

Hepatocellular carcinoma patients highly and specifically expressing XAGE-1 exhibit prolonged survival

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Abstract. XAGE-1 is classified into the group of a new family of cancer-testis antigens (CTA) and has the four transcript variants of XAGE-1a, XAGE-1b, XAGE-1c and XAGE-1d. Immunohistochemistry was used to investigate the expression of XAGE-1 transcript variants in Chinese patients with hepatocellular carcinoma (HCC). Reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR were used to analyze XAGE-1 gene expression, and XAGE-1 protein expression was examined by immunohistochemistry. Furthermore, the clinical correlation of XAGE-1 expression was analyzed. The expression of the XAGE-1 mRNA was investigated in the tissues of 96 HCC patients and all XAGE-1 isoforms were detected in these tissues. Three types of XAGE-1 transcript variants (XAGE-1b, XAGE-1c and XAGE-1d) showed high specific and frequent expression in HCC tissues, with the positive expression rate of XAGE-1b, XAGE-1c and XAGE-1d being 41.7% (40/96), 15.6% (15/96) and 26.0% (25/96), respectively. XAGE-1b was the dominant type, but none of the three were detected in adjacent non-HCC tissues. Only 2 cases of XAGE-1a mRNA expression were observed. Moreover, XAGE-1 protein was detected in 39 of 96 HCC patients, but none in the adjacent non-cancerous tissue and normal liver tissue. No relationship was found between the expression of XAGE-1 and clinical parameters, such as age, gender, tumor size, TNM staging, serum AFP level and infection with hepatitis virus. Patients with XAGE-1b-positive transcript variant exhibited shorter 2-year survival times. The high frequency and specificity of XAGE-1, particularly XAGE-1b, in HCC indicates that their products may predict the prognosis of HCC patients and be novel targets for antigen-specific immunotherapy to HCC.

Introduction

Human hepatocellular carcinoma (HCC) is a common malignancy, with high incidence and mortality rates worldwide, particularly in East Asia (1). Hepatic surgery is the normal treatment and has undergone improvement; however, recurrence and metastasis rates remain high (2,3). Therefore, it is crucial to identify and establish effective novel approaches to treat HCC. In the past two decades, immunotherapy has proven to be a treatment strategy, and active immunotherapy approaches using specific vaccines appear to have potential in the treatment of HCC patients.

In recent years, a new family of tumor-specific antigens has been identified, known as cancer/testis antigens (CTA). CTA is a family that includes various types of tumor-specific antigens primarily expressed in primitive germ cells, spermatogonia and certain human tumors. The malignant transformation of these antigens is frequently associated with the activation or depression of genes, resulting in the expression of the antigens. More than 50 types of CTA have been identified thus far, including MAGE, GAGE, PAGE, NY-ESO-1, SSX, SPANX and XAGE (4-8).

XAGE-1 was originally identified by database mining of expressed sequence tags and belongs to a new family of CTA with an expression pattern that is limited to the germ cells of the testis and a variety of neoplastic tissues with immunogenicity (9,10), but is abundantly expressed in breast, prostate and lung cancer as well as in Ewing's sarcomas and rhabdomyosarcomas (11-14). The XAGE-1 gene lies on the X chromosome and its encoded proteins have strong homology with members of the GAGE/PAGE family in the COOH terminus. XAGE-1 expression in testis and cancer tissues is regulated by methylation of the CpG island in the gene promoter (15). There are 4 transcript variants of XAGE-1, i.e., XAGE-1a, XAGE-1b, XAGE-1c and XAGE-1d (10,13,16), with XAGE-1b and XAGE-1d being specifically expressed in lung and prostate cancer. These variants are immunogenic and can therefore be used as immunotherapy targets. However, only limited research has been performed on XAGE-1 in HCC. The present study showed that XAGE-1b was expressed strongly with a high frequency in HCC, while XAGE-1d relatively less strongly with a lower frequency. Additionally, more information is provided on this cancer antigen family and its potential use in the diagnosis of or immunotherapy for HCC.

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Materials and methods

Patients and specimens. A total of 96 HCCs and corresponding adjacent non-cancerous liver tissues from patients at Peking University People's Hospital, China, were surgically resected and dissected into sections of $\sim 0.3 \times 0.3 \times 0.3$ cm and immediately stored in liquid nitrogen. Written informed consent was obtained from each patient for the use of the specimens and their clinicopathological characteristics. The study was approved by the Ethics Committee of Peking University People's Hospital. HCC diagnosis was confirmed by pathological examination and the TNM stage was determined on the basis of the criteria issued by UICC in 2002.

Total RNA isolation and cDNA synthesis. Total RNA was isolated from the frozen tumors and adjacent non-cancerous liver specimens using RNeasy mini kit (Qiagen, Hilden, Germany). A total of 2 μ g was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). In addition to 200 units M-MLV, the reaction mixture consisted of 1 μ l oligo (dT)₁₅ as a primer (Promega), 0.5 units RNase ribonuclease inhibitor (Promega), 4 μ l of RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂ and 50 mM DTT) and 1 μ l of 10 mM dNTPs (Pharmacia), with DEPC-treated water added to a final volume of 20 μ l. The mixture was incubated for 5 min at 70°C and 5 min on ice, 60 min at 42°C and 5 min at 95°C. The cDNAs were tested for integrity by amplifying the GAPDH gene.

Reverse transcription-polymerase chain reaction (RT-PCR) for XAGE-1 gene. A schematic representation of the structure of the XAGE-1 gene and transcripts, including locations of primers used in this study, is shown in Fig. 1. Primer pair sequences used for RT-PCR are listed in Table I, with X-7 and X-2 for XAGE-1a, X-1 and X-2 for the common region of XAGE-1b, X-4 and X-2 for XAGE-1c and X-6 and X-2 for XAGE-1d. The amplification program for XAGE-1 transcript variants was 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 30 cycles after 10 min at 94°C. The amplification program for GAPDH was 30 sec at 94°C, 45 sec at 57°C and 45 sec at 72°C for 30 cycles after 10 min at 94°C. The cycles were followed by a 10-min elongation step at 72°C. The PCR products were analyzed on 1.6% agarose gel electrophoresis. The positive PCR products were then recovered and sequenced. Data were analyzed by comparison with the respective sequences in GeneBank.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) for GAPDH and XAGE-1. Sequences of gene-specific primers for XAGE-1b and XAGE-1d GAPDH are shown in Table I. Relative quantification using real-time RT-PCR was performed using SYBR-Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions, and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification was performed for 42 cycles with 1 μ l cDNA (corresponding to 60 ng total RNA) solution extracted from tumor and non-cancerous specimens with 10 μ l PCR SYBR-Green Master Mix (Applied Biosystems) and 1 μ l 750 nM forward and reverse primers in a total volume of 20 μ l. The

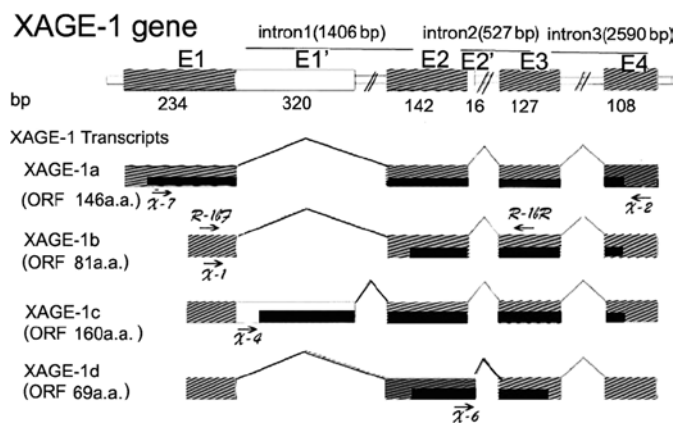


Figure 1. Schematic representation of the XAGE-1 gene and its transcripts and open reading frames (ORFs). Exons are shown as black boxes and introns as white boxes. ORFs are shown in hatched boxes. Arrows indicate the primers used in our study.

amplification conditions were: 95°C for 15 sec and 60°C for 60 sec.

Immunohistochemistry. Paraffin-embedded tumors and the adjacent non-cancerous tissues of 4 μ m were mounted on glass slides and deparaffinized with xylene and ethanol. For antigen retrieval, the tissues were water-bath heated in an antigen retrieval buffer [10 mM citrate buffer (pH 6.0)] for 10 min. Endogenous peroxidase was inactivated with 0.3% H₂O₂ for 10 min. Following pre-incubation with serum-free blocking solution, XAGE-1 mAb (Santa Cruz) was added at a concentration of 2 μ g/ml and incubated at 4°C overnight. Following PBS washing, biotin-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated avidin (Santa Cruz) were applied, respectively, and incubated for 30 min at room temperature for each step. The specimens were then visualized using 3,3'-diaminobenzidine (DAB) in H₂O₂ and counterstained with hematoxylin solution.

Statistical analysis. Associations between the XAGE-1 expression and the clinicopathological characteristics were analyzed using the Chi-square or Fisher's exact test, according to the test condition. The survival probabilities were estimated using the Kaplan-Meier method and were compared using the log-rank test. Overall survival time was defined as the time from the date of surgery to the date the patient succumbed to any cause. Patients who were alive at the date of the last follow-up were censored on that date plus 1 day. Confidence intervals of 95% were used throughout the analysis. Statistical significance was defined as $P < 0.05$. The statistical tests were performed using the Statistical Package, SPSS 13.0 for Windows (SPS Inc., Chicago, IL, USA).

Results

XAGE-1 mRNA expression in hepatocellular carcinoma. Expression of the four XAGE-1 transcript variants was investigated in 96 HCC and adjacent non-cancerous liver tissues by conventional 30-cycle RT-PCR using specific primer pairs (Fig. 1, Table I). The PCR product was analyzed in 1.6% agarose gel and sequenced. Fig. 2 shows a representative

Table I. PCR primers used in this study, including GAPDH and XAGE-1.

Primer	Sequence	Notes
GAPDHF	5'-GAAGGTGAAGGTCGGAGTC-3'	Forward, sense
GAPDHR	5'-GAAGATGGTGATGGGATTTC-3'	Reverse, anti-sense
X-7	5'-ACCTCAGTGCGCATGTTAC-3'	Forward, sense
X-1	5'-TTTCTCCGCTACTGAGACAC-3'	Forward, sense
X-4	5'-CTGGGAGTTGAAGTGTGAGT-3'	Forward, sense
X-2	5'-CAGGTGCTGGGAAGGGAAAT-3'	Forward, sense
X-6	5'-CAGCTTGCGTTGTTTCAGCT-3'	Reverse, anti-sense
R-1bF	5'-TACTGAGACACGGCGGAC-3'	Forward, sense
R-1bR	5'-TTCCATGTCGCGCACTG-3'	Reverse, anti-sense

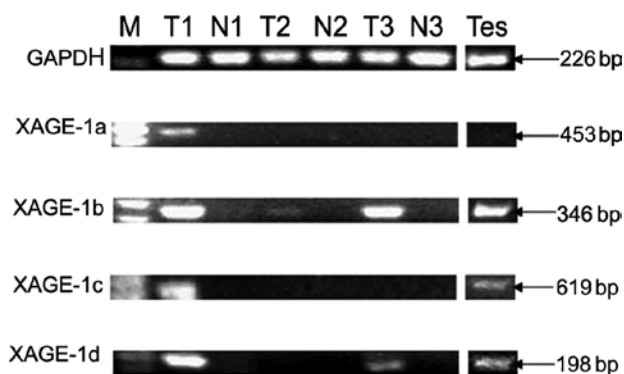


Figure 2. XAGE-1 mRNA expression in hepatocellular carcinoma, as well as adjacent non-cancerous and testis tissues. GAPDH was used for the internal controls. XAGE-1b was confirmed by the negative expression of XAGE-1a and nucleotide sequence. M, 50-bp marker; T, tumor; N, adjacent tissue and Tes, testis.

RT-PCR and Table II summarizes the results. The expression of XAGE-1b, XAGE-1d and XAGE-1c mRNA, but not XAGE-1a, was observed, with the exception of 2 cases. Histologically, XAGE-1b mRNA expression was observed in 40 of 96 (41.7%) HCC patients. By contrast, XAGE-1d mRNA expression was observed in 25 (15.6%) HCC specimens and XAGE-1c mRNA in 15 (26.0%) HCC specimens. XAGE-1a mRNA expression was observed in only 2 cases of HCC tissues. XAGE-1c and XAGE-1d mRNA expression, except for 1 case, was associated with XAGE-1b mRNA expression. No XAGE-1 mRNA expression was observed in the 96 adjacent normal liver tissues.

Quantitative real-time RT-PCR analysis of XAGE-1b and XAGE-1d mRNA expression in HCC, adjacent normal liver tissues and testis tissues. XAGE-1b and XAGE-1d mRNA expression was quantitatively analyzed by real-time RT-PCR using SYBR-Green PCR Mastermix with the procedure running on the ABI PRISM 7000 Sequence Detection System and specific primers shown in Fig. 1 and Table I. As shown in Fig. 3, compared to GAPDH expression, the XAGE-1b and XAGE-1d mRNA copy numbers ranged from 0.02 to 74.74% and 0.001 to 44.44%, respectively. Additionally, the mean values were 9.73 and 4.81%, respectively, and standard deviation was 14.48 and 9.85%, respectively, in the 40 and 25 HCC

specimens found to be positive for XAGE-1b and XAGE-1d mRNA expression by conventional RT-PCR. Compared to GAPDH expression, XAGE-1b and XAGE-1d mRNA copy numbers were 15.86 and 3.51%, respectively, in testis tissues.

XAGE-1 isoform expression in HCC cancer by immunohistochemistry. XAGE-1 protein expression was examined in 96 HCC specimens by immunohistochemistry using a XAGE-1 mAb that reacted against XAGE-1 isoforms, including XAGE-1b, XAGE-1c and XAGE-1d. Cases with staining in >10% of the cells were considered to be positive. Positive staining was observed in 39 of 40 (97.5%) of the XAGE-1b mRNA-positive specimens, while no positive staining was noted in mRNA-negative specimens (Fig. 4). No positive staining was observed in the adjacent non-cancerous, relatively normal liver tissues examined.

Correlation between XAGE-1b and clinicopathological characteristics. The various clinicopathological characteristics of the patients and their tumors were compared according to the XAGE-1b mRNA expression (Table II). No significant association was found between age, gender, HBV infection, tumor size, AFP level, TNM stage or differentiation. The effect of XAGE-1b mRNA expression on survival was examined. Adequate clinical follow-up information was available for the 96 cases. The mean follow-up of the 96 cases was 12.8 months, (range 1-29). A total of 68 patients (70.8%) survived, but 28 patients (29.2%) succumbed during the follow-up period. The 2-year survival rate of the patients with a positive and negative XAGE-1b expression was 37.1 and 67.3%, respectively, and the former group exhibited a relatively shorter 2-year survival rate ($P=0.045$). The Kaplan-Meier survival curves showed that patients with a positive XAGE-1b expression did not have a significant survival difference compared to patients with a negative XAGE-1b mRNA expression ($P=0.182$) (Fig. 5).

Discussion

The present investigation showed that XAGE-1 expression occurred in tumor samples from HCC patients, demonstrating a relatively high frequency expression of XAGE-1b and XAGE-1d (41.7 and 26.0%, respectively). Additionally, the XAGE-1 protein was detected in 39 (40.6%) HCC

Table II. Correlation between XAGE-1b, the dominant type, XAGE-1c and XAGE-1d mRNA expression and clinicopathological characteristics in HCC.

	XAGE-1b- positive	XAGE-1b- negative	P-value	One positive	Double positive	Triple positive	Tetra positive
Cases	40 (41.7%)	56 (58.3%)		16	9	14	1
Age (mean \pm SD)	54.3 \pm 12.7	51.3 \pm 11.1	0.97	16	9	14	46
≤50 years	18	25		8	5	4	0
>50 years	22	31		8	4	10	1
Gender			1.00				
Male	30	42		9	9	11	1
Female	10	14		7	0	3	0
HBV			0.53				
Positive	30	45		13	7	10	0
Negative	10	11		3	2	4	1
AFP level			0.29				
≤20 μ g/l	16	28		8	2	5	1
>20 μ g/l	24	27		8	7	9	0
Tumor size			0.70				
≤5 cm	22	33		10	2	9	1
>5 cm	18	23		6	7	5	0
TNM stage			0.88				
I/II	27	37		9	5	12	1
III/IV	13	19		7	4	2	0
Differentiation			0.20				
Well/moderate	32	50		15	6	10	1
Poor	8	6		1	3	4	0

One positive, only XAGE-1b is positive; double positive, XAGE-1b and XAGE-1a or XAGE-1b and XAGE-1d are positive; triple positive, XAGE-1b, XAGE-1c and XAGE-1d are positive; tetra positive, XAGE-1b, XAGE-1a and XAGE-1c and XAGE-1d are positive. SD, standard deviation.

tumor tissues, rendering them ideal candidate antigens for antigen-specific HCC immunotherapy. Moreover, our study qualitatively showed that XAGE-1b and XAGE-1d exhibited a higher expression in certain HCC cases compared to testis tissues. Concomitantly, we found no relationship between the expression of XAGE-1b and the clinical parameters. Furthermore, the HCC patient groups with a positive XAGE-1 mRNA expression had a relatively shorter 2-year survival rate compared to the negative group.

Studies have shown that XAGE-1 was highly expressed in numerous malignancies. Using automated high-throughput filter immunoscreening or cDNA phage surface display, XAGE-1 antigen was identified in prostate cancer, particularly in the serum of prostate cancer and lung adenocarcinoma patients (17-19). The molecular mechanism regulating the expression of this CTA gene may be epigenetic modulation of the gene promoter, histone deacetylase and DNA methyltransferase inhibitors (20). Epigenetic modulation of this antigen gene may be found in tumorigenic human mesenchymal stem cells that can potentially be utilized in cancer therapy (21).

Furthermore, the dominant XAGE-1 isoform, XAGE-1b, is known to be able to stimulate the immune response of patients suffering from non-small cell lung or prostate cancer

(22-24). In addition, antibody responses to recombinant L552S protein, an alternatively spliced isoform of XAGE-1, were observed in the pleural effusion fluids of lung cancer patients (25). The XAGE-1b protein can be processed, is present in the HLA-I molecule and stimulates the autologous CD4⁺ T-lymphocyte response (26,27). Patients with lung adenocarcinoma expressing both XAGE-1b and HLA class I antigens were associated with prolonged survival time (28).

Full-length XAGE-1b protein-pulsed dendritic cells induce a specific cytotoxic T-lymphocyte response *in vitro* that suggests a potential role of immunotherapy in various types of cancer (29). Therefore, XAGE-1b is a type of highly and specifically expressed XAGE-1 antigen in tumors that can be used as targets for immunotherapy. However, XAGE-1b expression and its correlations with the clinicopathological characteristics have yet to be adequately analyzed in human HCC cancer tissues. To the best of our knowledge, this is the first study regarding the relationship between XAGE-1 expression and clinical parameters in a larger sample of patients with HCC. Of note is that this study did not demonstrate statistical significance of the survival difference on the basis of XAGE-1b expression, which has been shown to be of prognostic significance in lung cancer, as reported by Kikuchi *et al*

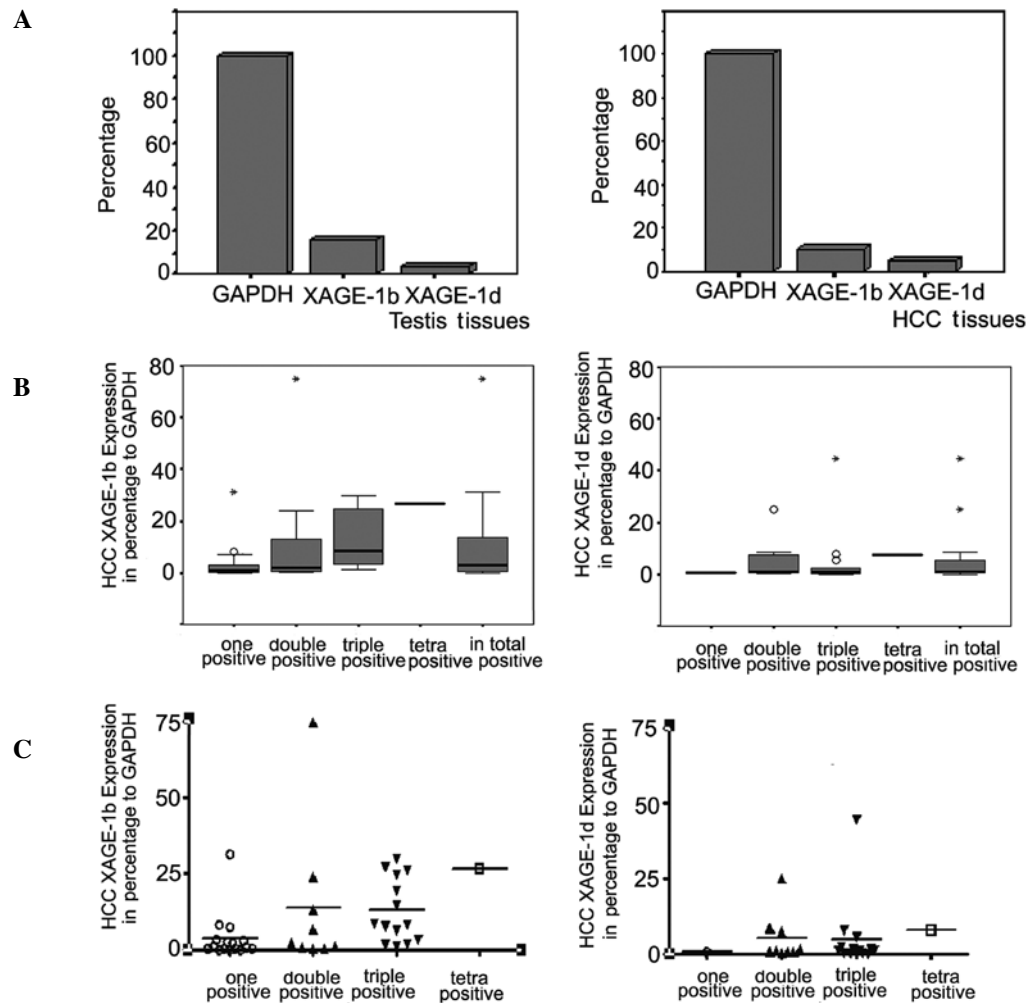


Figure 3. XAGE-1 mRNA expression in HCC and testis tissues. Histogram plots show i) the expression of the candidate internal control gene GAPDH as 100%, and ii) XAGE-1b and XAGE-1d compared to GAPDH in HCC and testis tissues. (A) The box bars show XAGE-1b and XAGE-1d expression compared to GAPDH in HCC tissues. (B) The data are presented in five groups: One positive, only XAGE-1b is positive; double positive, XAGE-1b and XAGE-1a or XAGE-1b and XAGE-1d is positive; triple positive, XAGE-1b, XAGE-1c and XAGE-1d is positive; tetra positive, XAGE-1b, XAGE-1a and XAGE-1c and XAGE-1d are positive; total positive included the four above-mentioned groups. (C) The distribution of the relative expression values compared to the internal control gene GAPDH is shown, with the groups divided as above.

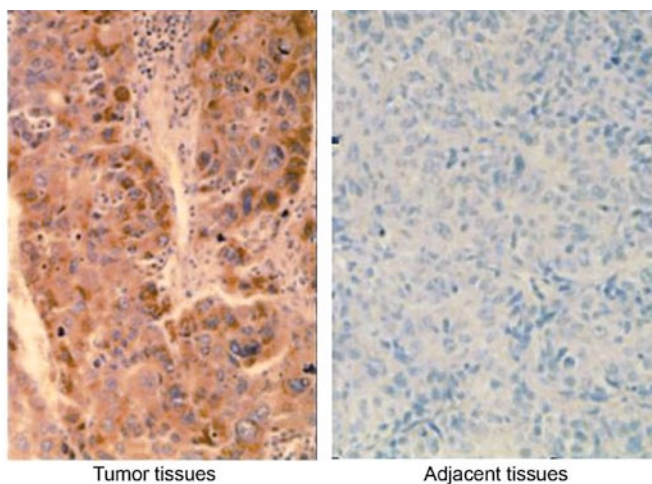


Figure 4. Immunohistochemical staining of XAGE-1 protein in liver tissues. In the XAGE-1b mRNA-positive HCC tissues, the cancer tissue cells were randomly and heterogeneously reactive for XAGE-1 protein, while in the XAGE-1b mRNA-negative HCC tissues, the cancer tissue cells showed a negative expression of XAGE-1.

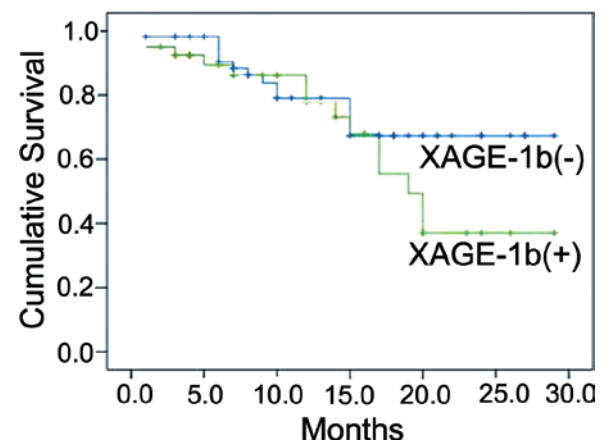


Figure 5. Overall survival curves following surgical therapy grouped by XAGE-1b expression and calculated using the Kaplan-Meier method. Statistical analysis showed that the positive XAGE-1b expression group (n=40, green line) had no significant survival difference from the negative XAGE-1b expression group (n=56, blue line) (P=0.182).

(28). This result may be due to the fact that our study did not simultaneously evaluate the MHC-I expression status.

Further prospective studies with a larger number of cases in a wide range of clinical settings are required to evaluate XAGE-1b expression in order for the unique role of XAGE-1b in HCC development and progression to be determined. Moreover, humoral immunity and autologous T-lymphocyte responses induced by XAGE-1b should also be assayed. The results suggest that potential strategies to reduce or antagonise the XAGE-1b expression may become a valuable therapeutic approach in the treatment of HCC patients. Furthermore, due to its highly specific expression and immunogenic effects, XAGE-1b may be used as a vaccine applicable in the immunotherapy of HCC. However, further investigations regarding the application of XAGE-1b are crucial.

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