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ORIGINAL ARTICLE

High Expression of Neuropilin-1 Associates with Unfavorable Clinicopathological Features in Hepatocellular Carcinoma

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Abstract As a co-receptor for vascular endothelial growth factor (VEGF), Neuropilin-1 (NRP-1) plays an important role in angiogenesis and malignant progression of many human cancers. However, the role of NRP-1 in hepatocellular carcinoma (HCC) is not well understood. The study aimed to detected the expression of Neuropilin-1 in HCC and investigate the association between its expression and the clinicopathological characteristics and prognosis of HCC. Quantitative real-time PCR (qRT-PCR), Western blot, Immunofluorescence and immunohistochemistry (IHC) analyses were performed to characterize the expression of NRP-1 in HCC cell lines and tissues. The association of NRP-1 expression with the clinicopathological characteristics and the prognosis was subsequently assessed. qRT-PCR and Western blot assays revealed that the expression of NRP-1 in HCC was significantly increased relative to that of normal live cells and tissues ($P < 0.05$,and <0.001, respectively). In addition, high expression of NRP-1 was significantly associated with intrahepatic metastasis ($P = 0.036$), Edmondson grade ($P = 0.007$), TNM classification ($P = 0.0031$), and portal vein invasion ($P = 0.004$). Furthermore, the HCC patients with high NRP-1 expression had shorter overall survival (OS), and recurrence-free survival (RFS), whereas, patients with low NRP-1 expression had better OS and RFS ($P = 0.0035$, and 0.0048, respectively). These data indicate that NRP-1 expression may play an important role in the progression of HCC, and that high NRP-1 expression suggests unfavorable clinicopathological characteristics and survival in HCC patients.

Keywords Hepatocellular carcinoma · Neuropilin-1 · Expression · Clinicopathology

Introduction

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, is the third leading cause of cancer mortality in the world, and the second in China [1, 2]. Although significant improvement in diagnosis and treatment of HCC has been achieved in the last two decades, HCC prognosis remains poor because of the high recurrence and metastasis rates [3], the overall 5-year survival rate of HCC patients has recently been reported to be only 16 % [4]. At present, alpha-fetoprotein (AFP) is the most acknowledged biomarker for early detection and the follow-up of HCC patients during treatment [5]. However, due to the existence of AFP-negative HCC patients, studies that focus on novel biomarkers correlating with the clinicopathological features and prognosis are urgently needed and of great importance.

Neuropilin-1 (NRP-1), which was originally identified as a semaphoring receptor for mediating neuronal guidance and axonal growth [6], is expressed on endothelial cells and acts as a co-receptor for vascular endothelial growth factor receptor (VEGFR), thereby being implicated in VEGF-induced angiogenesis [7]. NRP-1 is reported to be upregulated in several tumors, including neuroblastomas, astrocytomas, breast, prostate, lung, pancreas, bile duct, gastric, and colon cancers [8, 9]. Overexpression of NRP-1 is closely correlated with the

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infiltration and migration of tumors [9, 10]. **Bergé M**, et al. [11] detected the expression of NRP-1 in a mouse transgenic model of HCC, the results proved that increased NRP-1 expression in hepatocellular carcinoma tumor cells was significantly associated with primary HCC, its level was increased with disease progression, and blocking NRP-1 function with peptide N leaded to the inhibition of tumor growth in HCC mice. Cheng W, et al. [9] found that high expression of NRP-1 was an independent prognostic factor for overall survival in the patients with bladder cancer. Our previous studies showed that NRP-1 was highly expressed on the membrane of glioma cells and tissue, and high expression of NRP-1 was significantly associated with the grade of glioma [12]. However, the expression patterns and involvement of NRP-1 in HCC patients are not completely understood. Therefore, the aim of this study was to investigate the clinical significance of NRP-1 expression in HCC.

Methods

Materials

Anti-NRP-1 mAb (A6) was produced by our lab (designed by Dr. J. Yan) [13]. HRP labeled Goat anti-mouse IgG secondary antibody and FITC labeled goat anti-mouse IgG secondary antibody were obtained from Sigma-Aldrich (St Louis, MO, USA). Hoechst 33,258 was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Three human HCC cell lines (Bel-7402, SMMC-7721, and HepG2), and one human normal hepatic cell line (LO2) were purchased from Shanghai Cell Repository of Chinese Academy of Science (Shanghai, China). ELISA Reader was purchased from Bio-Rad, Inc. (Hercules, CA, USA); Centrifuge 5415R from Eppendorf, Inc. (Barkhausenweg, Hamburg, Germany); Image Station 4000R Molecular imaging system from Kodak, Inc. (Rochester, New York, USA); FV1000 laser scanning confocal microscope from Olympus, Inc. (Melville, New York, USA). The study protocol was approved by the Ethics Committee of Zhongshan Hospital Xiamen University. Written informed consent was acquired from all of the patients who were enrolled in this study.

Patient Tissue Samples and Follow-up

A total of 16 fresh HCC and matched adjacent normal liver tissue specimens were obtained from patients who underwent surgery between March 2013 and May 2014 in Zhongshan Hospital Xiamen University. The fresh tissue specimens were immediately frozen in liquid nitrogen until use. For immunohistochemical assay, a total of 105 paraffin-embedded, archival HCC tissues and matched adjacent normal liver tissues were collected from our hospital between July 2004 and January 2013. Before surgical therapy, none of the patients had received chemotherapy, radiotherapy or immunotherapy. Important clinical data, such as gender, age, tumor size, hepatitis B virus infection, serum AFP, intrahepatic metastasis, Edmondson grade, TNM classification, portal vein invasion, and follow-up data, were collected for further analyses. The TNM classification of all HCC samples was confirmed according to the 2002 American Joint Committee on Cancer/International Union Against Cancer TNM staging system [14].

Patients were followed up by clinic visit every 2 months during the first postoperative year and at least every 3–6 months thereafter, and received follow-up examination including liver function, serum AFP, and abdomen ultrasonography. If recurrence was suspected, CT or MRI scanning was performed immediately. Overall survival (OS) and recurrence-free survival (RFS) were defined as the interval between surgery and death or recurrence. If recurrence was not diagnosed, patients were censored on the date of death or the last follow-up assessment.

Cell Culture

Three human HCC cell lines (Bel-7402, SMMC-7721, and HepG2), and one human normal hepatic cell line (LO2) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, California, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco, Grand Island, New York, USA) and 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). The cells were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C, with the medium changed every two days. A 70-80 % confluent monolayer was detached by 0.1 % trypsin (Gibco) and dissociated into a single cell suspension for further cell culture.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from four cell lines (Bel-7402, SMMC-7721, HepG2, and LO2) or tissues using the TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA) according to the manufacturer's instructions. The cDNA was synthesized from 2 μg of total RNA from each sample using MMLV transcriptase (ToYoBo, Shanghai, China) with random hexamers. qRT-PCR were performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China). The sequences of the primers were as follows: human NRP-1 forward 5′-AAATGCGAATGGCTGATTCAG-3′, and reverse 5′-CTCCATCGAAGACTTCCACGTAGT-3′ [15]; and human β-actin forward 5′-CAAGAGATGG CCACGGCTGCT-3′, and reverse 5′-TCCTTCTGCATCTGTCGGCA-3′. The qRT-PCR procedures were described in our previous publication [16, 17]. Brief, the template cDNA was first denatured at 94 °C for 2 min. The amplification step included 35 cycles of

denaturation for 30 s at 94 °C, 30 s of annealing at 60 °C, and elongation at 72 °C for 30 s. The final extension step was at 72 °C for 7 min. All qRT-PCRs were performed in duplicate. The human β-actin gene was used as the endogenous control. All cDNA samples were normalized to the β-actin endogenous control; $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification of NRP-1 mRNA expression..

Western blot

Cells were lysed with lysis buffer (200 mM Tris-HCl (pH 7.5), 1.5 M NaCl, 10 mM EDTA, 25 mM sodium pyrophosphate, 10 mM glycerophosphate, 10 mM sodium orthovanadate, 50 mM NaF,1 mM PMSF, in combination with protein inhibitor cocktail). Cell lysates (40 μg) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Blots were probed with primary A6. After extensive washing, blots were then incubated with goat anti-mouse IgG HRP secondary antibody and visualized by chemiluminescence. β-actin blotting was used as an internal control.

Cellular Immunofluorescence Staining

Cellular immunofluorescence staining was performed as previously described [12]. Briefly, the cell-seeded coverslips were washed and fixed. The diluted (1:100) A6 and the diluted (1:50) fluorescence (TRITC)-labeled secondary antibody were Added. After the BEL-7402, SMMC-7721, and HepG2 HCC cells were fluorescence-labeled, the fluorescence-labeled secondary antibody was discarded, eluted and stained with Hoechst 33,258 staining solution, then observed under a confocal scanning microscope, in which, the excitation wavelength for Hoechst 33,258 was about 350 nm and the emission wavelength was about 460 nm; while the maximum absorption wavelength of light for TRITC was 550 nm and the maximum emission wavelength was 620 nm. The relevant images were shot.

Immunohistochemistry

The expression of NRP-1 in 105 HCC tissues and matched adjacent normal liver tissues was examined by immunohistochemistry (IHC). The samples were fixed by formalin, embedded in paraffin and cut into 4 μm thick sections. IHC analysis was performed as described previously [12]. In short, the tissue chip was dewaxed and hydrated. Endogenous peroxidase was inactivated with 0.3 % H_2O_2 for 10 min. The sections were microwaved for antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 20 min and were incubated with a primary A6 antibody (1:100 dilution) overnight at 4 °C. Subsequently, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody for 30 min at room temperature. Then according to the following steps: color-developing, redyeing, dehydration, making transparent and sealing.

Results determination: The degree of immunostaining was evaluated by two independent observers who were blind to the clinical data of the patients. Based on previous reports [9, 18], the expression of NRP-1 was scored according to intensity and percentage of NRP-1 positive cells. Staining intensity was scored as follows: absent staining (negative, 0), weak staining (1), moderate staining (2), and strong staining (3). The percent of positive cell was also scored following 4 categories, in which 1 was given for 0–10 %, 2 for 11–50 %, 3 for 51–80 %, and 4 for 81–100 %. The two scores were then multiplied to calculate the final score. NRP-1 expression was considered low if the final score was equal to or less than three; otherwise, NRP-1 expression was considered high.

Statistical Analysis

The statistical analyses were performed using the SPSS 18.0 (SPSS Company, Chicago, IL, USA). Comparisons of NRP-1 mRNA expression between the HCC cell lines and human normal hepatic cell lines were done using two tailed Student's t test. The correlations between the NRP-1 expression and clinicopathologic characteristics were analyzed with the chi-square test. The survival analyses was calculated by Kaplan-Meier method and compared by log-rank test. The significance level for statistical analysis was set at $P < 0.05$.

Results

Detection of NRP-1 Expression in HCC Cell Lines by Western blot and qRT-PCR Analysis

To explore the potential role of NRP-1 in HCC progression, firstly, Western blot assay was performed to determine the protein level of NRP-1 in three HCC cell lines with different metastatic capability including low metastatic cell lines HepG2 and SMMC-7721, high metastatic cell line Bel-7402 and one human normal hepatic cell line (LO2). As shown in Fig. 1a, b , Western blot results indicated that protein expression of NRP-1 was detected in three HCC cell lines with high intensities compared with extremely low expression of NRP-1 in LO2 (Fig. 1a). The relative protein level of NRP-1 in HCC was significantly higher than that of LO2 (1.20 ± 0.05, 0.99 ± 0.10, 0.7 ± 0.03, 0.34 ± 0.02, respectively, $P < 0.05$) (Fig. 1b). To elucidate the mechanism responsible for expression level of NRP-1 protein in these cell lines, we next examined the NRP-1 mRNA expression levels by qRT-PCR. Consistent with the results obtained from protein level, NRP-1 mRNA expression was significantly increased in Bel-7402, SMMC-7721, and HepG2 cell lines (48.83 ± 2.32, 34.64 ± 2.40, 12.08 ± 1.09, respectively) relative to that of

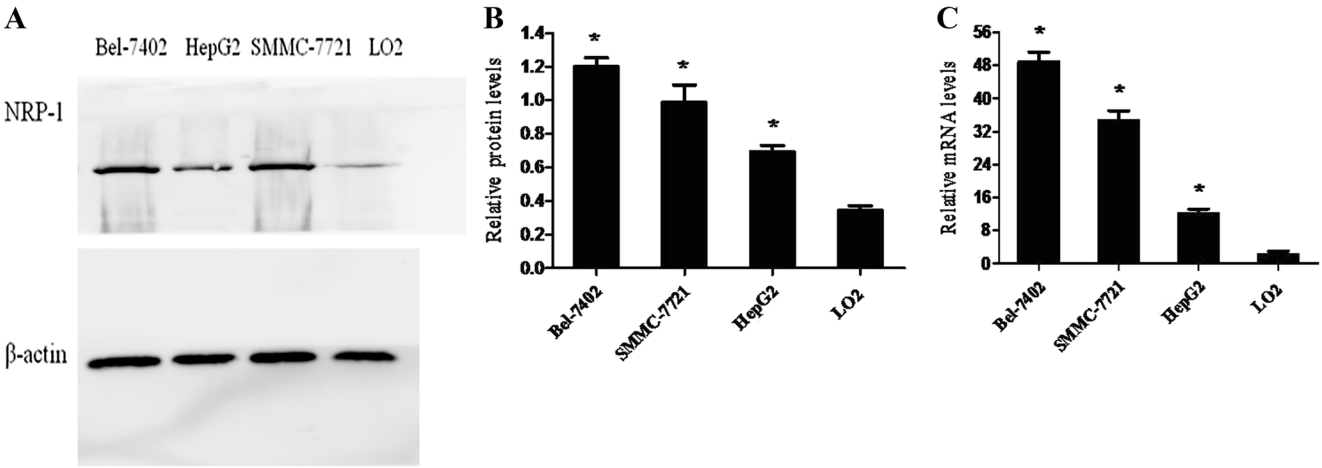

Fig. 1 NRP-1 expression in three HCC cell lines and one non-cancerous cell line. Western blot (**a**, **b**), and qRT-PCR (**c**) revealed that NRP-1 expression in the HCC cell lines Bel-7402, SMMC-7721, and HepG2 are significantly increased relative to the human normal hepatic cell line LO2. Data shown are mean ± SD of three independent experiments. The protein (**b**) and mRNA (**c**) expression of NRP-1 were normalized to the β-actin internal control. *$P < 0.05$

LO2 (2.20 ± 0.80), (difference significant at $P < 0.05$) (Fig. 1 c). Furthermore, NRP-1 expression in HepG2 was lower than that of SMMC-7721, and Bel-7402 ($P < 0.05$). The expression level of NRP-1 progressively increased from LO2, and HepG2, SMMC7721, to Bel-7402. The results clearly showed that NRP-1 expression correlated with malignant potential of HCC cell lines we selected, suggesting that NRP-1 might involve in HCC initiation and aggressive progression.

Detection of NRP-1 Expression in the Surface of HCC Cell Lines by Immunofluorescence

Results for confocal immunofluorescence microscopy detection were shown in Fig. 2. When 350 nm light was used as excitation to detect the Hoechst 33,258 fluorescence, the nucleus of three HCC cells (Bel-7402, SMMC-7721, and HepG2) were labeled blue. When 488 nm light was used as excitation to detect FITC fluorescence, no green fluorescence was detected in the three groups without A6, but clear green fluorescence was detected in the three groups with A6. In overlaying fluorescent image, no green fluorescence was detected on the membrane of cells in the three groups without A6, but green fluorescence could be detected in the three groups with A6, which was mainly detected on the membrane. These results indicated that A6 could bind well with NRP-1 receptor on the surface of the Bel-7402, SMMC-7721, and HepG2 cells. So it could be concluded that NRP-1 was expressed in the three cell lines. However, the intensities of green fluorescence in the three HCC cell lines were different, the intensities of green fluorescence in Bel-7402, SMMC-7721cells were stronger than those of HepG2, suggesting that the density of NRP-1 expression in the three HCC cell lines was different, Bel-7402 and SMMC-7721cells had more NRP-1 expression relative to HepG2.

Detection of NRP-1 mRNA and Protein Expression in HCC Tissues by qRT-PCR and IHC

Firstly, the expression of NRP-1 mRNA were detected and analyzed in 16 fresh HCC tissues and matched adjacent normal liver tissues by qRT-PCR. As shown in Fig. 3, the NRP-1 mRNA expression in HCC tissues was significantly higher than that of matched adjacent normal liver tissues (3.28 ± 0.35 vs. 0.76 ± 0.23, $P < 0.001$).

Next, IHC was executed to evaluate the NRP-1 protein expression in 105 paraffin-embedded, archival HCC and matched adjacent normal liver tissue samples. High NRP-1 expression was exhibited in 56 of 105 (53.3 %) HCC tissue samples, whereas only 22 cases of 105 matched adjacent normal liver tissues (21.0 %) showed high NRP-1 expression (Table 1).There was significant difference in high expression rate of NRP-1 protein between HCC tissues and matched adjacent normal liver tissues ($P < 0.05$). Positive staining was mainly detected in the membrane of HCC cells and the representative IHC staining for NRP-1 protein expression in HCC are shown in Fig. 4.

Correlation of NRP-1 Protein Expression with the Clinicopathologic Characteristics

To better understand the clinical significance of NRP-1 expression in HCC, we investigated the association between NRP-1 protein expression and clinicopathological characteristics. As shown in Table 2, high NRP-1 expression was significantly positively correlated with intrahepatic metastasis ($P = 0.036$), Edmondson grade ($P = 0.007$), TNM classification ($P = 0.0031$), and portal vein invasion ($P = 0.004$). In comparison, no significant relationship was demonstrated between NRP-1 expression and other clinical features, including gender, age, tumor size, hepatitis B virus infection (all $P > 0.05$).

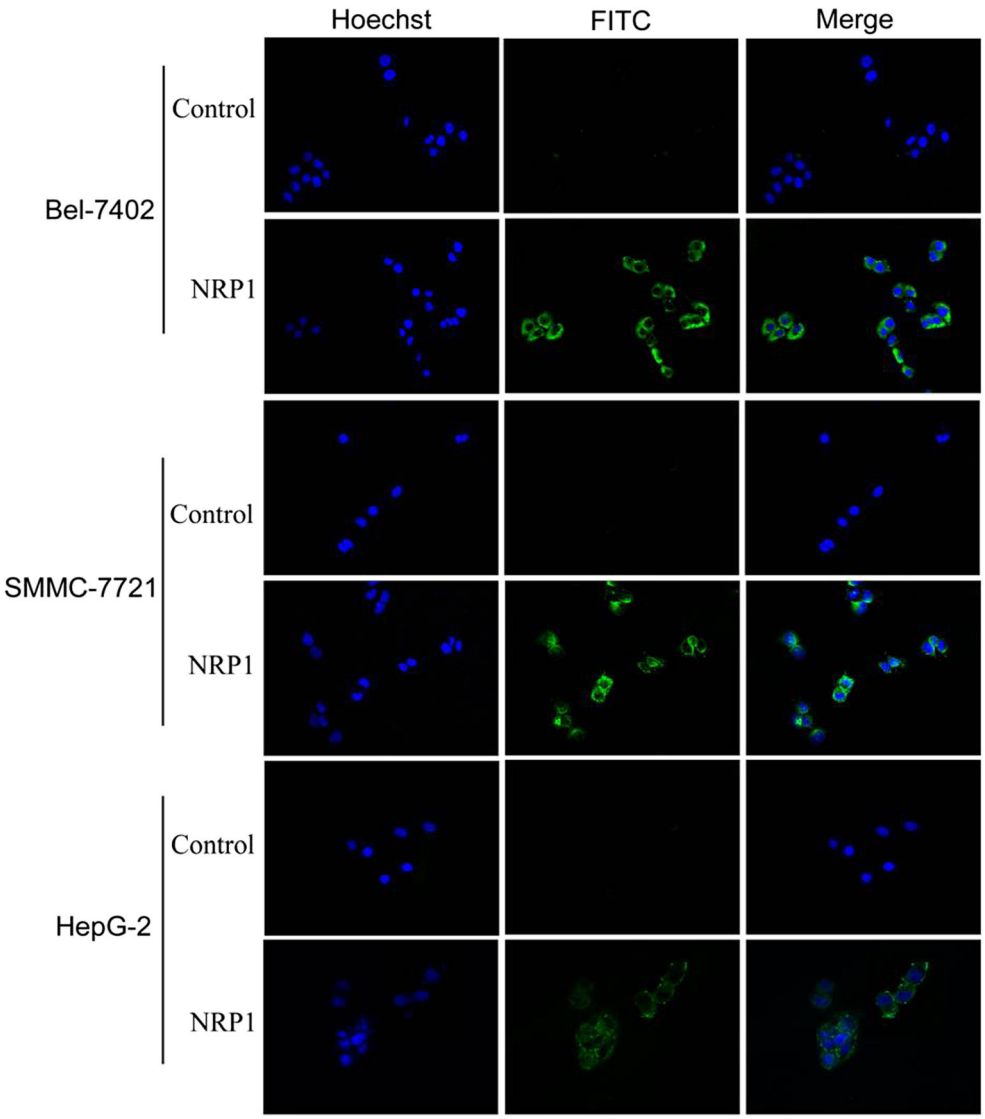

Fig. 2 The immunofluorescence of three HCC cell lines. NRP-1 was expressed on the three HCC cell lines at various density. The density of NRP-1 progressively increased from HepG2, SMM-7721 to Bel-7402

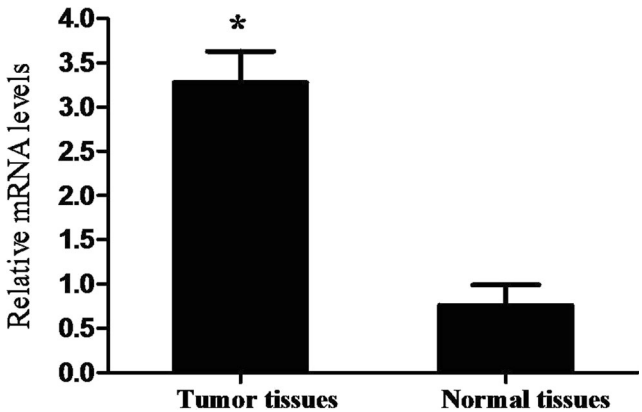

Fig. 3 Expression levels of NRP-1 mRNA in HCC tissues and matched adjacent normal liver tissues. qRT-PCR demonstrated that the expression of NRP-1 in HCC tissues were significantly higher than that in adjacent normal liver tissues (*$P < 0.001$), when normalized to the β-actin internal control

Correlation of NRP-1 Protein Expression with the Prognosis of HCC Patients

Kaplan-Meier survival curves were used to evaluate the effects of NRP-1 protein level on the prognosis of HCC patients. They showed that overall survival (OS) and recurrence-free survival (RFS) were significantly lower in patients with high NRP-1 expression than in those with low NRP-1 expression

Table 1 Expression NRP-1 in the HCC tissues and matched adjacent normal liver tissues

	No. of patients	NRP-1 protein	
	Cases n	Low expression n (%)	High expression n (%)
Tumor tissues	105	49 (46.7)	56 (53.3)
Normal tissues	105	83 (79.0)	22 (21.0)

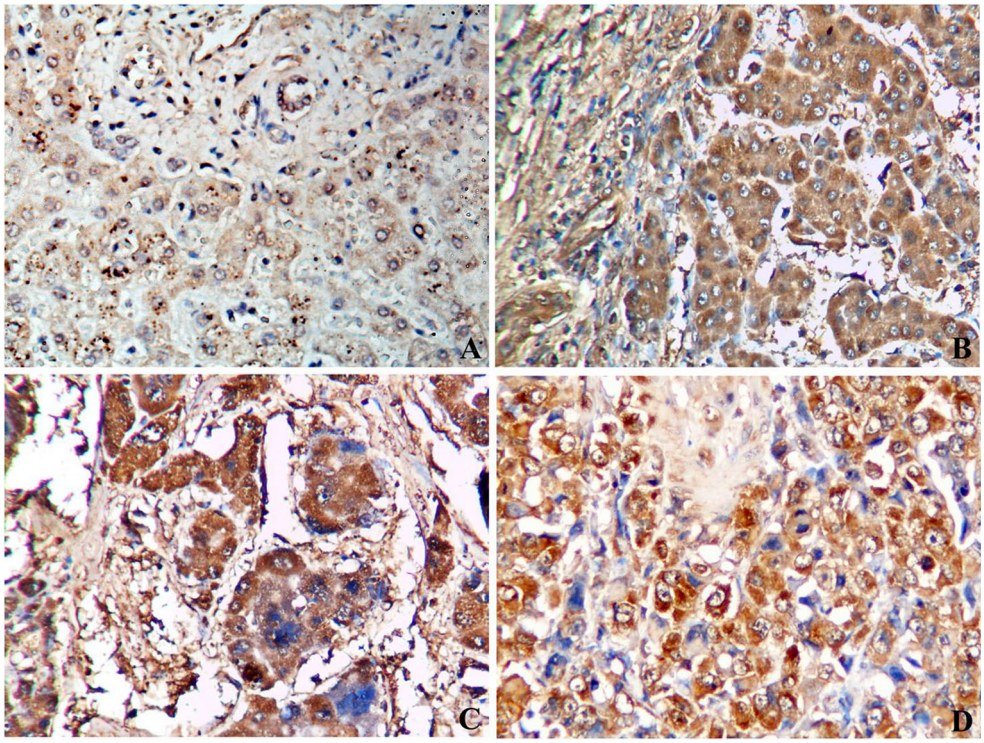

Fig. 4 Differential expression of NRP-1 in HCC tissues. **a** Negative staining of NRP-1 in HCC tissue; **b** Weak staining NRP-1 in HCC tissue; **c** Moderate staining of NRP-1 in HCC tissue; **d** Strong staining of NRP-1 in HCC tissue. Original magnification, × 400

(P = 0.0035, and 0.0048, respectively, Fig. 5a, b). These results indicated that NRP-1 expression was significantly correlated with HCC death and recurrence.

Discussion

HCC is the fifth most common cancer globally and has a poor prognosis mainly due to the high incidence of postoperative metastasis and recurrence. In recent years, although many efforts have been made to understand the molecular mechanisms underlying its invasion and metastasis, the detailed mechanisms involved in HCC malignancy and metastasis are not explicit. The identification of dependable tumor molecular biomarkers for HCC is critical to design personalized treatments and predict prognoses in HCC patients. A large body of evidence indicates that NRP-1 plays an essential role in the tumor growth and metastasis by regulating angiogenesis [8–10]. Bergé M, et al. [11] had detected NRP-1 expression in human HCC tissue microarrays, no further valuable information was applied for its clinical and prognostic significance for HCC patients. So far, the detailed mechanisms of NRP-1 underlying HCC progression have not been well elucidated.

The major finding of this study was that the overexpression of NRP-1 is associated with the progression of HCC. Furthermore, this study also demonstrated that NRP-1 mRNA and protein expression increases as tumor progresses to advanced clinicopathological characteristics and confers to the unfavorable prognosis in HCC patients.

In this study, we first investigated NRP-1 protein and mRNA expression in cell lines by Western blot and qRT-PCR analysis. The results revealed that elevated NRP-1 expression in three HCC cell lines (Bel-7402, SMMC-7721, and HepG2) compared to one human normal hepatic cell line (LO2), and the level of NRP-1 expression paralleled with malignant potential of HCC cell lines. These results illustrated a close association of NRP-1 expression with HCC initiation and progression. Next, the immunofluorescence showed that NRP-1 MAb could be well bound to NRP-1 on the surface of Bel-7402, SMMC-7721, and HepG2 cell lines and it could be positioned on the membrane. Subsequently, the expression of NRP-1 mRNA in fresh HCC tissues and matched adjacent normal liver tissues was evaluated by qRT-PCR. The results suggested a significantly higher level of NRP-1 expression in HCC tissues than that of matched adjacent normal liver tissues. Moreover, IHC analysis was performed to further prove that NRP-1 protein expression in HCC is also higher than that of matched adjacent normal liver tissues, with statistical significance. In a report by Bergé M, et al. [11], a high NRP-1 expression was detected in HCC endothelial cells lining the higher order vessels, whereas no or low expression was found in normal sinusoidal endothelial cells. Interestingly, NRP-1 was identified as a specific marker in determining the arterial or venous identity of blood vessels [19]. The upregulation of NRP-1 in HCC is, therefore, consistent with the enhancement of arterial blood supply in HCC and supports a phenotypic switch of hepatic vasculature towards the arterial phenotype. These data are consistent with our results and support our

Table 2 Relationship between NRP-1 expression in cancerous tissues and clinicopathological characteristics in HCC

Characteristics	No. of patients	NRP-1 expression		X^2	*P* value
	Cases n	Low expression n (%)	High expression n (%)		
Total	105	49 (46.7)	56 (53.3)		
Gender					
Female	28	18 (64.3)	10 (35.7)	0.223	0.637
Male	77	31 (40.3)	46 (59.7)		
Age (years)					
≥ 50	36	19 (52.8)	17 (47.2)	0.882	0.365
< 50	69	30 (43.5)	39 (56.5)		
Tumor size (cm)					
≥ 5	43	22 (51.2)	21 (48.8)	0.591	0.442
< 5	62	27 (43.5)	35 (56.5)		
Hepatitis B virus infection					
Positive	84	41 (48.8)	43 (51.2)	0.775	0.379
Negative	21	8 (38.1)	13 (61.9)		
Serum AFP (μg/L)					
≥ 400	65	27 (41.5)	38 (58.5)	1.391	0.238
< 400	40	22 (55.0)	18 (45.0)		
Intrahepatic metastasis					
Yes	33	12 (36.4)	21 (63.6)	4.413	0.036*
No	72	37 (51.4)	35 (48.6)		
Edmondson grade					
I	23	16 (69.6)	7 (30.4)	9.975	0.007*
II	57	27 (47.4)	30 (52.6)		
III	25	6 (24.0)	19 (76.0)		
TNM classification					
Stage I ~ II	59	33 (55.9)	26 (44.1)	4.645	0.031*
Stage III ~ IV	46	16 (34.8)	30 (65.2)		
Portal vein invasion					
Yes	48	15 (31.3)	33 (68.7)	8.444	0.004*
No	57	34 (59.6)	23 (40.4)		

AFP, α-fetoprotein; *TNM*, Tumor-node-metastasis; *$P < 0.05$

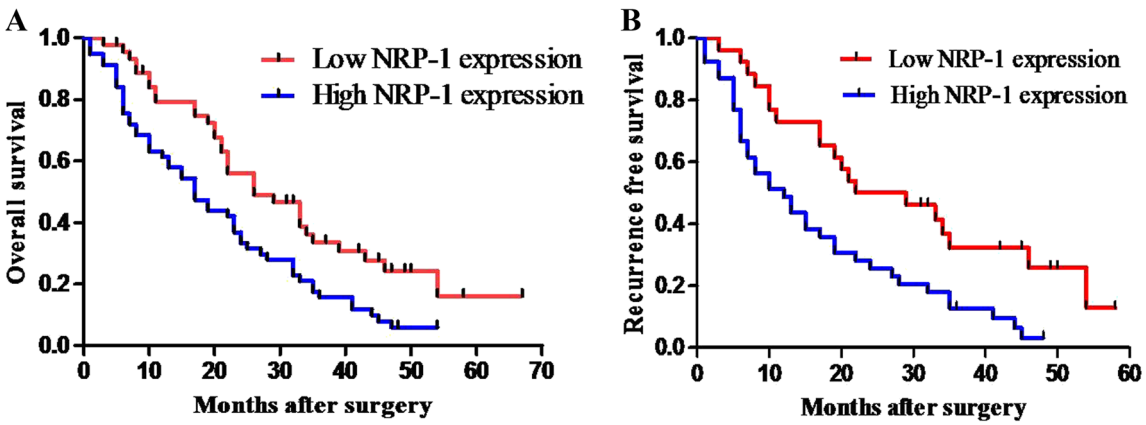

Fig. 5 Kaplan–Meier survival analysis of NRP-1 expression in HCC patients. **a** Overall survival rate in patients with high NRP-1 expression (*blue line*) was significantly lower than that in patients with low NRP-1 expression (*red line*) ($P = 0.0035$). **b** Recurrence free survival rate was significantly decreased in the patients with high NRP-1 expression (*blue line*) compared with the patients with low NRP-1 expression (*red line*) ($P = 0.0048$)

findings. More importantly, certain clinical parameters, including intrahepatic metastasis, Edmondson grade, TNM classification, and portal vein invasion were significantly correlated with the upregulation of NRP-1. Similarly, Zhu H, et al. [20] described that the upregulation of NRP-1 may be involved in the induction of local invasiveness of neoplasia and angiogenesis and have direct relevance to the progression of osteosarcoma. Xu Y, et al. [8] suggested that the enhanced expression of NRP-1 may be not only associated with oncogenesis, but also with nasopharyngeal cancer malignancy, and this molecule may be a targeting candidate for the treatment of nasopharyngeal malignancies. These findings suggest the possibility that upregulated expression of NRP-1 may provide a selective advantage in the HCC tumorigenic processes.

To our the best knowledge, there are no reports on the prognostic effect of NRP-1 on HCC patients. In the current study, we further investigated the correlation of NRP-1 expression with overall survival (OS) and recurrence-free survival (RFS) of patients. The patients with high NRP-1 expression exhibited unfavorable outcomes for both OS and RFS compared with those with low NRP-1 expression, suggesting that NRP-1 might be served as a novel prognostic biomarker for HCC patients. The unfavorable prognostic role of NRP-1 in HCC was similar to its prognostic effect on nasopharyngeal carcinoma [8], bladder cancer [9], and osteosarcoma [20]. However, Zhuang PY et al. [21] found that peritumoral NRP-1 expression was significantly higher than that of the tumoral tissue, and high peritumoral expression of NRP-1 prolonged time to recurrence (TTR) and extended OS of HCC patiients. Moreover, peritumoral NRP-1 expression was negatively correlated with peritumoral hypoxia, tumoral and peritumoral MVD (microvessel density), primary tumor size, and satellite lessions. These results indicated that abundant peritumoral NRP-1 expression may play a positive role by providing an infertile soil for endothelial cells and primary tumor and subclinical metastatic tumor cells. Furthermore, in colon cancer, Kamiya et al. [15] reported that the gene expression levels of NRP-1 in the tumor were significantly decreased compared to those in the extraneoplastic tissues, and the preserved NRP-1 expression provides colon cancer patients with a better prognosis. These results suggested that the effect of NRP-1 may be cancer type specific and the abnormal expression of NRP-1 may play key roles in tumor progression and tumor prognosis.

Conclusions

Taken together, our data suggest that NRP-1 expression was significantly high in HCC, and that NRP-1 overexpression was markedly associated with tumor malignant progression and poor survival in patients with HCC. These results suggested that NRP-1 could be recognized as a novel biomarker for HCC prognosis and optimization of clinical treatments.

VEGF Vascular endothelial growth factor, *NRP-1* Neuropilin-1, *HCC* Hepatocellular carcinoma, *qRT-PCR* Quantitative real-time PCR, *IHC* Immunohistochemistry, *OS* Overall survival, *RFS* Recurrence-free survival, *AFP* Alpha-fetoprotein, *TNM* Tumor-node- metastasis.

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Authors' Contributions YZ, XS and JY conceived and designed experiments and interpreted data. YZ and XD performed experiments. YZ, PL, YJ, CM, QF, WW, FS, and HT completed patient follow-ups and data collection. YZ and XS wrote the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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