Original Article

Cancer-Testis Antigen GAGE-1 Expression and Serum Immunoreactivity in Hepatocellular Carcinoma

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Abstract

Aim: To explore the use of cancer-testis antigen G antigen 1 (GAGE-1) in the diagnosis and potential therapeutic targeting of hepatocellular carcinoma (HCC), we measured the expression of GAGE-1 protein levels in HCC tissues and its serum immunoreactivity in HCC patients. **Materials and Methods:** We detected the expression of GAGE-1 protein in HCC by immunohistochemistry (IHC). We then analyzed the clinical significance of GAGE-1 expression in HCC with respect to clinicopathological parameters. We observed positive anti-GAGE-1 antibody reactivity in HCC patient serum, liver cirrhosis patients (LC), hepatitis B patients (HB), and normal human individuals (NHS) by enzyme-linked immunosorbent assay. **Results:** The IHC results showed that the positive rates of GAGE-1 protein expression in cancer tissues and adjacent tissues were 43.3% (26/60) and 5% (3/60), respectively. The expression level of GAGE-1 protein in HCC tissues was significantly higher than that in tumor-adjacent tissues ($P < 0.05$). Positive GAGE-1 protein expression was not correlated with clinicopathological parameters ($P > 0.05$). Positive serum anti-GAGE-1 antibody reactivity in HCC patients, LC, HB, and NHS was 23.33% (14/59), 13.1% (8/61), 3.3% (2/60), and 3.4% (2/59), respectively. The frequency of anti-GAGE-1 antibody-positive sera in HCC patients and LC was significantly different than that in HB and NHS ($P < 0.01$), but no significant differences were found between HCC patients and LC ($P = 0.485$) or between HB and NHS ($P = 0.410$). Positive anti-GAGE-1 antibody reactivity was not correlated with clinicopathological parameters ($P > 0.05$). **Conclusion:** These data illustrate that the GAGE-1 protein exhibits moderate cancer-restricted pattern of expression and immunogenicity, laying the foundation for the application of GAGE-1 in immunotherapy and for the diagnosis of HCC.

Keywords: *Cancer-testis antigen,G antigen 1, immunohistochemistry, serology*

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Introduction

Hepatocellular carcinoma (HCC) is one of the most commonly lethal cancers and the leading cause of cancer death worldwide. Although surgical resection or liver transplantation are common treatment modalities in the majority of patients, some patients may not be eligible for these treatments due to diagnosis occurring at an advanced stage.[1] Immunotherapy using cancer vaccines may represent a novel approach to improving outcomes in HCC patients.[2] However, only a limited number of target molecules are recognized by HCC-reactive T-cells, limiting the options for development of immunotherapies in HCC patients.

Cancer-testis (CT) antigens are a group of protein antigens expressed at a high frequency in various cancers, while having barely detectable levels in

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normal tissues with the exception of germ cells.[3] This cancer-restricted pattern of expression, together with the ability to elicit immune responses in patients, makes CTs attractive candidates for HCC immunotherapy.[4] The G antigen 1 (GAGE-1) gene was identified previously as one that codes for YRPRPRRY, an antigenic peptide that was presented on a human melanoma MZ2-MEL by HLA-Cw6 molecules and that was recognized by a clone of cytotoxic T lymphocytes derived from the patient bearing the tumor.[5] In healthy individuals, GAGE-1 expression is limited to germ cells, but transcription of GAGE-1 is activated in response to epigenetic dysregulation in cancer cells.[6,7] GAGE-1 gene transcripts exhibit universally high expression pattern in cancers, such as non-small cell lung cancer (26%), thyroid carcinomas (30%), breast cancer (26%), ovarian cancer (30%), malignant melanomas (24%–42%), and HCC (30%).[8-14] GAGE-1 expression levels typically correlate with poor prognosis in stomach cancer, esophageal carcinoma, and neuroblastoma, indicating that the gene plays an important role in tumorigenesis.[9,15,16] Furthermore, GAGE-1 protein expression has been identified in several cancers, including malignant melanoma, lung cancer, breast cancer, and thyroid cancers.[17] However, to date, GAGE-1 expression levels have not been clarified comprehensively in HCC tissues.

We previously showed that GAGE genes (1, 2, and 8) are present at high frequencies in HCC tissues (16/40),[18] suggesting that GAGE genes are potential targets for HCC immunotherapy. Here, we measured GAGE-1 protein expression in HCC tissues and its serum immunoreactivity in HCC patients to explore the possibility of using GAGE-1 in diagnosis and as a potential therapeutic target in HCC.

Materials and Methods

Ethics statement

All tissue and serum samples were collected with the informed consent of patients and control individuals. The Ethic Review Committee of the First Affiliated Hospital of Guangxi Medical University (Nanning, China) approved this study.

Tissues and serum

After histopathological examination, sixty formalin-fixed paraffin-embedded HCC tissues and adjacent tissues (the edge of cancer ≥5cm) used for this study were procured from the Department of Pathology of the Tumor Hospital affiliated with Guangxi Medical University (52 men and 8 women; mean age, 51.32 ± 11.27 years; age range, 31–74 years). HCC was confirmed by pathological diagnosis.

Serum from 59 HCC patients (50 men and 9 women; mean age, 56 ± 13 years; age range, 23–83 years), 61 liver cirrhosis patients (LC) (47 men and 14 women; mean age, 53.2 ± 11 years; age range, 21–77 years), and 60 hepatitis B patients (HB) (38 men and 22 women; mean age, 39.8 ± 14 years; age range, 20–84 years) were obtained from the First Affiliated Hospital of Guangxi University of Chinese medicine for GAGE-1 serology. Serum from 59 control normal human individuals (NHS) was also obtained from the Guangxi Medical University students.

Immunohistochemistry analysis

Immunohistochemistry (IHC) analysis was performed using an Elivision™ super HRP (Mouse/Rabbit) IHC Kit (Fuzhou Maixin Biotech Co., Ltd., China): paraffin-embedded tissue sections were heated at 65°C for 2 h. Environmental-protective dewaxing agents were used for deparaffinization four times for 5 min each.

Sections were deparaffinized with environmental-protective dewaxing agents, rehydrated, and treated in H_2O_2 to block endogenous peroxidase activity. Sections were subjected to antigen retrieval by autoclaving for 2.5 min in 1 mmol/LEDTA buffer, at pH 9.0. Next, sections were incubated with primary antibody (1:100) for 1.5 h at 37°C followed by detection using Elivision™ super HRP (mouse/rabbit) IHC Kit (Fuzhou Maixin Biotech Co., Ltd.) and 3,3'-diaminobenzidine as the chromogen. Slides were counterstained with hematoxylin and subsequently evaluated. Positive staining in any cancer cells, irrespective of the percentage of positive cells or intensity, was regarded as positive. Negative control staining was performed using phosphate-buffered saline (PBS) with no primary antibody.

Immunochemistry results were assessed by a semi-quantitative scoring system to evaluate the expression of GAGE-1. Briefly, staining intensity and percentage were used to determine the final score, i.e., negative (no staining) =0, weak (yellow) =1, moderate (light brown) =2, and strong (dark brown) =3. We randomly selected five areas at × 400 in one sample. The average percentage of positive cells was determined by counting the number of positive cells out of 200 cells. Samples were then grouped into the following categories: 0 (0%), 1 (1%–10%), 2 (11%–50%), 3 (51%–80%), and 4 (81%–100%). By multiplying the percentage and intensity to calculate the final score, a final score of ≥3 was considered high expression and a score <3 was regarded as low expression.

Enzyme-linked immunosorbent assay

The glutathione S-transferase (GST)/GAGE protein used for serology was purified according to the methods

described by Zhao *et al*.[19] The GST/GAGE-1 antigen was diluted at a final concentration of 2.0 mg/L in 0.05 mmol/L carbonate buffer. The wells of a PVC microtiter plate were coated with the antigen by pipetting 100 μL of the antigen dilution in the top wells of the plate and incubating overnight at 4°C. The antigen coating solution was then removed, and the plate was washed three times by filling the well with room temperature PBS with tween. The remaining protein-binding sites in the coated well were blocked with 300 μL blocking buffer in 5% nonfat dry milk per well for 1 h. 1:64 dilutions of serum and preimmune serum (the latter as the negative control) were created in blocking buffer. Then, 50 μL of each dilution was added into an antigen-coated well in triplicate for 1 h. The biotin-conjugated secondary sheep anti-human antibody was diluted in the ratio of 1:4000 at 37°C for 1 h. Next, 50 μL of substrate 3,3′,5,5′-tetramethylbenzidine solution was added to each well and incubated for 15 min, at which time an equal volume of stopping solution (1 M H_2SO_4) was added in order to read the optical density at 450 nm and 630 nm.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS 16.0 (SPSS Inc, SPSS for Windows, SPSS Inc., Ill Chicago, USA). The correlation between GAGE-1 expression/serum anti-GAGE-1 antibody and clinicopathological parameters was analyzed using the Chi-square test. The Kruskal–Wallis test was used to analyze differences in the frequency of sera anti-GAGE-1 antibody between HCC, LC, HB, and NHS. GAGE-1 diagnostic accuracy was analyzed using the receiver operating characteristics (ROC) curve. P <0.05 was considered statistically significant. The r value signifies the correlation coefficient.

Results

Expression levels of G antigen 1 protein in hepatocellular carcinoma tissues are significantly higher than that in tumor-adjacent tissues

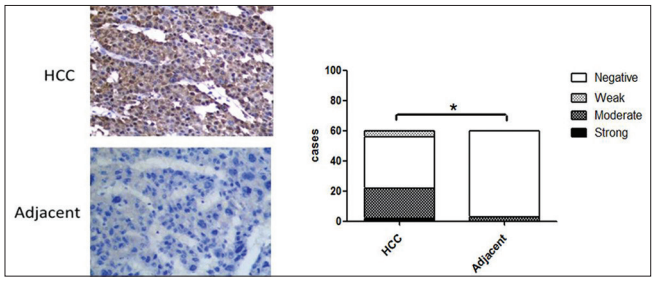

Figure 1: Immunohistochemical analysis of G antigen 1 expression in sixty hepatocellular carcinoma tissues and adjacent nontumor tissues. *Statistically significant, $P < 0.5$

Table 1: The correlation between pathological features of hepatocellular carcinoma and positive expression of G antigen 1

Parameters	Total	Positive (%)	Negative (%)	χ^2	P
Gender					
Male	52	12 (48.1)	40 (51.9)	2.272	0.132
Female	9	1 (12.5)	8 (87.5)		
Age					
≥50	30	15 (50)	15 (50)	1.086	0.297
<50	30	11 (36.7)	19 (63.3)		
TNM Stage					
I~II	43	16 (37.2)	27 (62.8)	2.318	0.128
III~IV	17	10 (58.8)	7 (41.2)		
Tumor size (cm)					
<5	24	12 (50.0)	12 (50.0)	0.404	0.525
≥5	36	21 (58.3)	15 (41.7)		
AFP (ng/ml)					
<200	35	15 (42.9)	20 (57.1)	0.008	0.930
≥200	25	11 (44.0)	14 (56.0)		

Table 2: Frequency of autoantibodies against G antigen 1 in human sera by enzyme-linked immunosorbent assay the cutoff value designating positive reaction was the mean optical density of 59 normal human sera plus 3 standard deviations

Type of sera	No. tested	Antibody against GAGE-1 (%)
HCC	59	14 (23.7)**
LC	61	8 (13.1)
HB	60	2 (3.3)
NHS	59	2 (3.3)

The prevalence of autoantibody against GAGE-1 was significantly higher in HCC than that in LC, CH and NHS. **Statistically significant, P<0.001

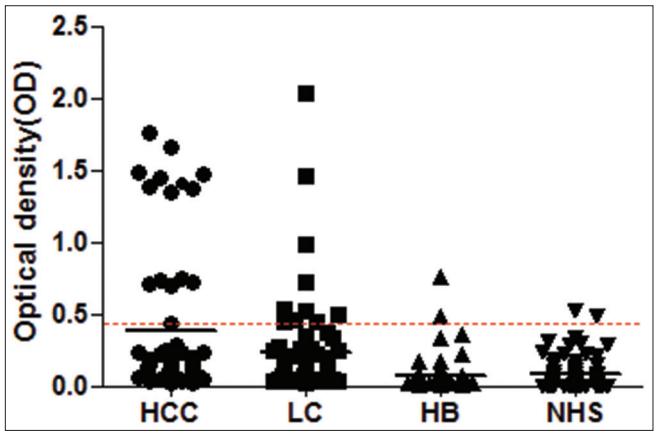

Figure 2: Titer of autoantibodies against G antigen 1 in human sera by enzyme-linked immunosorbent assay. The range of antibody titers is presented as optical density obtained by enzyme-linked immunosorbent assay. The mean ± standard deviation of G antigen 1 is shown in relationship to all serum samples. The red line indicates the cutoff value line for positive samples. The frequency of autoantibodies against G antigen 1 in human sera of hepatocellular carcinoma and liver cirrhosis samples was significantly different from that of hepatitis B and normal human individual samples ($P < 0.01$), but no significant differences were found between hepatocellular carcinoma and liver cirrhosis ($P = 0.485$) or between hepatitis B and normal human individuals ($P = 0.410$)

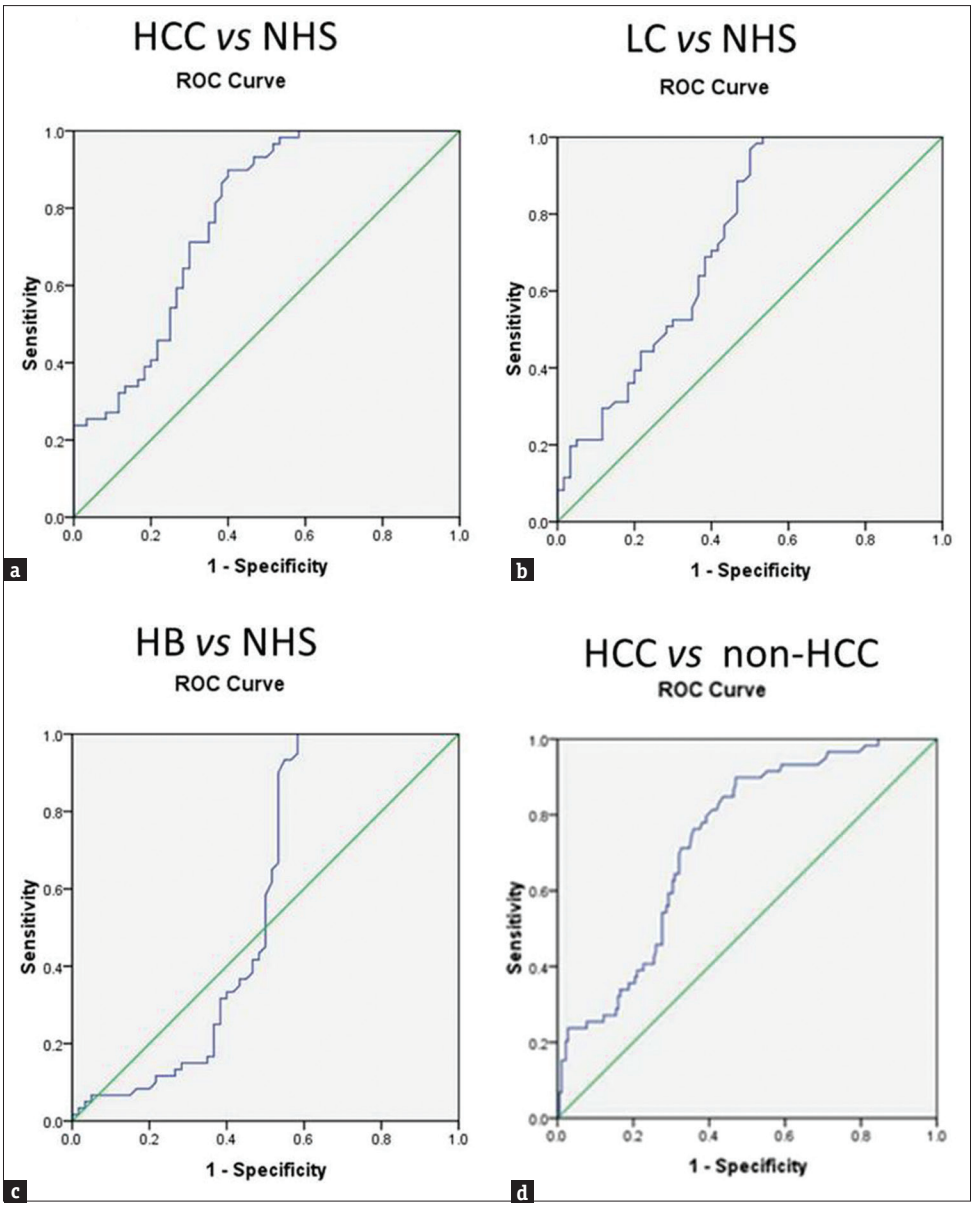

Figure 3: (a-d) Receiver operating characteristic curve used to predict the performance of serum anti-G antigen 1 antibody in distinguishing hepatocellular carcinoma with normal human individuals, liver cirrhosis with normal human individuals, hepatitis B with normal human individuals, and hepatocellular carcinoma with nonhepatocellular carcinoma controls. The receiver operating characteristic curve was generated using data from 59 hepatocellular carcinoma sera samples and 180 nonhepatocellular carcinoma sera samples (including 61 liver cirrhosis sera samples, 60 hepatitis B sera sample, and 59 normal human individual sera samples). The area under curve the receiver operating characteristics curve for G antigen 1 in distinguishing hepatocellular carcinoma with normal human individuals, liver cirrhosis with normal human individuals, hepatitis B with normal human individuals, and hepatocellular carcinoma with nonhepatocellular carcinoma controls was 0.776, 0.730, 0.566, and 0.733, respectively

IHC results revealed that positive GAGE-1 protein expression levels in HCC tissues and adjacent tissues were 43.3% (26/60) and 5% (3/60), respectively. Positive staining was primarily cytoplasmic and evenly distributed, with only small amounts observed in the nuclei. GAGE-1 protein expression in HCC tissues was significantly higher than that in tumor-adjacent tissues ($P < 0.001$) [Figure 1]. Notably, rather heterogeneous expression was present in a significant number of HCC samples, which varied from individual positive cells and foci of stained cells to uniform staining of tumor cells.

Analysis of the clinicopathological parameters by Chi-square test demonstrated that GAGE-1 protein expression is not correlated with clinicopathological parameters, such as sex, age, tumor node metastasis stage, tumor size, or alpha fetoprotein level [Table 1].

G antigen 1 protein immunogenicity observed in hepatocellular carcinoma patient serum

Here, we used a GST/GAGE-1 fusion protein to detect the autoantibody against GAGE-1 in serum from HCC, LC, HB, and NHS samples. The cutoff value of GAGE-1

Table 3: Correlation between clinicopathological features of hepatocellular carcinoma and the positive rate of anti-G antigen 1 antibodies

Parameters	Total	Positive (%)	Negative (%)	χ^2	*P*
Gender					
Male	50	25 (48.1)	27 (51.9)	2.272	0.132
Female	9	1 (12.5)	7 (87.5)		
Age					
≥50	37	9 (24.3)	28 (75.7)	1.086	0.297
<50	22	5 (22.7)	17 (77.3)		
AST					
≥45	35	16 (37.2)	27 (62.8)	2.318	0.128
<45	24	10 (58.8)	7 (41.2)		
ALT					
≥40	31	12 (50.0)	12 (50.0)	0.404	0.525
<40	28	21 (58.3)	15 (41.7)		

is shown in Table 2, and the prevalence of autoantibody against GAGE-1 was 23.7% (14/59) in HCC, significantly higher than that in LC (8/61, 13.1%), HB (2/60, 3.3%), and NHS (2/59, 3.4%). The average titer of anti-GAGE-1 antibodies from sera of HCC patients was higher than that in LC, HB, and NHS individuals [Figure 2]. The Kruskal–Wallis test results showed that the frequency of positive anti-GAGE-1 antibody sera in HCC and LC was significantly different from those of the HB and NHS groups ($P < 0.01$); however, no significant difference was found between HCC and LC ($P = 0.485$) or between HB and NHS ($P = 0.410$).

To evaluate whether serum anti-GAGE-1 antibody levels can be used as a potential diagnostic marker for HCC, ROC curve analysis was performed. The area under the ROC curve (AUC) for GAGE-1 was 0.733 [Figure 3d]. At the cutoff value of 0.386, the sensitivity and specificity for this marker was 71.2% and 67.4%, respectively. This result suggests that serum anti-GAGE-1 antibody levels may be a moderate serum marker in HCC. Furthermore, to explore the performance of serum anti-GAGE-1 antibody in distinguishing HCC from NHS, LC from NHS, or HB from NHS, we also performed ROC curve analysis on them. Their AUC for GAGE-1 was 0.776, 0.730, and 0.566, respectively [Figure 3a-c]. Hence, serum anti-GAGE-1 antibody levels are appropriate for distinguishing HCC from NHS.

Associations between serum anti-GAGE-1 antibody and clinicopathological parameters including sex, age, aspartate aminotransferase level, and alanine aminotransferase level were statistically evaluated. As shown in Table 3, no significant correlations were observed between serum anti-GAGE-1 antibody and any of the clinical parameters.

Discussion

GAGE-1 was originally identified as a gene coding for a tumor antigen in the melanoma cell line MZ2-MEL. Due to its ability to induce cellular and humoral immunity, GAGE-1 is considered one of the most immunogenic tumor antigens. Therefore, evaluation of the potential use of GAGE-1 proteins as targets for cancer-specific immunotherapy requires study of GAGE-1 expression. Our previous study demonstrated that GAGE-1 mRNA is expressed at high frequencies in HCC patients (40%, 16/40).[18] Here, the expression of GAGE-1 protein was detected by IHC in sixty HCC patients to assess the prognostic value of GAGE-1. IHC results showed that 43.3% of HCC patients expressed GAGE-1 protein, and GAGE-1 protein expression in HCC tissues was significantly higher than that in adjacent, nontumor tissues. GAGE-1 protein was primarily cytoplasmic in expression with small amounts in the nuclei. GAGE-1 expression in HCC tissues was higher than that in other malignant tumors, such as malignant melanoma, breast carcinoma, bladder carcinoma, lung carcinoma, thyroid carcinoma, mesothelioma, and germinal cell cancers. Despite GAGE-1 transcript levels having been correlated with a poor prognosis in stomach cancer, esophageal carcinoma, and neuroblastoma, we found no correlation between the levels of GAGE-1 protein expression in the tumors and clinicopathological parameters of the HCC patients. In 2000, Kobayashi reported that GAGE-1 gene transcripts were not significantly correlated with differentiation stage or size of the HCC. These results are in accordance with our results in this study. Although GAGE-1 gene expression levels had no relationship with clinicopathological parameters, GAGE-1 can be recognized as a potential target for cancer-specific immunotherapy due to its high frequencies in HCC patients (40%). However, due to a limited number of cases in this cohort, further studies are needed to confirm the prognostic value of GAGE-1 by enlarging the sample size.

Here, we observed that the level of GAGE-1 protein overexpression is significantly heterogeneous among samples, which could hamper the effectiveness of GAGE-1 targets as immunogenic treatments for HCC. The problem of heterogeneity could be overcome by including the inhibitors of DNA methyltransferases and histone deacetylases in the treatment regimen. Hence, limited GAGE-1 expression in HCC patients indicates that GAGE-1 can only be used as an immune target in a select group of HCC patients.

Although GAGE-1 eliciting abnormal and cellular immune response in patients with advanced cancer has been confirmed in melanoma, it is still unclear whether GAGE-1 can elicit a humoral response in HCC patients. Hence, the diagnostic value of

GAGE-1 needs to be further explored. In this study, we examined GAGE-1 antibodies in serum from liver cancer patients, LC patients, HB patients, and normal human control individuals to evaluate the use of GAGE-1 as a diagnostic marker in HCC. The enzyme-linked immunosorbent assay results showed that the prevalence of autoantibodies against GAGE-1 in HCC was significantly higher than that in LC, HB, and NHS. Anti-GAGE-1 antibodies were present in 14 of 59 sera samples (23.7%), demonstrating that HCC frequently exhibits a humoral immune response to GAGE-1.

We next performed ROC curve analysis to evaluate whether serum anti-GAGE-1 antibody levels can be used as a potential diagnostic marker for HCC. At first, all the serum was divided into two populations: one population with a disease (HCC) and the other population without the disease (LC, HB, and NHS). Our results showed that ROC curve is close to midline. Moreover, this will rarely make a perfect separation between the two groups. Therefore, we analyzed if serum anti-GAGE-1 antibody levels can be used to distinguishing HCC with NHS, LC with NHS, or HB with NHS. Due to the AUC for GAGE-1 in distinguishing HCC with NHS is the highest among the three group, serum anti-GAGE-1 antibody levels provided greater diagnostic performance in distinguishing HCC with NHS. Overall, serum anti-GAGE-1 antibody may not be a good independent diagnostic marker for HCC due to its moderate serum immunoreactivity.

There are some limitations in our study: first, expression of CT antigens within a tumor is coordinated commonly; thus, more than one is expressed in a single tumor. Our study is mainly focused on GAGE-1 protein expression levels and its serum immunoreactivity in HCC patients. Our results set the base for more CT antigen combination in detecting HCC in the future clinical application. The combined detection composed of several CT antigens is deserved to be exploring in the future research. Second, the sample size of our study is relatively small. More samples need to be enrolled for confirming the prognostic value of GAGE-1.

Conclusion

These data illustrate that the GAGE-1 protein exhibits moderate cancer-restricted pattern of expression and immunogenicity, laying the foundation for the application of GAGE-1 in immunotherapy and for the diagnosis of HCC.

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Conflicts of interest

There are no conflicts of interest.

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