Evaluation of MAGE-D4 Expression in Hepatocellular Carcinoma in Japanese Patients

HIDEKI TAKAMI, MD, MITSURO KANDA, MD, PhD,* HISAHARU OYA, MD, SOKI HIBINO, MD, HIROYUKI SUGIMOTO, MD, PhD, MASAYA SUENAGA, MD, SUGURU YAMADA, MD, PhD, YOKO NISHIKAWA, MIKAKO ASAI, TSUTOMU FUJII, MD, PhD, FACS, SHUJI NOMOTO, MD, PhD, FACS, AND YASUHIRO KODERA, MD, PhD, FACS

Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan

Background and Objectives: Though Melanoma-associated antigen (MAGE) family genes have received lots of attention as cancer-related genes and targets for immunotherapy, MAGE-D4 expression in hepatocellular carcinoma (HCC) has not yet been evaluated.
Methods: MAGE-D4 mRNA expression was assayed in nine HCC cell lines and 94 HCC surgical specimens obtained from Japanese patients by quantitative real-time reverse transcription polymerase chain reaction, and the correlations between MAGE-D4 mRNA expression and clinicopathological factors were evaluated. The expression and distribution of MAGE-D4b protein were evaluated immunohistochemically.
Results: MAGE-D4 mRNA was overexpressed in five of nine HCC cell lines and 34 of 94 primary HCCs (36.2%). Median overall survival (14.8 vs. 118 months, $P < 0.001$) and relapse-free survival (2.7 vs. 18.3 months, $P < 0.001$) were significantly shorter in patients with high than with low-moderate MAGE-D4 expression. Multivariate analysis for overall survival showed that MAGE-D4 overexpression was independently prognostic for survival (hazard ratio 2.88, $P = 0.009$) and significantly associated with high alpha-fetoprotein concentration ($P < 0.001$), poor tumor differentiation ($P = 0.003$) and vascular invasion ($P = 0.021$). MAGE-D4b protein expression patterns were consistent with those of MAGE-D4 mRNA.
Conclusions: Overexpression of MAGE-D4 may be a predictive marker of early recurrence and mortality in patients with HCC.
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KEY WORDS: MAGE-D4; hepatocellular carcinoma; progression; recurrence

INTRODUCTION

Hepatocellular carcinoma (HCC), the fifth most common type of cancer and the third leading cause of cancer-related deaths worldwide [1,2], is associated with poor prognosis. Despite advances in diagnostic and therapeutic modalities for chronic hepatitis, the incidence of HCC is still increasing in developed countries. In Japan, HCC ranks the fourth most common cancer and the incidence rate is approximately 7 per 100,000 persons [3]. About 30–40% of patients are diagnosed during early stages, when the disease is amenable to potentially curative treatments such as surgical modalities (e.g., resection, liver transplantation) and locoregional procedures (e.g., radiofrequency ablation and trancecatheter arterial chemoembolization) [4–6]. Patients diagnosed at an advanced stage or those who progress after locoregional therapy have a dismal prognosis, owing both to the underlying liver disease and the lack of effective treatment options [7,8].

Searches for agents targeting aberrant molecular pathways involved in carcinogenesis led to the development of sorafenib, an oral multikinase inhibitor that blocks tumor angiogenesis and tumor cell proliferation [9]. Two randomized, phase III clinical trials showed that sorafenib improves overall survival in patients with advanced HCC [10,11]. Despite these initially encouraging results, it is necessary to explore new diagnostic and therapeutic methods, including additional targeted agents, to further improve outcomes in patients with HCC.

Melanoma-associated antigen (MAGE)-A1 gene, the first member of the MAGE family of cancer testis genes to be identified, is expressed on melanoma cells and is recognized by cytotoxic T lymphocytes [12]. Because MAGE proteins are expressed in germ-line cells and tumors but not in normal cells [13,14], these proteins are increasingly being utilized as targets in immunotherapy [15–19]. To date, >60 genes encoding MAGE proteins have been identified; based on their sequence and expression patterns, they have been classified as types I and II [20]. Type I MAGE genes, which encode MAGE-A, -B and -C proteins, are located on the X-chromosome; these proteins are expressed during germ cell development, but not in normal mature somatic cells [13]. By contrast, the localization and expression of type II MAGE proteins, including MAGE-D, -E, -F, -G, and -H, are less clear [15,21–23], with some studies reporting that these proteins are universally expressed in mature tissues, whereas other studies found that type II MAGE proteins have distinct expression patterns during development and in adult tissues [24]. Interestingly, both type I and II MAGE proteins were found to be overexpressed in many types of malignancies including melanoman lung cancer and breast cancer [14,16,22].

Although four MAGE-D proteins, MAGE-D1 to -D4, have been identified to date, their biological roles remain unclear [24–26]. In particular, MAGE-D4, newly discovered in 2001, has been associated with several human malignancies, including gliomas, non-small-cell

Abbreviations: HCC, hepatocellular carcinoma; RT-PCR, reverse transcription-polymerase chain reaction; MAGE-D4, melanoma-associated antigen D4.

Conflict of interest: none.

*Correspondence to: Mitsuro Kanda, MD, PhD, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, University of Nagoya, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Fax: +81-52-744-2252.
E-mail: m-kanda@med.nagoya-u.ac.jp

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lung cancers, oral squamous cell carcinomas and breast cancers [27–31], but its expression in gastroenterological cancers, including HCC, has not been reported and the functional role of the encoded protein remains unclear. We have therefore assessed the expression of MAGE-D4 in HCCs and normal liver tissue, as well as the association between MAGE-D4 expression and patient outcomes.

MATERIALS AND METHODS

Ethics

This study conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for usage of clinical samples and data, as required by the institutional review board at Nagoya University, Japan, was obtained from all patients.

Cell lines and Surgical Specimens

Nine HCC cell lines (Hep3B, HepG2, HLE, HLF, HuH1, HuH2, HuH7, PLC/PRF/5 HepG2, and SK-Hep1) were obtained from the American Type Culture Collection (Manassas, VA), stored at −80°C with cell preservative solution (Cell Banker®, Mitsubishi Chemical Medience Corporation, Tokyo, Japan) and cultured in RPMI-1640 (Sigma–Aldrich, Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. Primary HCC tissues and corresponding non-cancerous tissues were collected consecutively from 94 patients undergoing liver resection for HCC at Nagoya University Hospital between January 1998 and July 2008. Specimens were classified histologically using the 7th edition of the Union for International Cancer Control (UICC) classification [32]. Background liver status, Pugh-Child's classification, hepatitis virus infection, preoperative serum tumor markers, tumor multiplicity and maximum size, pathological findings including tumor differentiation, vascular invasion and margin status l infiltration were investigated. Mean duration of patient follow-up was 41.2 ± 36.7 months (range 0.8–147 months). Postoperative follow-up examinations included physical examination and measurement of serum tumor markers every three months, and enhanced computed tomography scan (chest and abdominal cavity) every 6 months. Treatment after recurrence was generally selected from surgery, radiofrequency ablation, trancecatheter arterial chemoembolization and chemotherapy according to tumor status and liver function.

Collected tissue samples were immediately flash frozen in liquid nitrogen and stored at −80°C until RNA extraction (28 days in average). Approximately 5 mm square of tumor samples were extracted without necrotic component and confirmed to contain more than 80% tumor cells by definition. Corresponding non-cancerous liver tissue samples were obtained from the same patient and did not any contain regenerative or dysplastic nodules. Basically, non-cancerous tissues were collected >2 cm away from the edge of the tumors.

Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The levels of MAGE-D4 mRNA expression were analyzed by quantitative real time RT-PCR. Total RNA was isolated from the HCC cell lines, primary HCC tissue samples and corresponding non-cancerous tissues using RNeasy mini kits (Qiagen, Chatsworth, CA), and 10 μg aliquots of each were reverse described to generate complementary DNAs. Quantitative real-time RT-PCR was performed with the SYBR-Green PCR core reagents kit (Applied Biosystems, Foster City, CA). The amplification protocol consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The MAGE-D4 specific primers, 5′-GGCGATCTGAGGAAGCTCAT-3′ (sense) in exon 10 and 5′-CATACTCAGGTGGGTTGCTGT′ (antisense) in exon 11, complementary to sequences in all three MAGE-D4 isoforms, MAGE-D4a, -D4b, and -D4c, were used to amplify a 91 base pair product, indicating amplicons generated by this RT-PCR reaction reflect whole MAGE-D4 mRNA. SYBR-Green emission intensity was assessed using an ABI prism 7000 Sequence Detector (Applied Biosystems). For standardization, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (TaqManR, GAPDH control reagents, Applied Biosystems) was quantified in each sample. Each of the clinical samples was assayed in triplicate and all assays included samples without template as negative controls. The level of expression of MAGE-D4 mRNA in each sample was normalized relative to the level of expression of GAPDH mRNA. Tumor tissues with the levels of MAGE-D4 mRNA >3 times higher than their corresponding non-cancerous tissues were defined as overexpressing MAGE-D4 mRNA.

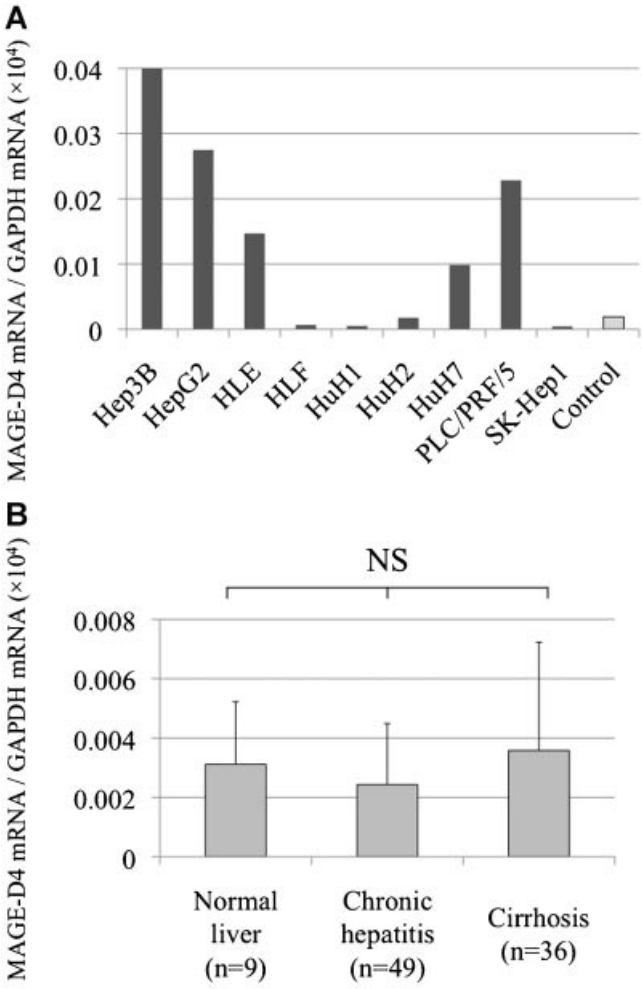

Fig. 1. **A:** MAGE-D4 expression in HCC cell lines and control (non-cancerous tissues of 94 surgical specimens) by quantitative real time RT-PCR. Expression of MAGE-D4 mRNA was markedly higher in five of the cell lines than control normal liver. **B:** Quantitation of total MAGE-D4 expression by real-time quantitative RT-PCR in non-cancerous tissues. There were no significant differences in expression among these patient groups classified by background liver status. NS, not significant.

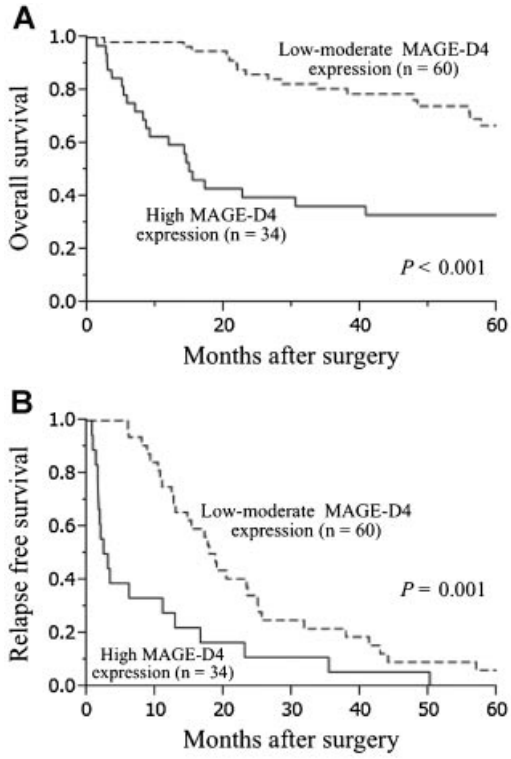

Fig. 2. Kaplan–Meier analysis of survival in 94 patients with hepatocellular carcinoma, categorized as having low-moderate and high expression of MAGE-D4. *P* value was calculated using log-rank test. **A, B:** Graphs showing that overall survival (A) and relapse free survival (B) were significantly shorter in patients with high than low-moderate MAGE-D4 mRNA expression. **B:** Relapse free survival rates, which were significantly shorter in the high than in the medium-low expression group.

Immunohistochemical Staining

We used immunohistochemical staining to investigate expression and localization of MAGE-D4b protein, which has been reported to be a dominant isoform of MAGE-D4 [30,31], in 30 representative HCCs. Formalin-fixed, paraffin-embedded tissue samples were dewaxed in xylene twice for 5 min, rehydrated in grading alcohols 100%, 90%, and 70% to H_2O for 2 min each and subsequently treated with 3% H_2O_2 to inhibit endogenous peroxidases, followed by retrieval with 10 mM citrate buffer at 95°C for 5 min, five times. The samples were incubated with Histofine SAB-PO(R) (Nichirei, Tokyo, Japan) for 5 min to limit nonspecific reactivity, and were then incubated for 1 hr with a rabbit antibody to MAGE-D4b, the dominant form of MAGE-D4b in several types of malignancy (HPA003554, Sigma Aldrich), diluted 1:1000 in ChemMatet antibody diluent (Dako). Samples were then washed with phosphate buffered saline, followed by a 10 min incubation with biotinylated secondary antibody (Histofine SAB-PO(R), Nichirei). Sections were subsequently developed for 1 min using liquid 3,3′-diaminobenzidine (DAB) as the substrate (Nichirei). Staining properties were determined using vessels as internal controls, and staining patterns were compared in HCCs and corresponding non-cancerous tissues. Presence or absence of overexpression in the cancerous tissues was mainly evaluated. To avoid subjectivity, specimens were randomized and coded before analysis by two independent observers, blinded to the status of the samples. Each observer evaluated all specimens at least twice within a given time interval to minimize intra-observer variation.

Statistical Analysis

Relative mRNA expression levels (MAGE-D4/GAPDH) in different groups were compared using the Mann–Whitney *U*-test. The associations between MAGE-D4 expression and clinicopathological parameters were evaluated using the χ^2 test. Overall and relapse free survival rates were calculated using the Kaplan–Meier method, and differences in survival curves were compared using the log-rank test. We performed multivariable regression analysis to detect prognostic factors using Cox proportional hazards models, and variables with a *P* value of <0.05 were entered into the final model. All statistical analysis was

TABLE I. Prognostic Factors in 94 Patients With Hepatocellular Carcinoma

		Univariate			Multivariable		
Variable	n	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age (≥65)	47	1.71	0.93–3.24	0.086			
Gender (male)	77	1.18	0.55–2.93	0.684			
Background liver (cirrhosis)	36	1.54	0.83–2.82	0.170			
Pugh-Child's classification (B)	7	1.27	0.31–3.53	0.702			
AFP (≥20 ng/ml)	47	1.89	1.03–3.55	0.040*	0.90	0.40–2.01	0.798
PIVKA II (≥40 mAU/ml)	54	2.04	1.09–4.00	0.026*	1.32	0.64–2.86	0.455
Tumor multiplicity (multiple)	24	1.78	0.91–3.33	0.090			
Tumor size (≥3.0 cm)	67	2.11	1.04–4.73	0.038*	1.27	0.55–3.18	0.588
Tumor differentiation (well)	28	0.58	0.27–1.15	0.121			
Growth type (invasive growth)	17	1.29	0.60–2.56	0.495			
Serosal infiltration	25	2.28	1.14–4.36	0.021*	1.54	0.73–3.13	0.248
Formation of capsule	68	0.99	0.52–2.01	0.966			
Infiltration to capsule	54	1.08	0.59–2.04	0.800			
Septum formation	34	0.92	0.50–1.76	0.797			
Vascular invasion	23	2.94	1.54–5.45	0.002*	1.92	0.92–3.92	0.081
Margin status (positive)	24	2.20	1.15–4.07	0.018*	1.62	0.82–3.13	0.164
MAGE-D4 expression (high)	34	3.26	1.76–6.04	<0.001*	2.88	1.30–6.44	0.009*

CI, confidence interval; AFP, alpha-fetoprotein; PIVKA, protein induced by vitamin K antagonists. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model.
*Statistically significant ($P < 0.05$).

performed using JMP® 10 software (SAS Institute, Inc., Cary, NC), with $P < 0.05$ defined as statistically significant.

RESULTS

Patient Characteristics

The ages of the 94 patients ranged from 34 to 84 years (64.5 ± 10.0 years, mean $\pm$ SD), and the male-to-female ratio was 77:17. Twenty-six patients had hepatitis virus B infection and 53 had hepatitis C virus. In terms of background liver, number of patients with normal liver, chronic hepatitis and cirrhosis were nine, 49 and 36, respectively. Eighty-seven patients were in the Pugh-Child's class A and 12 patients were in class B. When classified by the 7th edition of the UICC classification, 11, 44, 29, and 10 patients were in stages I, II, III, and IV, respectively.

MAGE-D4 Expression in HCC Cell Lines

Quantitative real-time RT-PCR analysis of MAGE-D4 expression in nine HCC cell lines showed that the normalized level of MAGE-4D was higher in five of these cell lines, Hep3B, HepG2, HLE, HuH7, and PLC/PRF/5, than in non-cancerous liver tissues of the surgical specimens (median value) as reference samples (Fig. 1A).

Correlation Between MAGE-D4 Expression and Background Liver in Non-cancerous Tissue Samples of HCC Patients

Mean relative mRNA expression level, MAGE-D4/GAPDH ($\times 10^4$), of 94 non-cancerous liver tissues were 0.00293 ± 0.00281 (standard deviation; SD). Non-cancerous tissue samples of HCC patients were classified into normal liver (n = 9), chronic hepatitis (n = 49), and cirrhosis (n = 36) by histopathologic examination, and relative MAGE-D4 mRNA expression levels were 0.00312 ± 0.00211, 0.00243 ± 0.00206 and 0.00358 ± 0.00365, respectively (mean $\pm$ SD). No difference in the level of MAGE-D4 mRNA was found between these three types, suggesting that the expression of MAGE-D4 mRNA in non-cancerous liver is not affected by inflammation or fibrosis (Fig. 1B). Additionally, 23 patients with relatively high (the top quartile) MAGE-D4 mRNA expression level in non-cancerous tissues were compared with the others, however, there was no significant association with age, gender, background liver and hepatitis virus (data not shown).

Prognostic Impact of Overexpression of MAGE-D4 mRNA

Overexpression of MAGE-D4 mRNA was observed in tumor samples from 34 of the 94 (36.2%) patients with HCC. Median overall survival (14.8 vs. 118 months, $P < 0.001$, Fig. 2A) and relapse-free survival (2.7 vs. 18.3 months, $P < 0.001$, Fig. 2B) were significantly shorter in patients with high MAGE-D4 mRNA expression than in those with low to moderate expression.

Univariate analysis showed that alpha-fetoprotein (AFP) $\geq$0 ng/ml, protein induced by Vitamin K antagonists (PIVKA) II $\geq$40 mAU/ml, tumor size $\geq$3.0 cm, serosal infiltration, vascular invasion, positive margin status, and high MAGE-D4 mRNA expression were significantly associated with shorter overall survival. Multivariate analysis showed that high MAGE-D4 expression was the only independent prognostic factor for overall survival (hazard ratio 2.88, $P = 0.009$, Table I).

Association Between MAGE-D4 mRNA Expression and Clinicopathological Factors

We found that overexpression of MAGE-D4 mRNA in HCCs was significantly associated with high AFP concentration ($P < 0.001$), poor tumor differentiation ($P = 0.003$) and vascular invasion ($P = 0.021$, Table II).

TABLE II. Association Between Expression of MAGE-D4 mRNA and Clinicopathological Parameters in 94 Patients With Hepatocellular Carcinoma

Clinicopathological parameters	High MAGE-D4 expression in tumor tissue (n)	Others (n)	*P*-value
Age			
<65 year	17	30	1.000
≥65 year	17	30	
Gender			
Male	26	51	0.308
Female	8	9	
Background liver			
Normal liver	2	7	0.515
Chronic hepatitis	17	32	
Cirrhosis	15	21	
Pugh-Child's classification			
A	31	56	0.705
B	3	4	
Hepatitis virus			
Absent	5	10	0.748
HBV	11	15	
HCV	18	35	
AFP (ng/ml)			
<20	7	40	<0.001*
≥20	27	20	
PIVKA II (mAU/ml)			
<40	12	28	0.282
≥40	22	32	
Tumor multiplicity			
Solitary	23	47	0.258
Multiple	11	13	
Tumor size			
<3.0 cm	7	20	0.182
≥3.0 cm	27	40	
Differentiation			
Well	4	24	0.003*
Moderate to poor	30	36	
Growth type			
Expansive growth	27	50	0.637
Invasive growth	7	10	
Serosal infiltration			
Absent	21	48	0.058
Present	13	12	
Formation of capsule			
Absent	11	15	0.447
Present	23	45	
Infiltration to capsule			
Absent	15	25	0.818
Present	19	35	
Septum formation			
Absent	22	38	0.894
Present	12	22	
Vascular invasion			
Absent	21	50	0.021*
Present	13	10	
Margin status			
Negative	23	47	0.258
Positive	11	13	
UICC pathological stage†			
I, II	17	38	0.209
III, IV	17	22	

HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein; PIVKA, protein induced by vitamin K antagonists.
*Statistically significant ($P < 0.05$).
†Classified by the 7th edition of the Union for International Cancer Control (UICC) classification.

Immunohistochemical Staining

The level of expression of MAGE-D4b protein was evaluated immunohistochemically in 30 representative samples, including those showing overexpressed, underexpressed, and equivalent MAGE-D4 mRNA in cancerous relative to corresponding non-cancerous tissues. Strong expression of MAGE-D4b protein was observed in the membrane and cytoplasm of cancerous tissues showing overexpression of MAGE-D4 mRNA (Fig. 3A). By contrast, MAGE-D4b protein was undetectable in all tumors showing equivalent (Fig. 3B) and reduced expression of MAGE-D4 mRNA relative to adjacent, non-cancerous liver tissue.

DISCUSSION

Expression of human MAGE family genes has recently been reported in several types of cancer [12,15,20]. MAGE-A1, the first of these proteins discovered, is a tumor-specific antigen [12,33] extensively studied as an attractive target for anti-tumor immunotherapy [17–19,24]. To date, many MAGE family genes have been identified and classified as types I and II, based on differences in gene structure and tissue-specific gene expression [15,20,13].

The MAGE-D genes have been classified as type II MAGE genes. One of these genes, MAGE-D4 (previously known as MAGE-E1), is located on chromosome Xp11 and has three splice forms: MAGE-D4a, -D4b, and -D4c [27,34]. The MAGE-D4 gene is specifically expressed in normal brain and ovary, with high expression levels observed in several malignancies [27–31].

This study was designed to analyze the expression of MAGE-D4 in HCCs. High expression of MAGE-D4 mRNA was observed in five of nine HCC cell lines, suggesting that the MAGE-D4 genes are associated with the development of HCC cells. In analyzing MAGE-D4 mRNA expression in HCC tissue samples and adjacent normal tissue, we found that the expression of this gene was similar in non-cancerous tissue samples from patients with normal liver, chronic hepatitis, and cirrhosis, indicating that MAGE-D4 expression was not affected by the degree of inflammation or fibrosis. In contrast, aberrant expression of several cancer-related genes has been reported to depend on background liver status, indicating that chronic inflammation and fibrosis may have affected their level of gene expression [35,36]. Because MAGE-D4 expression was not affected by the status of background liver, the overexpression of this gene in HCC samples suggests that MAGE-D4 may play a role specific to carcinogenesis and cancer progression. We also found that overexpression of MAGE-D4 correlated with high AFP concentration, poor tumor differentiation and vascular invasion. Kaplan–Meier survival analysis showed that overexpression of MAGE-D4 was associated with early HCC recurrence and poor prognosis after surgical resection, and multivariate analysis showed that MAGE-D4 expression was the only factor independently prognostic for survival. Taken together, these findings suggest that MAGE-D4 may play an important role in HCC progression and could serve as a strong prognostic marker of HCC.

Three alternative spliced variants of MAGE-D4 have been identified, MAGE-D4a, -D4b, and -D4c with the expression patterns of MAGE-D4a and/or b being unique, whereas that of MAGE-D4c is similar to that of other MAGE family genes [27]. The intracellular distributions of MAGE-D4a and -D4b, but not MAGE-D4c, were similar to those of tubulin, with these proteins concentrated in the central spindle and in the midbody from telophase to the postmitotic phase [28]. Thus, MAGE-D4 may colocalize with tubulin in a cell cycle specific manner and be involved in cell division.

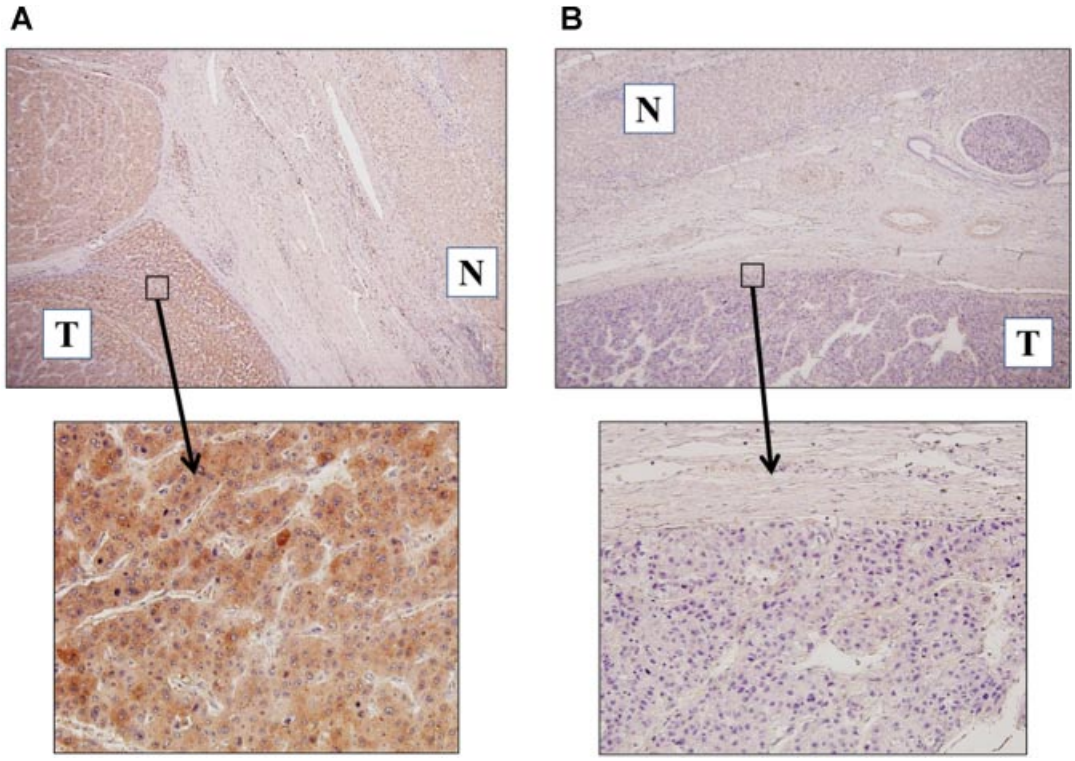

Fig. 3. Immunohistochemical staining of MAGE-D4b protein in representative patients with HCC tiumors. **A:** Unequivocal expression of MAGE-D4b protein observed only in the cancerous tissue components of patients showing overexpression of MAGE-D4 mRNA (upper 40×, lower 200×). **B:** Absence of MAGE-D4b expression from cancerous and non-cancerous tissue components in patients without overexpression of MAGE-D4 mRNA (upper 40×, lower 100×). N, non-cancerous tissue component; T, tumor tissue component.

Because MAGE-D4b has been reported to be the clinically dominant isoform in breast cancers and oral squamous cell carcinoma [30,31], we compared the expression patterns of MAGE-D4b protein with that of MAGE-D4 mRNA. Consistent with findings of quantitative real time PCR, immunohistochemical staining showed that MAGE-D4b protein was expressed only in HCC samples showing overexpression of MAGE-D4 mRNA, indicating that MAGE-D4b may be the clinically dominant isoform in HCC as well in breast cancers and oral squamous cell carcinomas. Moreover, the results showing weak MAGE-D4b protein expression in non-cancerous liver tissues add liver tissues to the list of normal adult tissues that express little MAGE-D4.

This study is limited by its lack of functional analysis of the MAGE-D4 gene. Further studies including pathway analysis in hepatocarcinogenesis and functional analysis are expected to clarify the molecular mechanisms underlying the biological activities of MAGE-D4 in HCC.

CONCLUSIONS

The expression of MAGE-D4 mRNA is correlated with early recurrence and poor prognosis in patients with HCC. MAGE-D4 may serve as a novel prognostic marker for HCC.

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