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NRAGE is a potential diagnostic biomarker of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers and a leading cause of cancer-related deaths worldwide. Early diagnosis of HCC remains a great challenge in clinic. Novel and effective biomarkers are in urgent need in early diagnosis of HCC.

Serum levels of neurotrophin-receptor-interacting melanoma antigen-encoding gene homolog (*NRAGE*) were measured for 107 patients with HCC, 98 patients with benign liver diseases, and 89 healthy controls using quantitative real-time polymerase chain reaction. Receiver operating characteristic curve was applied to evaluate the diagnostic capacity of serum *NRAGE* in HCC.

NRAGE expression was significantly higher in patients with HCC than in controls (all, $P<.05$). Moreover, its expression was tightly correlated with TNM stage ($P=.004$). *NRAGE* could distinguish patients with HCC from healthy controls with the area under the curve (AUC) of 0.874, yielding a sensitivity of 81.3% and a specificity of 78.7%. Additionally, in differentiation between benign liver diseases and HCC, the AUC value of *NRAGE* was 0.726, with a sensitivity of 63.6% and a specificity of 73.5%. Meanwhile, alpha-fetoprotein also could distinguish patients with HCC from benign liver disease cases, with an AUC of 0.677, a sensitivity of 64.4%, and a specificity of 60.2%.

NRAGE could be a potential biomarker for HCC early diagnosis.

Abbreviations: AFP = alpha-fetoprotein, AUC = area under the curve, GAPDH = glyceradehyde-3-phosphate dehydrogenase, HCC = hepatocellular carcinoma, IHC = immunohistochemistry, LncRNA = long noncoding RNA, *NRAGE* = neurotrophin-receptor-interacting melanoma antigen-encoding gene homolog, OPN = osteopontin, P75NTR = p75 neurotrophin receptor, PIVKA = vitamin K absence II, qRT-PCR = quantitative real-time polymerase chain reaction, ROC = receiver operating characteristics, TNM = tumor node metastasis, UICC = International Union Against Cancer.

Keywords: diagnosis, hepatocellular carcinoma, *NRAGE*

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent human malignancies, representing a major cause of cancer-related deaths, particularly in Africa and Asia.[1,2] HCC development is a multistep and multistage carcinogenetic process. Several risk factors have already been confirmed to be associated with the etiology of HCC, including the infection of hepatitis B or C virus, heavy alcohol intake, prolonged dietary exposure to aflatoxin or vinyl chloride, and primary hemochromatosis.[3] Due to the lack of diagnostic biomarkers, only 30% to 40% of patients with HCC are diagnosed in time, possibly receiving curative intervention. Despite the application of surgical resection, liver transplantation and chemoembolization, the 5-year survival rates among patients with HCC remain dismal during the past several decades.[4] To improve the management of HCC, various blood-based biomarkers have been explored,[5,6] such as glycomic profile,[7] alpha-fetoprotein (AFP), lens culinaris agglutinin-reactive AFP (AFP-L3), des-carboxy prothrombin, glypican-3, osteopontin (OPN), squamous cell carcinoma antigen, as well as microRNAs and long noncoding RNAs, etc.[8,9] AFP is a most widely used biomarker for HCC in clinic. It has been reported that increased level of serum AFP was positively associated with HCC clinical features, including tumor size, TNM stage and invasion depth. Serum AFP may be a potential biomarker for patients with HCC.[10,11] However, the sensitivity and specificity of AFP are far from satisfactory. Thus, more effective biomarkers with high sensitivity and specificity are urgently needed for early diagnosis of HCC.

Neurotrophin-receptor-interacting melanoma antigen-encoding gene homolog (*NRAGE*), also known as *MAGED1* or *Dlxin-1*, was recently identified as a new member of the melanoma antigen family, and encodes a tumor-specific antigen. It is involved in diverse molecular pathways. It could regulate gene transcription by binding to *MSX2* and *DLX5*,[12] and induce the apoptosis of neural cells via binding to p75 neurotrophin receptor (P75NTR) during neural development.[13] Given its functions in cellular processes, *NRAGE* is regarded to be closely associated with cancer development and progression.[14] However, to the best of our knowledge, the diagnostic capacity of serum *NRAGE* in HCC has not been investigated yet.

In the present study, we examined the relative expression of *NRAGE* in patients with HCC using the quantitative real-time

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polymerase chain reaction (qRT-PCR) method, and analyzed the correlation of serum *NRAGE* expression with clinicopathologic characteristics of patients with HCC. We also explored whether *NRAGE* could serve as a diagnostic biomarker for HCC.

2. Materials and methods

2.1. Patients and samples

The study was approved by the Ethic Committee of Affiliated Hospital of Changchun University of Traditional Chinese Medicine. All the participants signed informed consents.

A total of 294 individuals were enrolled in this study, including 107 patients with HCC in a preoperative status, 98 patients with benign liver diseases, and 89 healthy controls. HCC tissue specimen and adjacent normal ones were also collected from the patients with HCC in surgery. The patients were all histologically confirmed with HCC. The tumor type and differentiation grade were evaluated according to the WHO classification system, while the pathologic stage was determined based on the International Union Against Cancer (UICC) TNM classification. The clinical characteristics of the subjects are listed in Table 1.

Peripheral blood was obtained from all subjects early in the morning. Serum was separated through centrifugation at 3000*g* for 15 minutes, and stored at −80°C for further assay.

2.2. Quantitative real-time polymerase chain reaction

Total RNA was isolated from serum samples using Trizol regents (Invitrogen, New York, IL) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Reverse transcription was performed with SuperScript Fist-strand synthesis system (Gibcol BRL, New York, IL). qRT-PCR was performed using SYBR Green PCR core reagent kit (Applied Biosystems, New York, IL; Thermo Fisher Scientific, Inc). Glyceradehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA (TaqMan *GAPDH* Control Reagents; Applied Biosystems; Thermo Fisher Scientific, Inc, New York, IL) was used as internal control. The relative level of *NRAGE* expression was calculated with the $2^{-\Delta\Delta CT}$ method.[15] All experiments were performed in triplicate.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was used to detect the levels of *NRAGE* protein in 107 pairs of HCC tissues and adjacent normal ones. In brief, after deparaffinization and rehydration, tissue sections were incubated with 0.01 M citric acid buffer (pH 6.0) at 98°C for 10 minutes, and then air-dried at room temperature. Next, the sections were incubated with primary antibody at 4°C overnight. Then they were washed thrice using phosphate-buffered saline. After then, the sections were incubated with the Biotin-labeled 2nd antibody at 37°C for 30 minutes. At last, staining signaling was conducted with DAB. The IHC results were estimated using cells' staining percentage (0–100%). Tissues with staining percentage <10% or no staining were considered to be negative, otherwise, they would be positive. The sections were blocked and reserved for later use.

2.4. Statistical analysis

All statistical analyses were carried out using SPSS 18.0 (SPSS Inc, Chicago, IL), and graphs were plotted with GraphPad prism 5. Student *t* test was used to assess the difference in *NRAGE* levels between patients with HCC and the controls. The association

Table 1

Association between serum *NRAGE* expression and clinicopathologic characteristics of patients with HCC.

Characteristics	No. of cases (n=107)	*NRAGE* expression Low (n=52)	*NRAGE* expression High (n=55)	χ^2	*P*
Age, y					
<50	51	25	26	0.007	.934
≥50	56	27	29		
Gender					
Female	75	38	37	0.430	.512
Male	32	14	18		
Tumor size, cm					
<5	67	33	34	0.031	.861
≥5	40	19	21		
Liver cirrhosis					
Absent	71	35	36	0.041	.839
Present	36	17	19		
Serum AFP, ng/mL					
<20	49	25	24	0.212	.645
≥20	58	27	31		
Tumor differentiation					
Well	31	17	14	1.226	.531
Moderate	42	21	21		
Poor	34	14	20		
TNM stage					
I	15	10	5	13.222	.004
II	35	20	15		
III	39	20	19		
IV	18	2	16		

AFP=alpha-fetoprotein, HCC=hepatocellular carcinoma, NRAGE=neurotrophin-receptor-interacting melanoma antigen-encoding gene homolog, TNM=tumor node metastasis.

between serum *NRAGE* expression and clinicopathologic factors of patients with HCC was analyzed using Chi-squared test. Receiver operating characteristic (ROC) curve was employed to evaluate the diagnostic role of *NRAGE* in HCC. Area under the curve (AUC) with corresponding sensitivity and specificity were used to estimate the diagnostic accuracy of *NRAGE*. $P<.05$ was defined as statistical significance.

3. Results

3.1. Baseline characteristics of the included subjects

The HCC group included 75 females and 32 males, with an average age of 56.48±16.25 years. The benign liver disease group was composed of 58 women and 40 men, and their mean age was 52.98±18.12 years. Additionally, there were 55 females and 34 males in the healthy control group, and the average age was 54.18±14.32 years. The HCC group was matched by both the benign liver disease group and the healthy control group in age and gender ($P>.005$ for all). AFP detection was only performed for HCC and benign liver disease patients in our study. The average value of AFP was 49.84±43.10 ng/mL in patients with HCC and 29.97±21.89 ng/mL in benign liver disease patients. AFP level in patients with HCC was significantly higher than that in benign liver disease patients ($P<.001$).

3.2. Expression of NRAGE in patients with HCC and the controls

The qRT-PCR was performed to investigate the expression of serum *NRAGE* in 107 patients with HCC, 98 benign liver disease patients, and 89 healthy controls. As shown in Figure 1, the average value of *NRAGE* expression was 0.917±0.295 (mean± standard deviation) in patients with HCC, 0.569±0.207 in benign liver disease patients, and 0.362±0.182 in healthy controls. Serum *NRAGE* was significantly increased in patients with HCC compared with the controls (all, $P<.05$).

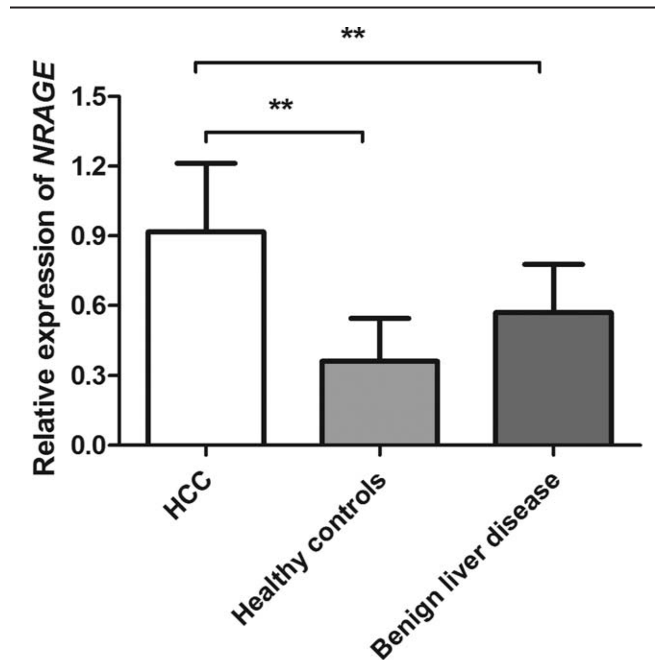

Figure 1. The relative expression of serum *NRAGE* in patients with hepatocellular carcinoma (HCC), benign liver disease patients and healthy controls. The results showed that serum *NRAGE* expression was significantly higher in patients with HCC than in the controls (all, $P<.01$).

In addition, IHC assay was performed to investigate whether blood *NRAGE* mRNA level was consistent with *NRAGE*

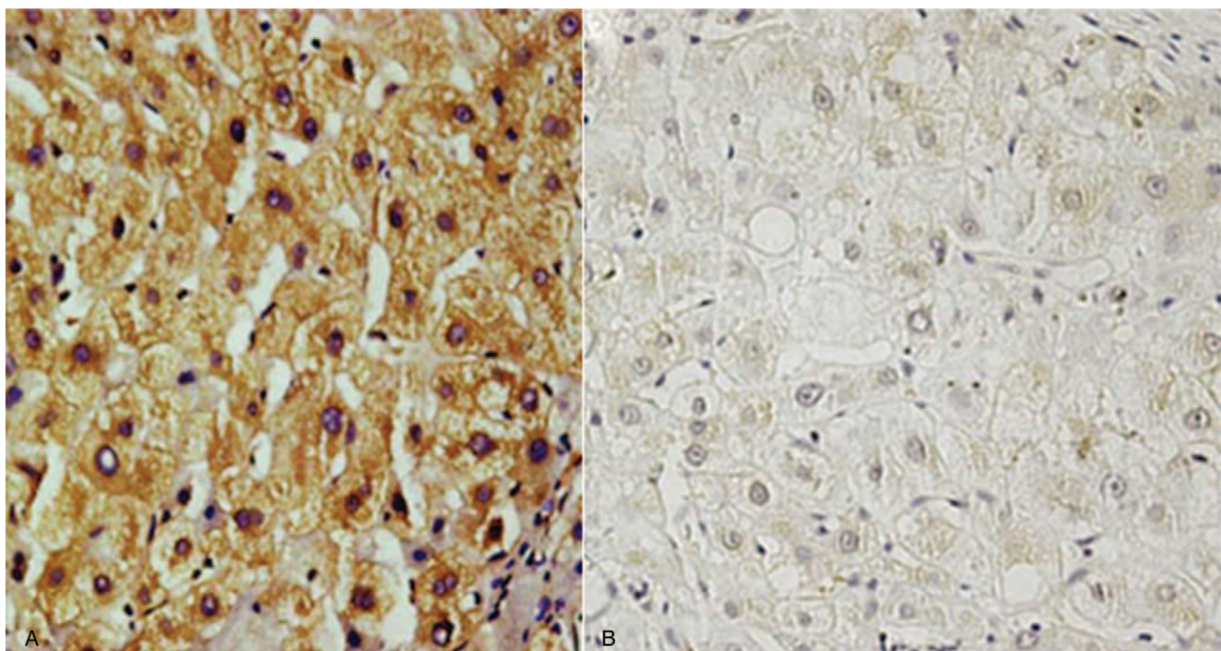

Figure 2. The expression profile of *NRAGE* in hepatocellular carcinoma (HCC) tissues and adjacent normal ones detected using immunohistochemistry assay. (A) Positive expression of *NRAGE* in HCC tissues (×400). (B) Negative *NRAGE* expression in noncancerous tissues (×400). Among the included 107 patients, positive staining was observed in 92.53% (99/107) HCC tissues, while the positive rate was only 4.67% (5/107) in adjacent normal ones. The positive rate was significantly higher in HCC tissues than in noncancerous ones ($P<.001$).

expression in tissue samples. Figure 2 shows representative images of *NRAGE* expressions in HCC tissues and adjacent normal ones. Analysis results suggested that for the included 107 patients, positive staining was observed in 92.53% (99/107) of the HCC tissues while the positive rate was only 4.67% (5/107) in adjacent normal tissues. The positive rate was significantly higher in HCC tissues than in noncancerous ones ($P<.001$). The results were highly consistent with blood detection.

3.3. Correlation of serum NRAGE expression with clinicopathologic features of patients with HCC

About 107 patients with HCC were divided into high and low expression groups according to their median value of *NRAGE* expression. The results showed that high *NRAGE* expression was strongly associated with TNM stage ($P=.004$). Unfortunately, no relationships were detected for *NRAGE* expression with age, gender, tumor size, liver cirrhosis, serum AFP, or tumor differentiation (all $P>.05$) (Table 1).

3.4. Diagnostic efficacy of serum NRAGE in HCC

The ROC curve was drawn to evaluate the diagnostic significance of *NRAGE* in HCC. In the light of the ROC curve, *NRAGE* could discriminate between patients with HCC and healthy controls with an AUC of 0.874, yielding a sensitivity of 81.3% and a specificity of 78.7% (Fig. 3). Moreover, we evaluated the diagnostic significance of *NRAGE* based on HCC cases and benign liver disease patients, and obtained an AUC value of 0.726, with a sensitivity of 63.6% and a specificity of 73.5% (Fig. 4).

In addition, ROC curve was also plotted according the AFP levels of patients with HCC and benign liver disease patients. The results shown in Figure 4 demonstrated that AFP could distinguish patients with HCC from the benign liver disease cases, with an AUC value of 0.677, a sensitivity of 64.4%, and a specificity of 60.2%.

4. Discussion

The HCC is the major type of liver cancers, with high mortality and morbidity rates.[16–18] Early and accurate diagnosis is necessary to improve clinical outcome of patients with HCC. Currently, serum AFP has been widely used for HCC diagnosis; however, its diagnostic sensitivity and specificity are not satisfactory.[19,20] Exploring novel and accurate diagnostic biomarkers may be an effective approach to improve HCC prognosis.

To date, extensive efforts have been made to explore potential biomarker for HCC diagnosis. Ji et al found that the methylation

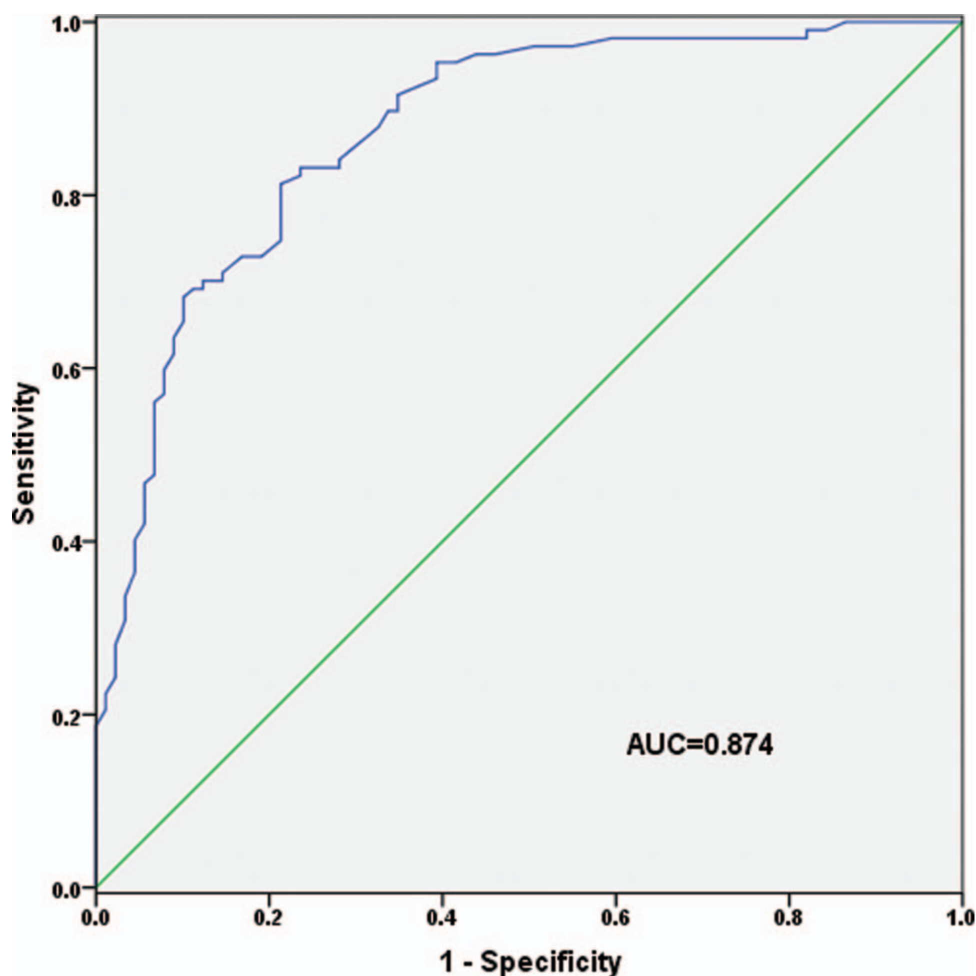

Figure 3. Diagnostic performance of serum *NRAGE* based on patients with hepatocellular carcinoma (HCC) and the healthy controls using ROC curve. *NRAGE* was a valuable diagnostic biomarker to distinguish patients with HCC from the healthy controls (AUC=0.874).

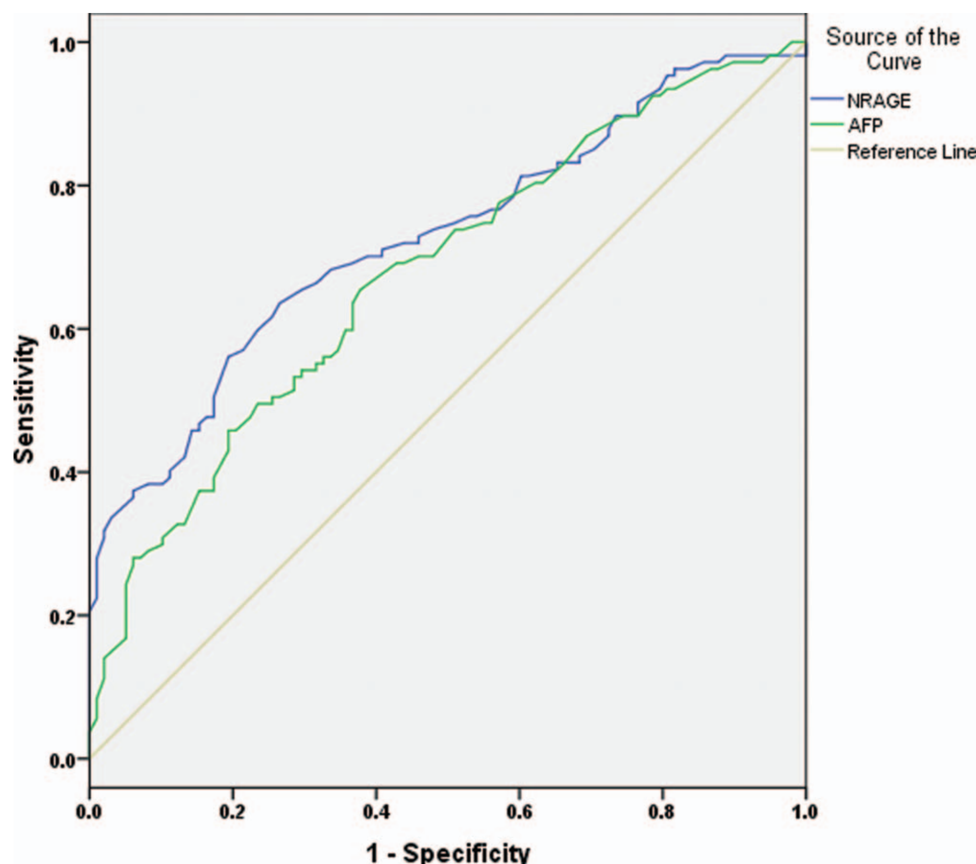

Figure 4. The clinical values of *NRAGE* and AFP in discriminating between benign liver disease cases and patients with hepatocellular carcinoma (HCC). The diagnostic efficiency of *NRAGE* (AUC=0.726, with a sensitivity of 63.6% and a specificity of 73.5%) was higher than that of AFP (AUC=0.677, with a sensitivity of 64.4% and a specificity of 60.2%).

of *MT1M* and *MT1G* promoters could be used as a serum biomarker for HCC diagnosis.[21] Serum *miR-143* and *miR-215* was observed to be upregulated in patients with HCC, suggesting their potential as circulating biomarkers due to their reasonable sensitivity and specificity.[22] Serum OPN levels were much higher in patients with HCC than in healthy subjects, and ROC analysis indicated that OPN was a useful diagnostic biomarker for HCC.[23] Serum DKK1 levels were significantly higher in patients with HCC than in controls, and further analysis indicated that *DKK1* together with AFP could improve the early diagnosis of HCC.[24] Besides, the serum levels of chromogranin A[25] and fibronectin[26] have also been reported to hold the capacity for HCC diagnosis. Recently, Gao and Song showed that the combined application of AFP, AFP-L3, and PIVKA could significantly improve HCC early detection.[27] Early article revealed upregulated expression of *NRAGE* in patients with HCC[28]; however, whether *NRAGE* could be used as a diagnostic marker was not further investigated. The present study was aimed to explore potential diagnostic role of *NRAGE* in HCC.

The *NRAGE*, identified by Salehi et al[13] in a 2-hybrid screen as bait, is a signaling cascade component that mediates apoptosis through interacting with p75NTR to antagonize its association with nerve growth factor receptor tropomyosin receptor kinase A. *NRAGE* is expressed in almost all adult tissues,[29] and may be an apoptosis and cell death stimulator.[12,13,30] Increasing reports have demonstrated that *NRAGE* promotes apoptosis through the ubiquitination of AATF,[31,32] therefore indicating that *NRAGE* functions as a tumor suppressor via inducing tumor cell apoptosis. Chu et al reported that *NRAGE* overexpression in melanoma and pancreatic cancer cells could significantly suppress the metastasis of the tumor cells in vitro and in vivo.[33] Du et al demonstrated that *NRAGE* played important roles in regulating the proliferation, migration and invasion of breast cancer cells. And enhanced *NRAGE* expression could reverse malignant phenotypes of breast cancer cells.[34] However, *NRAGE* overexpression promotes the proliferation and migration of esophageal cancer cells through interacting with PCNA.[35] In addition, a genome-wide association study demonstrated that *NRAGE* initiation through JNK pathway was correlated with the death of nonsmall-cell lung cancer cells.[36] Xue et al proved a relationship between enhanced *NRAGE* expression and increased radioresistance of esophageal carcinoma cells.[37] All the above mentioned studies indicate that *NRAGE* functions as either an inhibitor or promoter depending on cell types.

In the present study, we found that serum *NRAGE* expression was significantly upregulated in patients with HCC compared with benign liver disease cases and with healthy controls. The result was in line with those from a previous study reported by Shimizu et al which showed that *NRAGE* expression affected HCC progression via its interaction with AATF.[28] In addition, *NRAGE* was strongly related to TNM stage as well, suggesting the involvement of its abnormal expression in the development of HCC. Additionally, *NRAGE* expression could distinguish patients with HCC from benign liver disease cases and from

healthy individuals, suggesting its application value in HCC early diagnosis. Currently, AFP is widely used as a serum biomarker for HCC diagnosis. In our study, we compared the clinical values between AFP and *NRAGE* for HCC early diagnosis. The results demonstrated that both of them could discriminate between benign liver diseases and HCC. The diagnostic sensitivity and specificity in HCC early detection for *NRAGE* was 63.6% and 73.5%, with an AUC value of 0.726. Meanwhile, the AUC value for AFP was 0.677, with a sensitivity of 64.4% and a specificity of 60.2%. The AUC values and sensitivity were similar between AFP and *NRAGE*, but *NRAGE* exhibited higher diagnostic specificity. Thus, *NRAGE* might be better than AFP in HCC early detection. Therefore, *NRAGE* might be a specific and sensitive biomarker for HCC, potentially improving the malignancy diagnosis.

In conclusion, *NRAGE* is significantly upregulated in patients with HCC and associated with the development of HCC, possibly be a novel diagnostic biomarker for HCC early detection. Future multicenter clinical studies should be performed to verify our findings.

Author contributions

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Validation: Junfeng Cui, Zhong Ren.
Visualization: Zhong Ren.
Writing – original draft: Zhong Ren.
Writing – review & editing: Zhong Ren.

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