGA2 overexpression promotes metastasis and impacts survival of colorectal cancer patients [24]. Langelotz et al. [25] reported that HMGA2 mRNA expression in the peripheral blood of metastatic breast cancer patients was an independent indicator of poor overall survival. These observations supported our findings that HMGA2 overexpression is an independent poor prognostic indicator in HCC.

In conclusion, our results shown that HMGA2 expression was increased in hepatocellular carcinoma and that overexpression of HMGA2 correlated with tumor progression and poor prognosis in HCC.

Acknowledgments We are grateful to Dr. Xinyin Li for his comments. We also thank the patients who participated in this study.

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