RESEARCH ARTICLE

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Hepatic Expression of *NTN4* and Its Receptors in Patients with Hepatocellular Carcinoma

Jyothi Vennela[1], Biju Pottakkat[1]*, Bala subramaniyan Vairappan[2], Surendra Kumar Verma[3], Victor Mukherjee[2]

Abstract

Background: Angiogenesis contributes to hepatocellular carcinoma (HCC) progression by promoting tumor growth and metastasis. Netrin-4 (NTN4) is a secreted glycoprotein that has been reported to control angiogenesis and preserve endothelial homeostasis. Macrovascular invasion of the portal vein, referred to as portal vein invasion (PVI) is associated with poor prognosis in HCC patients. In this work, we sought to understand more about the systemic and hepatic level expression of NTN4 and its receptors in HCC patients with and without portal vein invasion. **Methods:** A total of 154 patients with HCC, and 90 healthy volunteers were recruited in this case-control study. Patients with HCC were further subdivided into those with portal vein invasion (PVI) (n=68), and those without portal vein invasion (NPVI) (n=86). Clinical characteristics and liver function parameters were recorded among the study subjects PVI and NPVI. The serum levels of NTN4 (pg/ml) were estimated by ELISA. HCC tissues and normal non-tumorous liver tissues (controls) were collected for gene expression analysis of NTN4 and its receptors. **Results:** ALT, ALP, and GGT levels were significantly elevated in the serum of HCC patients with PVI compared to NPVI and control subjects. Systemic NTN4 was significantly reduced in both PVI and NPVI patients compared to control subjects. At the tissue level, the hepatic NTN4 followed a similar trend with significantly lower mRNA expression in both patients with PVI and NPVI compared to control subjects. **Conclusions:** Systemic and hepatic NTN4 levels were reduced in both PVI and NPVI subjects. The hepatic expression of NTN4 receptors Neogenin and UNC5B were markedly elevated in patients with HCC with PVI compared to NPVI. Future experimental studies might shed the role of NTN4 and its receptors in the development of PVI in HCC.

Keywords: Angiogenesis- Hepatocellular carcinoma- Netrin-4- Portal vein invasion- Biomarker

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Introduction

Hepatocellular carcinoma (HCC), a primary liver malignancy, is one of the leading causes of cancer-related deaths worldwide (Llovet et al., 2021). Indeed, with a 5-year survival rate of 18%, it is second only to pancreatic cancer (Llovet et al., 2021). By the year 2025, it is estimated by the International Agency for Research on Cancer that more than 1 million individuals worldwide will be affected by HCC every year (Llovet et al., 2021; McGlynn et al., 2021). Owing to its multiple risk factors, including infection with hepatitis viruses, exposure to carcinogens, chronic alcohol abuse, and metabolic syndrome, HCC is a highly heterogeneous tumor, and its management has evolved into a global health challenge. HCC is a highly vascular solid tumor, where angiogenesis plays a vital role in disease progression (Yang and Poon, 2008). Being an aggressive malignancy, early identification is paramount in improving the long-term survival of patients. Despite rigorous surveillance through biochemical monitoring and clinical imaging, many cirrhotic patients already have advanced-stage HCC when they are initially diagnosed (Yang and Poon, 2008).

The maximum tumor diameter (MTD), the total number of tumors, the presence of portal vein thrombosis (PVT), and the quantity of alpha-fetoprotein (AFP) in the blood all affect the prognosis of HCC patients. PVT is the most significant tumor factor in HCC, as it represents tumor aggressiveness (migration, invasion, and propensity for metastasis), restricts the alternatives for curative surgery or transplantation, and may also deteriorate remaining liver function (Akkiz et al., 2018). Like metastasis, multiple tumors, high tumor markers, and cirrhosis, portal vein invasion (PVI) is also a feature of advanced disease. PVI, along with metastases, numerous tumors, elevated tumor markers, and cirrhosis, is a sign

*[1]Department of Surgical Gastroenterology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. [2]Department of Biochemistry, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. [3]Department of Pathology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. *For Correspondence: bijupottakkat@gmail.com*

of advanced disease (Sinn et al., 2021).

Notably, the angiogenic status correlates heavily with the prognosis of the disease (Yang and Poon, 2008). In HCC, neoangiogenesis is promoted and maintained primarily by vascular endothelial growth factor (VEGF) and angiopoietins (Moawad et al., 2020). Netrins are a laminin-related, extracellular, conserved family of proteins that have been widely reported for their role in controlling cell and axonal guidance during embryogenesis (Moore et al., 2007). Netrin-4 (NTN4), a member of the netrin family and a glycoprotein from endothelial cells, has been identified to play a role in the regulation of endothelial proliferation which promotes angiogenesis or anti-angiogenesis (Bruikman et al., 2019; Eveno et al., 2013; Lejmi et al., 2014; Nojima et al., 2018; Xu et al., 2017). The regulation of angiogenesis by NTN4 has been reported to happen owing to its ability to engage with various NTN4 receptors such as DCC, UNC5H, and Neogenin (Reuten et al., 2016). Emerging evidence indicates the inhibitory role of NTN4 in angiogenesis and showed decreased expression in a variety of malignancies affecting breast, pancreas, prostate, cervical, and colon cancers (Esseghir et al., 2007; Eveno et al., 2011; Eveno et al., 2013; Latil et al., 2003; Liu et al., 2019; Xu et al., 2017; Zhang et al., 2013). However, as a bi-functional modulator in the angiogenic process, NTN4 exhibits both pro-and anti-angiogenic activity that differs according to the micro-environment (Eveno et al., 2011; Eveno et al., 2013; Lejmi et al., 2008). Although the expression of NTN4 in various cancers is studied in animals, the significance of its involvement in humans remains uncertain. Very little information is available on the role of NTN4 in HCC. Nacth et al. noted that the in vitro growth of the liver tumor cell line (Hep3B) was found to be inhibited by NTN4 (Nacht et al., 2009). There are presently no studies that have explored the expression of NTN4 and its receptors in human HCC. Due to its close involvement in regulating angiogenesis and cell proliferation, which are key pathophysiological factors dysregulated in HCC, NTN4 could likely play an important role in PVI. The present study aims to explore the alterations in the expression of NTN4 and its receptors in patients with HCC with PVI and NPVI.

Materials and Methods

Study procedure

154 consecutive patients with HCC were recruited in the study and compared with healthy controls (n=90). Patients with HCC were further subdivided into those with portal vein invasion (n=68), and those without portal vein invasion (n=86). This case-control study was conducted in the Surgical Gastroenterology, Biochemistry, and Pathology departments from October 2017 to June 2022 at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER). The study was conducted after obtaining approval from the Institute's Ethics Committee for human studies based on the Ethical Guidelines of the Helsinki Declaration and the intramural fund was utilized for human studies (JIP/IEC/2017/0126). The purpose of the study was explained to the eligible participants and written informed consent was obtained.

Sample and data collection

Socio-demographic characteristics, clinical characteristics, behavioral characteristics, comorbidity, treatment history, and outcome were recorded. About 5ml of venous blood sample was collected and centrifuged at 3500 rpm for 10 minutes. The serum was separated, routine biochemistry tests were performed immediately, and the remaining samples (serum) were stored at -80°C for NTN4 measurements. HCC tissues and normal non-tumorous liver tissues (controls) were collected for gene expression analysis. HCC patients in whom biopsy was not done and those not taken up for surgery were excluded from tissue-based studies. Similarly, patients with suspicious diagnoses of HCC, patients with a rare variance of HCC like fibrolamellar carcinoma, patients who were critically ill patients, and patients in whom a Computed Tomography (CT) scan was not done were also excluded from the study.

Measurement of liver function markers and NTN4

The serum was examined by AU680 Beckman Coulter autoanalyzer (United States), for biochemical parameters including aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), albumin, total protein (TP), and bilirubin (Total) contents. At the time of diagnosis, the ADVIA Centaur CP immunoassay system (Siemens) was used to measure the serum AFP level using chemiluminescence. In patients with HCC and control subjects, the serum levels of NTN4 were determined by ELISA using a commercial kit (Genxbio Health Sciences Pvt. Ltd., India).

Gene expression studies by RT-PCR

The gene expression of NTN4, receptors of NTN4 (Neogenin, UNC5B, and DCC), and angiogenesis markers (VEGF and VEGFR) were measured among the patients with HCC tissues and normal non-tumorous liver tissues (controls). The RNA extraction was performed using an RNeasy Mini kit (Qiagen, Inc., USA). The procedure was performed according to the manufacturer's instructions. The RNA pellet was washed twice with 70% ethanol and dissolved in nuclease-free water. The concentration of the RNA isolated was estimated using a Nanodrop 2000/2000c Spectrophotometer. Briefly, 750ng of RNA template was mixed with PrimeScript RT reagent kit components, including Prime Script RT Enzyme Mix I, PrimeScript 5X buffer, Oligo dT primers, random hexamers, and RNase-free water. The reaction was set up in Bio-Rad s1000 thermal cycler with the incubation steps amplifying the first strand at 42˚C for 15 minutes and enzyme deactivation at 85˚C for 30 sec. The amplified cDNA with a purity of 1.8 was used as the template for qRT-PCR analysis using CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The reaction mixture comprised SYBR Premix Ex Taq II, 10µM of primers, and 200ng of cDNA template and was made up to 10µL using nuclease-free water. The expression of the target genes was calculated using $2^{-\Delta\Delta Ct}$, and normalized to the GAPDH housekeeping gene. The primers used in the study have been listed in Table 1.

Statistical analysis

GraphPad Prism 6.0 and 9.0 (San Diego, CA) were used to conduct the statistical analysis. Categorical variables were described using frequency (n) and percentage (%) while continuous variables were presented as mean ± SD or median [IQR]. The comparison between more than two groups was performed by one-way ANOVA followed by Tukey's multiple comparisons posthoc test or by the Kruskal-Wallis test. Statistical significance was defined as a p-value less than 0.05.

Results

Demographic features among patients with HCC with PVI, NPVI, and controls

Table 2 illustrates the demographic features among patients with HCC with PVI, NPVI, and controls. Among the 154 HCC participants, 68 were diagnosed with PVI and 86 were diagnosed as NPVI. The mean age of the study participants with PVI was higher than NPVI and control groups, and the difference between them was statistically significant ($p<0.001$). On post hoc analysis with the Bonferroni test, no significant association was found between the PVI and NPVI groups. About three-fourths (86.8%) of the male study participants were diagnosed with PVI and one-fourth (18.6%) of the female study participants were diagnosed with NPVI. Risk factors such as alcohol consumption and hypertension were found to be statistically significant between the groups ($p<0.001$, 0.010 respectively). More than half (51.2%) of the study participants with NPVI were diabetic.

Comparison of biochemical parameters in patients with HCC with PVI, NPVI, and controls

Table 3 illustrates the hepato-biliary profile of patients with HCC with PVI, NPVI, and controls. A significant increase in serum total bilirubin and direct bilirubin was detected in the NPVI group, whereas albumin was lower in PVI and NPVI groups compared to controls and was statistically significant. The total protein levels, AST, ALT, ALP, and GGT were significantly higher among the PVI and NPVI compared to controls. Post hoc Kruskal-Wallis one-way ANOVA (K-samples) showed that the difference in ALT (IU/L), ALP (IU/L), and GGT (IU/L) levels and was statistically significant between the PVI & NPVI groups. Whereas, statistical difference was not observed with AST (IU/L) and total protein levels between PVI & NPVI groups.

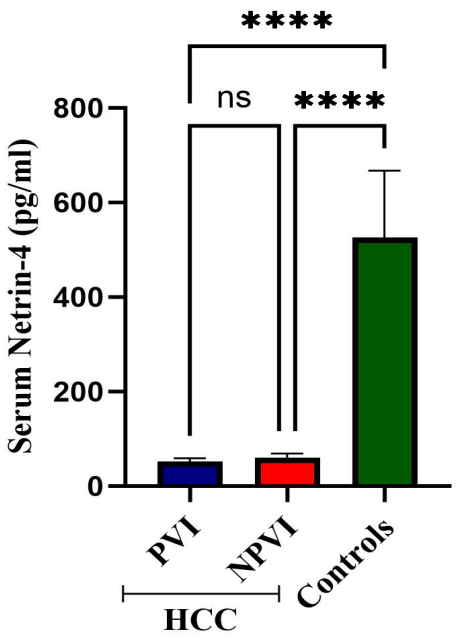

Figure 1. Serum NTN4 Levels in HCC Patients with PVI, NPVI, and Controls. Values are expressed as Median (IQR) (****$p<0.0001$, PVI vs. NPVI vs. controls) Graph Pad Prism Version 9.0.

Serum Levels of NTN4 in patients with HCC with PVI, NPVI, and controls

Compared to control subjects, the serum NTN4 levels

Table 1. Primers Used for Real-Time PCR

S.No	Gene		Primer sequence
1	*GAPDH*	Forward	AGCCACATCGCTCAGACAC
		Reverse	GCCAATACGACCAAATCC
2	*Netrin-4*	Forward	TCAGCACAACACAGAAGGACAGTATTG
		Reverse	GGATGGCAGGAACACGGTTTG
3	*Neogenin*	Forward	ATGGTGACCAAAGGTCGAAG
		Reverse	AGTCACATCCTTGGGTGGAG
4	*UNC5B*	Forward	ACTCATCTGCTGCCCTGACT
		Reverse	ATTTTGTCGGTGGAGTCCTG
5	*DCC*	Forward	ACTTGGGGTGGTGAAGTCAG
		Reverse	CCAAGACAGGGACCACATCT
6	*VEGF*	Forward	GCAGCTTGAGTTAAACGAACG
		Reverse	GGTTCCCGAAACCCTGAG
7	*VEGFR*	Forward	GAACATTTGGGAAATCTCTTGC
		Reverse	CGGAAGAACAATGTAGTCTTTGC

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; UNC5B, Unc-5 Netrin receptor B; DCC, deleted in colorectal cancer; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor and its receptor

Table 2. Comparison of Demographic Features among Patients with PVI, NPVI, and Controls

Variable	Category	Total	HCC (Cases)		Controls (n=90)	p-value
			PVI (n=68)	NPVI (n=86)		
Age Mean ± SD*			58.50 ± 12.79	56.44± 11.31	35.57± 7.63	<0.001
Gender# n (%)	Male	189	59 (86.8)	70 (81.4)	60 (66.7)	0.006
	Female	55	9 (13.2)	16 (18.6)	30 (33.3)	
Tobacco usage# n (%)	Yes	61	20 (29.4)	24 (27.9)	17 (18.9)	0.236
	No	183	48 (70.6)	62 (72.1)	73 (81.1)	
Alcohol Intake# n (%)	Yes	75	26 (38.2)	37 (43)	12 (13.3)	<0.001
	No	168	42 (61.8)	49 (57)	78 (86.7)	
Diabetic mellitus# n (%)	Present	71	17 (25)	44 (51.2)	10 (11.1)	<0.001
	Absent	173	51 (75)	42 (48.8)	80 (88.9)	
Hypertension# n(%)	Present	45	18 (26.5)	19 (22.1)	8 (8.9)	0.01
	Absent	199	50 (73.5)	67 (77.9)	82 (91.1)	

*, Mean ±SD; # , Frequency and percentage; PVI, portal vein invasion; NPVI, non-portal vein invasion; SD, standard deviation; n, number;%, percentage; p-value<0.05statistically significant

Table 3. Comparison of Liver Function Test Parameters among HCC Patients with PVI, NPVI, and Controls

Variable	HCC (Cases)		Controls	p-value	Post Hoc Kurskal-Wallis Test PVI vs NPVI
	PVI (n=68) Median (IQR)	NPVI (n=86) Median (IQR)	(n=90) Median (IQR)		
Total bilirubin (mg/dL)	1.09 (0.75-2.03)	1.46 (0.83-2.29)	0.54 (0.38-0.64)	0.001	1.00
Direct bilirubin (mg/dL)	0.39 (0.21-0.78)	0.4 (0.30-0.80)	0.16 (0.10-0.20)	0.001	0.957
Total protein (mg/dL)	7.06 (6.30-7.60)	6.9 (6.17-7.50)	6.6 (5.67-7.02)	0.011	0.987
Albumin (g/dL)	3.1 (2.65-3.60)	3.2 (2.77-3.50)	3.9 (3.20-4.30)	0.001	1.00
AST (IU/L)	87.5 (49.2-177.0)	63.5 (44.7-89.2)	23 (18-26.2)	0.001	0.214
ALT (IU/L)	52.5 (34.25-93.5)	38 (26.5-65.5)	22 (16.0-30.0)	0.001	0.015
ALP (IU/L)	207 (131.7-298.2)	133.5 (99.7-209)	91.5 (67 - 126.5)	0.001	0.001
GGT (IU/L)	142.5 (91 - 278.5)	69 (35.5-122)	22 (16-28)	0.001	0.001

AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; IQR, interquartile range; p-value <0.05

were significantly decreased in both HCC patients with PVI (23.50 (9.50-96.0) pg/ml) and NPVI (22.5 (7.75-85.75) pg/ml). Post hoc analysis confirmed the statistical significance between PVI vs. controls and NPVI vs. controls (Table 4) (Figure 1).

Gene expression analysis of NTN4 and its receptors

The mRNA expression of NTN4 in HCC tissues was significantly downregulated compared to non-tumorous liver tissues. Gene expression of NTN4 between PVI and NPVI groups of HCC was not significant (Figure 2A). The mRNA expression of Neogenin showed a significant increase in HCC liver tissues compared to non-tumorous liver tissues (Figure 2B). The mRNA expression of UNC5B was significantly upregulated in HCC liver tissues compared to non-tumorous liver tissues (Figure 2C). Notably, we found a significant elevation in the PVI group compared to the NPVI group for both Neogenin and UNC5B (Figures 2B and 2C). The mRNA expression of DCC showed significant downregulation in HCC liver tissues compared to non-tumorous liver tissues. However, mRNA expression of DCC between PVI and NPVI groups of HCC liver tissues was not statistically significant (Figure 2D).

Gene expression analysis of VEGF and its receptor

The mRNA expression of VEGF and VEGFR exhibited a significant increase in HCC liver tissues

Table 4. Association of Serum NTN4 Levels among HCC Patients with PVI, NPVI and Controls

Variable	Study group	n (%)	Median (IQR)	p-value	Post Hoc Kurskal-Wallis Test		
					PVI vs NPVI	PVI vs Controls	NPVI vs Controls
NTN4 pg/ml	PVI	68 (28)	23.5 (9.50-96.0)	0.0001	1.00	0.001	0.001
	NPVI	86 (35)	22.5 (7.75-85.75)				
	Controls	90 (37)	147.5 (106.7-317.2)				

NTN4, netrin-4; HCC, hepatocellular carcinoma; PVI, portal vein invasion; NPVI, non-portal vein invasion; n, numbers; %, percentage; IQR, inter-quartile range; p-value<0.05 statistically significant

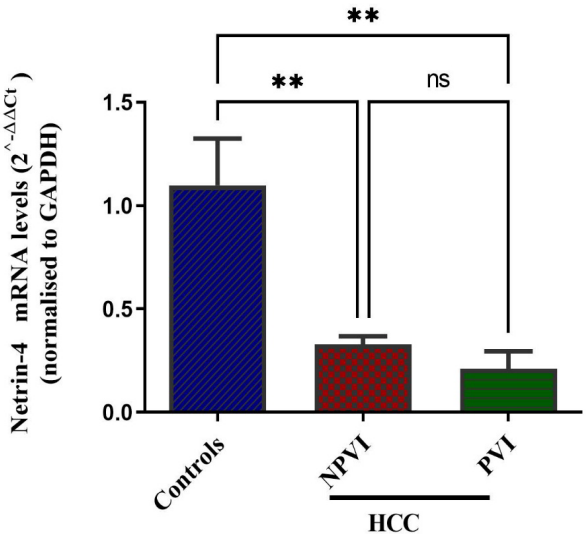

Figure 2A. mRNA Expression of NTN4 in HCC Liver and Control Liver Tissues. Values are expressed as mean ± SEM. Symbols represent **p<0.01, Multiple comparisons were performed using Dunnett's test. GAPDH was used as an endogenous internal control.

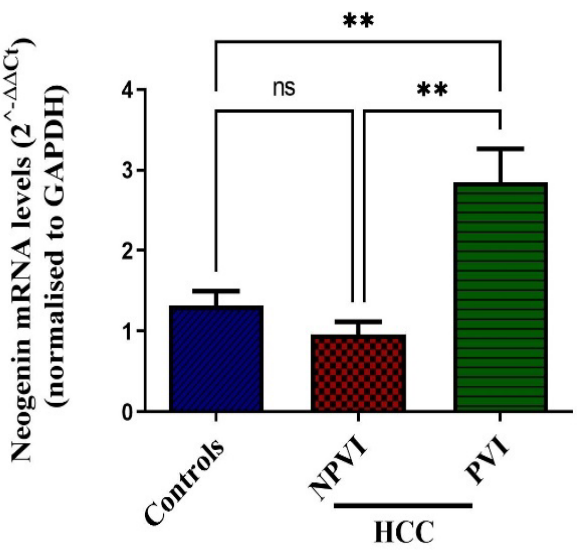

Figure 2B. mRNA Expression of Neogenin in HCC Liver and Control Liver Tissues. Values are expressed as mean ± SEM. Symbols represent **p<0.01, Multiple comparisons were performed using Dunnett's test. GAPDH was used as an endogenous internal control.

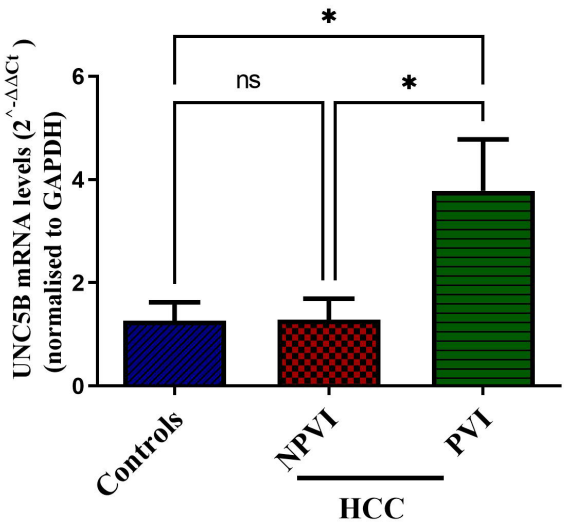

Figure 2C. mRNA Expression of UNC5B in HCC Liver and Control Liver Tissues. Values are expressed as mean ± SEM. Symbols represent *p<0.05, Multiple comparisons were performed using Dunnett's test. GAPDH was used as an endogenous internal control.

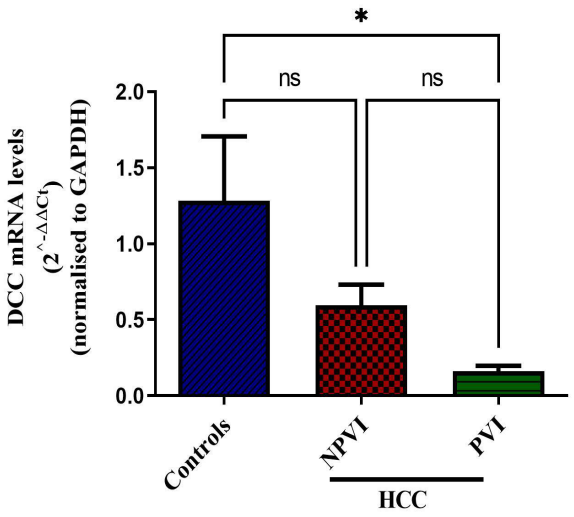

Figure 2D. mRNA Expression of DCC in HCC Liver and Control Liver Tissues. Values are expressed as mean ± SEM. Symbols represent *p<0.05, Multiple comparisons were performed using Dunnett's test. GAPDH was used as an endogenous internal control.

compared to non-tumorous liver tissues. Hepatic VEGF expression did not differ significantly between the PVI and NPVI groups of HCC (Figure 3A). However, hepatic VEGFR was significantly higher in the PVI group when compared to the NPVI group of HCC (Figure 3B).

Discussion

Vasculogenesis-driven hypervascularity has been demonstrated to have a substantial role in the pathogenesis of HCC (Fernández et al., 2009). Hypervascularity is crucially involved in the tumor invasion of the portal vein, an event that markedly affects the prognosis of HCC (Forner et al., 2010). In the present study, our findings showed for the first time that the tissue expression of NTN4 at the mRNA level was significantly downregulated in HCC liver tissues compared to non-tomorous liver tissues. However, both the mRNA and systemic levels of NTN4 were not altered between patients with HCC with PVI and without PVI (NPVI). Of interest, we noted that the mRNA expressions of NTN4 receptors Neogenin and UNC5B were considerably elevated in patients with HCC

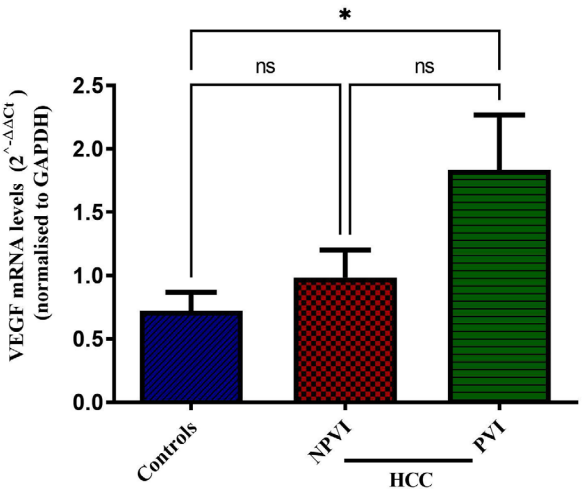

Figure 3A. mRNA Expression of VEGF in HCC Lliver and Control Liver Tissues. Values are expressed as mean ± SEM. Symbols represent *p<0.05, Multiple comparisons were performed using Dunnett's test. GAPDH was used as an endogenous internal control.

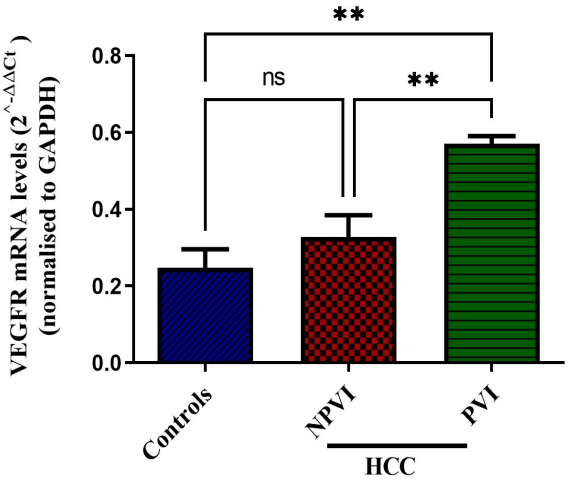

Figure 3B. mRNA Expression of VEGFR in HCC Liver and Control Liver Tissues. Values are expressed as mean ± SEM. Symbols represent **p<0.01, Multiple comparisons were performed using Dunnett's test. *GAPDH* was used as an endogenous internal control.

with PVI compared to NPVI. These findings were in line with the mRNA expression pattern of the VEGF receptor.

In the present study, among the 154 HCC patients, 44% of the patients had PVI, while the remaining did not manifest PVI. Some of the most often utilized functional markers in the clinical diagnosis of hepatic dysfunction and damage include total bilirubin, AST, ALP, and GGT (Stepien et al., 2016). 90% of patients with HCC were found to have elevated levels of AST, ALT, and GGT, and 50% of these patients also had elevated levels of bilirubin or liver-specific ALP, suggesting that these markers can be used as additional but highly non-specific markers in HCCs (Lopez et al., 1996). In our study, the liver function markers ALT, ALP, and GGT were significantly elevated in PVI patients compared to the NPVI group. Through ELISA we also estimated the systemic levels of NTN4 and observed for the first time that NTN4 was reduced in both PVI and NPVI patients compared to control subjects. Notably, no alteration in NTN4 was observed between systemic levels of PVI and NPVI patients. In our previous study (published article: Serum Levels of Netrin-4 and Its Association With Hepatocellular Carcinoma: Results From a Case-Control Study) we reported that NTN4 could be a good marker for HCC, further assessment is required to understand its discriminatory ability between PVI and NPVI patients.

NTN4 may have a bi-functional role in angiogenesis, similar to how they do in the neurological system (Lambert et al., 2012; Lejmi et al., 2008). Indeed, various evidences have implicated the involvement of NTN4 signalling in regulating angiogenesis (Dakouane-Giudicelli et al., 2014; Eveno et al., 2011; Larrieu-Lahargue et al., 2010; Lejmi et al., 2008; Xu et al., 2017). In the current study, we investigated the expressional behavior of NTN4 in HCC tissues with PVI and NPVI. Our findings indicated that the mRNA expression of NTN4 in both PVI and NPVI HCC tissues was markedly downregulated compared to non-cancerous tissues. Notably, this finding of reduced NTN4 in cancerous tissue is in similar line with a batch of evidence indicating decreased expression of NTN4 in breast (Esseghir et al., 2007; Xu et al., 2017), colorectal (Eveno et al., 2011; Eveno et al., 2013), prostate (Latil et al., 2003), pancreas (Hao et al., 2020), and cervical cancers (Zhang et al., 2013) tissues. However, a study on gastric cancer found that NTN4 is highly expressed in both serum samples and tumor tissues (Lv et al., 2015). It is likely that the effect of NTN4 could be concentration dependent, as at low physiological ligand concentrations, NTN4 has been observed to promote cell survival and migration, whereas, at high concentrations, it behaves as an anti-angiogenic agent to inhibit tumor growth (Villanueva et al., 2017). Indeed, overexpression of NTN4 after surgical resection in liver metastasis rodent model reduced tumor recurrence, and metastasis most likely due to an anti-angiogenic effect of NTN4 (Eveno et al., 2013).

Notably, NTN4 was observed to inhibit angiogenesis via binding to the Neogenin receptor and through the recruitment of the Unc5B receptor (Lejmi et al., 2008). Interestingly, the inhibition of endothelial cell migration by NTN4 was eliminated when either Neogenin or Unc5B was silenced, indicating that both receptors are necessary for the NTN4 function in vitro (Lejmi et al., 2008). Neogenin appears to exhibit differential expressions and functionality depending on the type of cancer (Dakouane-Giudicelli et al., 2012; Villanueva et al., 2017). Our study results confirmed for the first time that the mRNA expression of Neogenin was significantly upregulated in HCC patients with PVI compared to NPVI and nontumorous liver tissues. These findings could be indicative that the upregulation of Neogenin could be associated with aggressive cancers.

UNC5B a candidate tumor suppressor gene varies in expression depending on the type of cancer. The mRNA expression of UNC5B in our study was significantly

upregulated in HCC liver tissues of patients with PVI compared to NPVI and nontumorous liver tissues. However, our findings were not in line with the previous studies done in HCC where UNC5B was downregulated in HCC samples (Zhang et al., 2009). However, it should be that Zhang et al. did not assess the UNC5B levels in HCC tissues with PVI. Moreover, Huang et al., 2021 showed that the proliferation, migration, and EMT of Hepatocellular carcinoma cells were encouraged by UNC5B. Additionally, in the present study, in accordance with the pattern of expression of UNC5B and Neogenin, the gene expression of angiogenesis markers such as VEGF and VEGFR were found to be upregulated in HCC liver tissues with PVI compared to NPVI and noncancerous liver tissues.

The Deleted in Colorectal Cancer (DCC) is considered to be a tumor suppressor gene related to cell adhesion receptors. Staquicini et al., identified through a rodent model that astrocyte-derived NTN4 bound with DCC receptor in neural stem cells (Staquicini et al., 2009). Previous research showed that the propensity for nodal metastasis was connected with decreased mRNA expression of DCC, which might occur at an early stage in gastric cancer and a late stage in colorectal cancer (Kataoka et al., 1995). No studies have been done so far that demonstrate the levels of DCC in HCC. For the first time, we reported that the mRNA expression of DCC was significantly downregulated in HCC liver tissues compared to nontumorous liver tissues which are consistent with the results of research in other malignancies. We also noted that DCC is reduced in HCC patients with PVI compared to the NPVI group. Reduction of DCC, a tumor suppressor in HCC may participate in the progression of HCC, which needs to be confirmed in upcoming experimental studies. Further, since the environment surrounding tumor cells differs for each organ, future studies are warranted to determine the stage at which suppressor genes such as DCC are altered in HCC, which would greatly aid in determining the carcinogen or factors that contribute to malignant potential.

In conclusion, PVI is associated with poor prognosis in HCC patients. We investigated the systemic and hepatic level expression of NTN4 and its receptors in patients with HCC with PVI and NPVI. Of interest, we noted that the systemic and hepatic NTN4 levels were reduced in both PVI and NPVI subjects when compared to controls. Meanwhile, the hepatic expression of NTN4 receptors Neogenin and UNC5B were markedly elevated in HCC patients with PVI compared to NPVI, suggesting their involvement in PVI pathogenesis. There have not been any reports prior about the systemic and hepatic expression of NTN4 and its receptors in HCC patients. The low sample size for tissue studies is one of the limitations of the present study. Further human studies with larger sample sizes are warranted to better understand the role of NTN4 and its receptors in the development of PVI in HCC. Experimental studies using disease models of HCC could also shed light on the mechanisms through which NTN4 and its receptors are regulated in the HCC microenvironment.

Author Contribution Statement

BP designed the study. VJ carried out the experiments and drafted the manuscript. BP, VJ, BV, VM, and SKV contributed to the analysis and interpretation of the results. BV, SKV, and VM contributed to the final version of the manuscript. BP approved the final manuscript.

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Institutional review board

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the JIPMER scientific advisory committee and Institutional Human Ethics Committee.

Ethics approval and consent to participate

The study was reviewed and approved by the Institutional Human Ethics Committee. (JIP/IEC/2017/0126). All patients were provided with informed written consent regarding the data collection and scientific publication.

Availability of data

Data are available upon request.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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