XAGE-1b expression is associated with the diagnosis and early recurrence of hepatocellular carcinoma

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Received October 18, 2013; Accepted June 26, 2014

DOI: 10.3892/mco.2014.336

Abstract. *XAGE-1b* is a 470 bp transcript of the *XAGE-1* gene, which belongs to the cancer-testis antigens that exhibit a restricted pattern of expression in normal tissues. Recently, the expression of *XAGE-1b* has been shown to be frequent in patients with hepatocellular carcinoma (HCC). However, the underlying mechanism is not fully understood. To investigate the role of *XAGE-1b* in HCC diagnosis and postoperative evaluation, the expression level of *XAGE-1b* was first examined in the tissue and peripheral blood of HCC patients and controls by using quantitative polymerase chain reaction. Subsequently, the associations between *XAGE-1b* and the clinical variables were assessed using χ^2 or Kaplan-Meier tests. The data showed that HCC tissues had increased *XAGE-1b* expression when compared to paired non-tumorous tissues. The blood samples from the HCC patients showed upregulated *XAGE-1b* mRNA, as compared to non-HCC patients. The patients with portal vein tumor thrombus or higher tumor-node metastasis stages (II~IV) were more likely to have increased levels of *XAGE-1b* mRNA. Furthermore, the 1-year recurrence rate of the patients with a high level of *XAGE-1b* mRNA was significantly greater compared to the patients with a low level. All these findings indicate that *XAGE-1b* is associated with the aggressive biological behavior of HCC cells and it may be a potential biomarker for HCC diagnosis and prognosis.

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Key words: *XAGE-1b*, hepatocellular carcinoma, diagnosis, prognosis, cancer biomarker

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide and is the second leading cause of cancer-related fatalities in China (1,2). Resection or liver transplantation are the optimal treatments for a potential cure (3). Although advances in early diagnosis, surgical techniques, imaging modalities and perioperative management have improved the long-term survival rate in certain patients, the high incidence of intrahepatic and/or extrahepatic recurrence postoperatively remains a major challenge in HCC therapy (4). Therefore, it is of great importance to expand the current knowledge of novel diagnostic biomarkers for early diagnosis or evaluation of metastatic potentials.

A category of tumor-associated antigens, known as cancer-testis (CT) antigens, has recently been proposed as a new cluster of liver tumor biomarkers as members of the CT antigens have been shown to aid in screening and evaluating HCC (5). CT antigens have an expression pattern that is predominantly restricted to testis in normal tissues, however, they are expressed in numerous types of cancers, including melanomas, lung tumors, bladder carcinomas and liver cancers (6). The present collection of CT antigens contains 44 distinct CT-antigen families, a number of which have multiple members, including melanoma-associated antigen (MAGEA), prostate associated gene (PAGE) and G antigen (GAGE) (7). In HCC patients, a number of CT antigens have been found to be expressed with a high percentage and specificity in tumor tissue or peripheral blood. MAGE-1, synovial sarcoma X-1 (SSX-1), CTp11 and HCA587 were detectable in >50% HCC tissue samples and ~90% of HCC tissues positively expressed at least one member of the CT antigens (8). Additionally, MAGE-1, SSX-1 and CTp11 were also detectable in ~30% peripheral blood samples from HCC patients (8). Furthermore, SSX-2, SSX-5, NY-ESO-1 and MAGE-C2 were also frequently expressed in HCC samples (9-11).

Despite the growing evidence for a link between CT antigens and HCC, the role of the XAGE family remains poorly defined. *XAGE* was identified by 'homology walking' using the *PAGE4* sequence. Similar to the majority of the CT antigen family members, the *XAGE-1* gene is located on the

X-chromosome, and it is highly expressed in germ cells and frequently expressed in several cancers, including Ewing's sarcoma, rhabdomyosarcoma, breast cancer and germ cell tumor (12). *XAGE-1b*, a 470 bp transcript of the *XAGE-1* gene, was first isolated from melanoma metastases. There are four alternative splicing variants, *XAGE-1a*, *b*, *c* and *d*, and of these *XAGE-1b* mRNA is dominantly expressed in cancer (13). The open reading frame of this variant encodes a protein of 81 amino acids (14). Previous studies have shown that *XAGE-1b* is frequently expressed in acute leukemia, lung cancer, gastric cancer and HCC tissues (13,15). These studies indicate that a high-level of *XAGE-1b* expression may confer significant biological potential. However, the biological function of *XAGE-1b* in these tumors remains poorly understood.

In the present study, the correlation was evaluated between the expression of *XAGE-1b* and the clinical parameters, including age, gender, tumor size, tumor-node metastasis (TNM) staging and serum α-fetoprotein (AFP) level. Subsequently, the association of the serum *XAGE-1b* mRNA level with regards to the HCC diagnosis was assessed.

Materials and methods

Patients and specimens. Fifty-nine HCC and adjacent normal liver tissue specimens were surgically obtained from patients at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China). Peripheral blood was obtained from 108 HCC patients, 34 benign liver tumor patients, 23 liver cirrhosis patients and 45 healthy donors. Postoperative sera samples were collected from 39 patients after 1, 7 and 30 days. The clinical staging of tumors was determined according to the TNM staging systems. The histological grade of tumor differentiation was assigned using the Edmondson-Steiner grading system (16). Informed consent was obtained from each patient for the use of liver specimens and sera in the study. The study was approved by the Ethics Committee of the Second Military Medical University.

RNA extraction and quantitative polymerase chain reaction (qPCR). Tissues from cancer and normal liver samples were obtained during the surgery. All the tissue samples were snap-frozen in liquid nitrogen within 30 min and stored at -80˚C. The tissue sample RNAs were isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and peripheral blood sample RNAs were isolated by QIAamp RNA Blood Mini kits (Qiagen, Hilden, Germany). A total of 2 μg total RNA was synthesized into complementary DNA (cDNA) with random primers following the manufacturer's instructions (Fermantas, Vilnius, Lithuania). qPCR was performed using a standard TaqMan PCR kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The relative expression levels of *XAGE-1b* mRNA were compared to the levels of the reference gene [glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)] by the comparative cycle threshold method: Fold difference = $2^{-(\Delta\Delta Ct)}$. The primers used were *GAPDH*-forward, 5'-GGGCTGCTTTTAACT CTGGTAAAG-3' and *GAPDH*-reverse, 5'-CCATGG GTGGAATCATATTGG-3'; probe, FAM-CCTCAA CTACATGGTTTAC-MGB; *XAGE-1b*-forward, 5'-GCT GAAAGTCGGGATCCTACA-3' and *XAGE-1b*-reverse, 5'-CTTCCATGTCGCGCACTG-3'; and probe, TET-CTG GGCAGCAGACAG-MGB. To analyze the *XAGE-1b* mRNA expression, the recombinant pAdTrack-1b vector containing *XAGE-1b* or the T-GAPDH vector containing reference gene *GAPDH* cDNA were constructed by PCR cloning and were used as standard samples. The expression levels of *XAGE-1b* mRNA were calculated by dividing the copy number of *GAPDH* mRNA by that of *GAPDH* mRNA.

Follow-up. The patients were assessed every 2-3 months during the 3 years. The diagnostic criteria for HCC recurrence were the same as for preoperative criteria. Among the 59 HCC patients, 18 patients succumbed or had HCC recurrence within 6 months after the surgery. These patients were not included in the follow-up study.

Statistical analysis. All the statistical analyses were performed with SPSS version 10.0 software (SPSS, Inc., Chicago, IL, USA). The χ^2 test was used to analyze the correlation between the *XAGE-1b* expression in the HCC tissue samples and clinicopathological variables. The associations with the continuous variables were tested using the Wilcoxon rank-sum test. Kaplan-Meier analysis was used to determine the correlation between recurrence and *XAGE-1b* expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Dysregulation of XAGE-1b expression in human HCC tissues. To evaluate the potential role of *XAGE-1b* in human HCC, the mRNA expression level of *XAGE-1b* was first examined in specimens of tumor and non-tumorous hepatic tissues of 59 HCC patients. As shown in Table I, the 59 patients were categorized into two groups based on the mRNA expression levels of *XAGE-1b*: Upregulation (known as positive) (38/59, 64.4%) and downregulation (21/59, 35.6%) in HCC tissues (known as negative), indicating that *XAGE-1b* is frequently upregulated in HCC patients. The *XAGE-1b* mRNA expression was also analyzed in 14 benign liver tumors, including five focal nodularhyperplasia, three hepatic angiomyolipoma, four hemangioma, one inflammatory pseudotumor and one liver cyst. None of the benign liver tumors exhibited *XAGE-1b* upregulation. The elevated expression of *XAGE-1b* was also observed in intrahepatic cholangiocarcinoma (2/6) and liver metastases from colorectal cancer tissues (1/6), whereas it was not observed in liver metastases from breast cancer and certain digestive system cancers, including gastric cancer, pancreatic cancer and gastric stromal tumor.

Association of XAGE-1b expression with clinicopathological characteristics. The clinical features of the 59 HCC patients are summarized in Table I. The correlation between the *XAGE-1b* expression level in HCC tissues and the clinicopathological characteristics was further assessed by the χ^2 test. The data indicated that a high expression of *XAGE-1b* was significantly correlated with portal vein tumor thrombus (PVTT) and TNM stage ($P<0.05$). The upregulated levels of *XAGE-1b* mRNA were found in 77.4% of patients with PVTT and in 50% of patients without PVTT (Table I). Additionally, only 47.8% of patients in stage I exhibited *XAGE-1b* upregulation, whereas

Table I. Association between *XAGE-1b* and clinicopathological features.

Variable	Positive, n (n=38)	Negative, n (n=21)	P-value	χ^2	Note
Gender					
Male	33	15			
Female	5	6	0.1445	2.1184	
Age, years					
<40	6	8	0.0609	3.5125	<40 vs. ≥40 and <60
≥40 and <60	25	10	0.9299	0.0077	≥40 and <60 vs. ≥60
≥60	7	3	0.1883	1.7310	<40 vs. ≥60
Diameter, cm					
≥5	21	19			
<5	17	12	0.6138	0.2545	
Tumor number					
Single	27	16			
Multiple	11	5	0.6708	0.1806	
Encapsulation					
Yes	10	4			
No	28	17	0.5298	0.3948	
PVTT					
Yes	24	7			
No	14	14	0.0281	4.8248	
Differentiation					
I-II	3	2			
III-IV	35	19	0.8297	0.0463	
TNM					
I	11	12			
II	15	4			
III	10	5			
IV	2	0	0.0335	4.5205	I vs. IV
Liver cirrhosis					
Yes	19	12			
No	19	9	0.5988	0.2767	
HBsAg					
Positive	31	19			
Negative	7	2	0.3627	0.8282	
AFP					
Positive	24	15			
Negative	14	6	0.5205	0.4129	

PVTT, portal vein tumor thrombus; TNM, tumor-node metastasis; HBsAg, surface antigen of the hepatitis B virus; AFP, α-fetoprotein.

75% of patients in stage II~IV showed *XAGE-1b* upregulation. Taken together, these results strongly indicate that *XAGE-1b* may be involved in the invasive and metastatic features of HCC cells.

Association of XAGE-1b expression with prognosis. Of the 59 patients, 18 succumbed or suffered from recurrent HCC within 6 months after the hepatectomy. The remaining 41 patients were followed up and analyzed in the study. At the end of 1 year, recurrence was found in 13 patients. Approximately 73.3% of patients with increased *XAGE-1b* mRNA expression suffered from HCC recurrence, whereas only 28.6% of patients with decreased *XAGE-1b* mRNA expression had recurrence (Table II). However, at the end of 3 years, the patients with increased *XAGE-1b* mRNA expression had no significantly different recurrence rate compared to those with decreased *XAGE-1b* expression (data not shown). This indicates that *XAGE-1b* is a prognostic factor of early recurrence.

Table II. Association between *XAGE-1b* mRNA and recurrence at 1 year.

HCC	Positive, n (n=26)	Negative, n (n=15)	P-value	χ^2
Recurrence	11	2		
Non-recurrence	4	5	0.0467	3.9559

HCC, hepatocellular carcinoma.

Table III. Serum *XAGE-1b* mRNA levels in hepatocellular carcinoma (HCC) and control patients.

Group	Patients, n	*XAGE-1b* levels P50 (P25, P75) x10^{-5}	Kruskal-Wallis test (χ^2)	Wilcoxon test (Z)	P-value
HCC (i)	108	3.80 (0.90, 12.6)		(i) vs. (ii) -6.8035	<0.01
				(i) vs. (iii) -4.3211	<0.01
				(i) vs. (iv) -7.7728	<0.01
Benign liver tumor (ii)	34	0 (0, 0)	97.6639 (P<0.01)	(ii) vs. (iii) 2.4216	0.0155
				(ii) vs. (iv) -0.6930	0.4833
Liver cirrhosis (iii)	23	0 (0, 0.56)		(iii) vs. (iv) 2.2434	0.0249
Health control (iv)	45	0 (0, 0)			

Serum XAGE-1b expression in human HCC patients. Serum tumor markers have been widely studied in HCC diagnosis and prognosis (17-20). Thus, the serum *XAGE-1b* levels and the associations of its expression with HCC diagnosis and prognosis were further analyzed. The serum *XAGE-1b* mRNA levels were measured in 108 HCC patients, 34 benign liver tumor, 23 liver cirrhosis and 45 healthy controls. The *XAGE-1b* mRNA expression was significantly higher in HCC patients compared to the other three groups (Table III), indicating that *XAGE-1b* can be used as a serum biomarker for diagnosis of HCC. The combination of serum AFP and *XAGE-1b* has a sensitivity rate of 91.7% in HCC diagnosis (Table IV). The dynamic change of the *XAGE-1b* mRNA expression was investigated in the peripheral blood of 39 HCC patients prior and subsequent to hepatectomy. The data showed that serum *XAGE-1b* was strongly reduced by the 7th and 30th day postoperation (Table V). Thus, serum *XAGE-1b* mRNA is a potential serum tumor marker for HCC diagnosis and prognosis.

Table IV. Sensitivity of AFP and *XAGE-1b* as biomarkers for diagnosis in hepatocellular carcinoma patients.

Biomarker	Positive, n	Negative, n	Positive rate, %
AFP	57	51	52.80
XAGE-1b mRNA	86	22	79.60
AFP+*XAGE-1b* mRNA	99	9	91.70

AFP, α-fetoprotein.

Discussion

XAGE-1 is a member of the CT antigen family that is predominantly expressed in testis and is present in a wide range of cancers, including breast, prostate, lung, ovarian, melanoma and glioblastoma (6). In the present study, *XAGE-1b* was shown to be frequently upregulated in HCC tissues as compared to paired non-tumorous tissues. The data are in accordance with a previous study that showed the high expression of *XAGE-1b* had a high frequency in HCC but was undetectable in all the adjacent non-HCC tissues (21). A differential expression of *XAGE-1b* mRNAs was also observed in human primary liver cancer, benign liver tumor and certain digestive system cancers. Notably, the serum *XAGE-1b* mRNA is a more sensitive marker for HCC when compared to serum AFP and its specificity is increased when combined with serum AFP. Those findings strongly indicate that *XAGE-1b* is a potential diagnostic biomarker to differentiate HCC from chronic liver disease and benign liver tumors.

XAGE-1b was shown to be a risk factor affecting PVTT, TNM staging and the 1-year recurrence. PVTT in HCC patients is a well-known major complication that is correlated with a poor prognosis (22). The patients with PVTT or higher TNM stages (II~IV) were found to be more likely to have increased *XAGE-1b* mRNA levels. Additionally, the recurrence rates at 1 year in patients with *XAGE-1b* overexpression and underexpression (73.3% and 28.6%, respectively) indicated that HCC recurrence is more likely to occur in patients with overexpressed *XAGE-1b*. Thus, these findings strongly suggest that *XAGE-1b* is associated with the aggressive biological

Table V. Preoperative and postoperative serum *XAGE-1b* mRNA levels in 39 hepatocellular carcinoma patients.

Group	*XAGE-1b* levels P50 (P25, P75) x10^{-5}	Kruskal-Wallis test (χ^2)	Wilcoxon test (Z)	P-value
Preoperative (i)	3.88 (1.42, 2.24)		(i) vs. (ii) -1.5918	0.1161
			(i) vs. (iii) -5.2588	<0.01
			(i) vs. (iv) -4.9533	<0.01
Postoperative (ii) (day 1)	2.67 (3.11, 6.23)	42.7301 (P<0.01)	(ii) vs. (iii) 3.4704	<0.01
			(ii) vs. (iv) -3.7439	<0.01
Postoperative (iii) (day 7)	0.13 (0, 0.90)		(iii) vs. (iv) -1.1464	0.2516
Postoperative (iv) (day 30)	0 (0, 0.55)			

behavior of HCC cells and may be an indicator of poor prognosis. However, no differences were found between the two groups and the 3-year recurrence rate. The prognostic value appears limited due to the limited number of studied patients.

In addition to the diagnostic and prognostic value in HCC patients, the XAGE-1b protein is present in 1 hepatic sarcoma and 1 hepatic malignant fibrous histiocytoma (MFH) by immunohistochemistry (data not shown). Presently, a biomarker that reliably detects these two tumors is not available. Therefore, studying XAGE-1b in patients with hepatic sarcoma or MFH in the near future is required.

In conclusion, the present study assessed the correlation between the tissue or serum level of *XAGE-1b* and the clinicopathological features, diagnosis and recurrence in HCC patients. *XAGE-1b* was demonstrated to play a significant role in HCC cell proliferation. These results strongly indicate that *XAGE-1b* expression in HCC is a potential biomarker for diagnosis and prognosis evaluation.

Acknowledgements

The present study was supported by the Innovation Program of Shanghai Municipal Education Commission (grant no. 12ZZ077), the National Science Foundation of China (grant no. 31300126) and the Natural Science Foundation of Anhui Province (grant nos. 1208085QC71 and KJ2014A161).

References

1. Huether A, Höpfner M, Baradari V, Schuppan D and Scherübl H: EGFR blockade by cetuximab alone or as combination therapy for growth control of hepatocellular cancer. Biochem Pharmacol 70: 1568-1578, 2005.
2. Jia HL, Ye QH, Qin LX, *et al*: Gene expression profiling reveals potential biomarkers of human hepatocellular carcinoma. Clin Cancer Res 13: 1133-1139, 2007.
3. Budhu A, Jia HL, Forgues M, *et al*: Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 47: 897-907, 2008.
4. Pan HW, Ou YH, Peng SY, *et al*: Overexpression of osteopontin is associated with intrahepatic metastasis, early recurrence, and poorer prognosis of surgically resected hepatocellular carcinoma. Cancer 98: 119-127, 2003.
5. Grizzi F, Franceschini B, Hamrick C, Frezza EE, Cobos E and Chiriva-Internati M: Usefulness of cancer-testis antigens as biomarkers for the diagnosis and treatment of hepatocellular carcinoma. J Transl Med 5: 3, 2007.
6. Egland KA, Kumar V, Duray P and Pastan I: Characterization of overlapping XAGE-1 transcripts encoding a cancer testis antigen expressed in lung, breast, and other types of cancers. Mol Cancer Ther 1: 441-450, 2002.
7. Simpson AJ, Caballero OL, Jungbluth A, Chen YT and Old LJ: Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer 5: 615-625, 2005.
8. Sun YM, Zhang Y, Zeng LQ, *et al*: Broad profiling of DNA-binding transcription factor activities improves regulatory network construction in adult mouse tissues. J Proteome Res 7: 4455-4464, 2008.
9. Riener MO, Wild PJ, Soll C, *et al*: Frequent expression of the novel cancer testis antigen MAGE-C2/CT-10 in hepatocellular carcinoma. Int J Cancer 124: 352-357, 2009.
10. Xu Y, Zhang M, Wang Y, *et al*: A systems approach to mapping transcriptional networks controlling surfactant homeostasis. BMC Genomics 11: 451, 2010.
11. Wu LQ, Lu Y, Wang XF, Lv ZH, Zhang B and Yang JY: Expression of cancer-testis antigen (CTA) in tumor tissues and peripheral blood of Chinese patients with hepatocellular carcinoma. Life Sci 79: 744-748, 2006.
12. Brinkmann U, Vasmatzis G, Lee B and Pastan I: Novel genes in the PAGE and GAGE family of tumor antigens found by homology walking in the dbEST database. Cancer Res 59: 1445-1448, 1999.
13. Sato S, Noguchi Y, Ohara N, *et al*: Identification of XAGE-1 isoforms: predominant expression of XAGE-1b in testis and tumors. Cancer Immun 7: 5, 2007.
14. Zendman AJ, van Kraats AA, den Hollander AI, Weidle UH, Ruiter DJ and van Muijen GN: Characterization of XAGE-1b, a short major transcript of cancer/testis-associated gene XAGE-1, induced in melanoma metastasis. Int J Cancer 97: 195-204, 2002.
15. Iimuro Y and Fujimoto J: TLRs, NF-κB, JNK, and liver regeneration. Gastroenterol Res Pract 2010: 598109, 2010.
16. Edmondson HA, Steiner PE: Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. Cancer 7: 462-503, 1954.
17. Zinkin NT, Grall F, Bhaskar K, *et al*: Serum proteomics and biomarkers in hepatocellular carcinoma and chronic liver disease. Clin Cancer Res 14: 470-477, 2008.
18. Oka H, Saito A, Ito K, *et al*; Collaborative Hepato-Oncology Study Group of Japan: Multicenter prospective analysis of newly diagnosed hepatocellular carcinoma with respect to the percentage of *Lens culinaris* agglutinin-reactive alpha-fetoprotein. J Gastroenterol Hepatol 16: 1378-1383, 2001.
19. Shimauchi Y, Tanaka M, Kuromatsu R, *et al*: A simultaneous monitoring of *Lens culinaris* agglutinin A-reactive alpha-fetoprotein and des-gamma-carboxy prothrombin as an early diagnosis of hepatocellular carcinoma in the follow-up of cirrhotic patients. Oncol Rep 7: 249-256, 2000.
20. Qin LX and Tang ZY: Recent progress in predictive biomarkers for metastatic recurrence of human hepatocellular carcinoma: a review of the literature. J Cancer Res Clin Oncol 130: 497-513, 2004.
21. Gong L, Peng J, Cui Z, *et al*: Hepatocellular carcinoma patients highly and specifically expressing XAGE-1 exhibit prolonged survival. Oncol Lett 1: 1083-1088, 2010.
22. Guo W, Xue J, Shi J, *et al*: Proteomics analysis of distinct portal vein tumor thrombi in hepatocellular carcinoma patients. J Proteome Res 9: 4170-4175, 2010.