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Original Article

Low expression of trafficking protein particle complex 4 predicts poor prognosis in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Tumor recurrence and metastasis are major factors that contribute to the poor outcome of patients with HCC. However, it is difficult to predict the prognosis of hepatocellular carcinoma. Trafficking Protein Particle Complex 4 (Trappc4), is associated with tumorigenesis. The present study aimed to detect Trappc4 expression in HCC and its association with clinicopathological patient data. More importantly, this study reveals the relationship between Trappc4 and the prognosis of hepatocellular carcinoma. A total of 148 HCC tissues were assessed for expression of Trappc4 mRNA and protein with (reverse transcription polymerase chain reaction) RT-PCR (n=36), Western blotting (n=4) and immunohistochemistry (n=148), respectively. The data show that Trappc4 mRNA and protein are expressed at low levels in HCC tissues compared to adjacent tissues. Immunohistochemical analysis revealed that 148 cases of HCC showed different degrees of positive expression. Statistical analysis showed that expression of Trappc4 was associated with histological differentiation, TNM stage, and vascular invasion ($P < 0.05$), but did not correlate with the patient's age, gender, tumor size ($P > 0.05$). Most importantly, HCC patients with low expression of Trappc4 had shorter survival time compared to patients with high expression. Trappc4 might be involved in the pathogenesis of HCC and could be an important prognostic marker in HCC patients.

Keywords: Trafficking protein particle complex 4, prognosis, hepatocellular carcinoma, biomarker

Introduction

Hepatocellular carcinoma (HCC) ranks as the fifth and seventh most common cancer in men and women, respectively, and it is the third most common cause of cancer-related mortality worldwide [1]. HCC is a global health burden, and its prevalence varies worldwide [2]. HCC development is often related to the presence of a chronic liver inflammation [3], which represents one of the most important risk factors [4]. In particular cirrhosis, which can occur as a consequence of chronic viral hepatitis, excessive alcohol intake, nonalcoholic fatty liver disease, or genetic diseases (e.g., hemochromatosis), is a frequent setting for HCC onset as well as a cause of liver dysfunction [5]. This disease is characterized by highly recurrent rate after curative resection and resistance to chemotherapy [6]. Surgery and liver transplantation (OLT) represent the only radical treatments of this disease until now [7], but are not feasible in cases of advanced disease or significant hepatic dysfunction [8]. Sorafenib is the only standard treatment available for advanced HCC [9]. Despite advances in the diagnosis and treatment of HCC, the prognosis remains poor and lacks predictive factors for prognosis [10, 11]. Therefore, further clarification of the mechanisms underlying its development and finding new predictive factors for prognosis for HCC becomes particularly important [12].

At present, the prognosis of HCC is predicted based on imaging findings and biomarkers [13, 14]. Some biomarkers such as α-fetoprotein (AFP), AFP-L3 [15] and Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II)

[16] have been discovered. However, accurate indicators for the prognosis of HCC are scarce. Consequently, there is an urgent need to develop a novel biomarker for the prognosis of HCC. Trappc4, the human ortholog of yeast Trs23p, also known as synbindin, is generally known as a neuronal cytoplasmic protein originally identified by yeast two-hybrid screening using the syndecan-2 (belonging to a family of cell-surface heparan sulfate proteoglycans that regulates cell behavior through signal transduction pathways) [17, 18] using the cytoplasmic domain as bait [19]. Trappc4 is a member of the trafficking protein particle (TRAPP) family of proteins and is implicated in vesicle-mediated transport, a process carried out by virtually every cell and is required for the proper targeting and secretion of proteins [17]. Recent studies have shown that Trappc4 not only regulates activation of the extracellular signal-regulated kinase 2 (ERK2), but also affects the subcellular distribution of activated phospho-ERK2 in colorectal cancer (CRC) cells [20]. Furthermore, it also contributes to malignant phenotypes of gastric cancer (GC) by activating ERK signal pathway on the Golgi [21]. Previous studies have shown that Trappc4 is up-regulated in colon cancer and gastric cancer [20, 21]. However, the role and function of Trappc4 in HCC remain unknown, therefore, this study aimed to investigate the significance of Trappc4 expression and its prognostic value for HCC patients.

Material and methods

Patients and tumor tissues

A total of 148 HCC tissue samples and 117 para-carcinoma tissues were obtained from 148 HCC patients undergoing resection in the Nantong Third People's Hospital between September 2009 and December 2016. The distance between the para-carcinoma tissue and carcinoma was more than 2.5 cm. These tissue specimens were immediately frozen at -80°C or fixed in 10% formalin and then embedded in paraffin until use. The diagnosis of all patients was confirmed independently by two pathologists and no patient received chemotherapy or radiotherapy before surgery. This study was approved by the Ethics Committee of Nantong Third People's Hospital, and all of the patients gave signed informed consent.

RNA isolation and real-time fluorescent quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from the tissues and cells using TRIzol reagent (Takara Bio, Dalian, China). The quality and quantity of isolated RNA were evaluated using the UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). mRNA was converted to cDNA using PrimeScript RT Reagent Kit (Perfect Real Time) (Takara Bio) as the instruction. qPCR was performed using a SYBR Green PCR Master Mix (Vazyme Biotech, NanJing, China) as the direction. The sequences of primers for PCR analysis were as follows: β-actin, forward primer: 5'-GGACTTCGAGCAAGAGATGG-3'; reverse primer: 5'-AGGAAGGAAGGCTGGAAGA-3'; Trappc4, forward primer: 5'-GGGAAAGAGGTGCTGGAGTAT-3'; reverse primer: 5'-GCAAAGAGCGAGTGGAACAT-3'. The PCR was performed at 95°C for 5 min, then subjected to 40 cycles of amplification at 95°C for 10 s and at 60°C for 30 s. Expression of Trappc4 was normalized to the β-actin. Fold amplification for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Approximately 20 mg HCC tissue was lysed with RIPA lysis buffer (including Phosphatase Inhibitor Cocktail) (Beyotime, Shanghai, China). The supernatant was harvested and the protein concentration was measured using BCA Protein Assay Kit (Beyotime). Equal amounts of protein were electrophoretically separated by SDS-PAGE and transferred onto nitrocellulose membranes at 100 V for 90 min. Expression of Trappc4 was analyzed using specific antibodies (Abcam, Cambridge, MA; 1:1000). After overnight incubation at 4°C, the membranes were incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Kangchen, Shanghai, China; 1:3000) at room temperature for 1 hour, and then they were washed three times with tris-buffered saline with 0.1% (V/V) Tween 20. The ECL system (Thermo Scientific, New York, USA) was used to visualize the results. Grey values of Western blotting were analyzed using Image J software (National Institutes of Health, Bethesda, MD).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded HCC tissues

Table 1. Association between Trappc4 expression and clinicopathological parameter data

Parameters	Total	Trappc4 expression		*P*-value
		High expression (n=73)	Low expression (n=75)	
Age (years)				0.1882
≤54	74	32	42	
> 54	74	41	33	
Gender				0.1508
Male	104	47	57	
Female	44	26	18	
TNM				0.0016**
I and II	65	42	23	
III and IV	83	31	52	
Histological differentiation				< 0.001***
I and II	76	55	21	
III and IV	72	18	54	
Vascular invasion				0.0157*
Negative	123	55	68	
Positive	25	18	7	
tumor diameter (cm)				0.7190
≤5	105	53	52	
> 5	43	20	23	

TNM stage was according to 2002 TNM Classification of Malignant Tumors by the International Union Against Cancer; Histological differentiation was classified by the Edmondson-Steiner grade. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

using antibodies against Trappc4 (Abcam; 1:50), the procedure was performed as previously described with minor modifications. Slides were deparaffinized through a series of xylene baths and graded alcohols. Antigen retrieval was done with microwave in citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 for 15 min. Then the sections were subsequently incubated with rabbit anti-human Trappc4 (1:50 dilution) overnight at 4°C. Horseradish peroxidase (HRP) conjuncted-anti-rabbit secondary antibody was applied for 30 min in room temperature. After diaminobenzidine (DAB) staining for appropriate time, hematoxylin staining was performed. Stained sections were observed by pathologists.

Expression level of Trappc4 in each section was scored on a scale of 0-3 according to the intensity: 0, negative expression; 1, weak expression; 2, moderate expression; and 3, strong expression. A single unbiased blinded pathologist analyzed all samples.

Statistical analysis

Data are expressed as mean ± standard deviation. The differences between groups were determined by analysis of variance and comparison. Between two groups was carried out using the t test. The survival time was compared between HCC patients with high or low expression of Trappc4 using the log rank single factor test, and Kaplan-Meier survival curves were made using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Cox proportional hazards regression model was performed using the SPSS 21.0 statistical software package (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics of HCC patients

The complete clinical and follow-up data of 148 HCC patients (receiving no antitumor treatment before surgery) were screened for 104 male and 44 female patients, age ranged from 28 to 80 years (mean 54 years). Twenty five patients had vascular invasion. One hundred and twenty three had no vascular invasion. Seventy two had highly differentiated HCC, and seventy six patients had lowly differentiated HCC. The tumor diameter was ≤5 cm in 105 patients. According to the seventh edition of the TNM Classification of Malignant Tumors (TNM-7) [22], 65 patients were classified as TNM stage I or II, and 83 patients are on stage III or IV. All clinical data are summarized in **Table 1**.

Low expression of Trappc4 in HCC patients

qPCR was used to detect expression of Trappc4 in HCC (n=36) tissue and para-carcinoma tissue. Trappc4 mRNA declined dramatically in

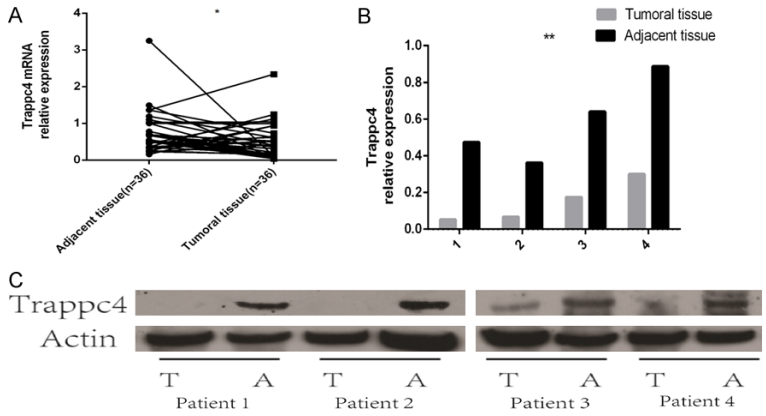

Figure 1. Expression of Trappc4 in HCC tissues (T) and adjacent tissues (A). A: Real-time fluorescent qPCR for detection of Trappc4 mRNA expression in HCC (n=36) tissues and adjacent tissues (n=36); B: Statistical analysis of Trappc4 expression in HCC tissues and adjacent tissues by Western blotting ($^{**}P < 0.01$); C: Detection of Trappc4 expression in HCC tissues and adjacent tissues by Western blotting.

HCC tissues (0.46 ± 0.48) compared to adjacent tissue (0.72 ± 0.55) (**Figure 1A**). Western blotting was then used to detect the level of Trappc4 protein. Trappc4 protein was highly expressed in adjacent tissues, whereas it was weakly detected in HCC tissues (**Figure 1B**, **1C**). In the end, immunohistochemical staining was used to detect expression of Trappc4 in HCC and adjacent tissues. Trappc4 was expressed in cytoplasm in HCC cells. There was strongly positive Trappc4 protein expression in 117 adjacent tissues, and weak or no expression in 148 HCC tissues. Statistical analysis showed a significant difference ($P < 0.01$) (**Figure 2**).

Association of Trappc4 expression with clinicopathological parameters in HCC patients

We detected a correlation of Trappc4 expression with clinicopathological parameters in HCC patients. We found a significant association between Trappc4 protein expression and TNM stage, histological differentiation, and vascular invasion (all $P < 0.01$). However, Trappc4 expression was not associated with age, sex, and tumor size in HCC patients (**Table 1**).

Correlation of Trappc4 expression with patient survival

All patients were classified into two groups according to immunohistochemical scores (0 or 1 was considered as low expression, and 3 or 4 was considered as high expression). We investigated the correlation between prognostic effect of Trappc4 expression and overall survival of HCC patients. The Kaplan-Meier curve showed that low Trappc4 protein level was a significant prognostic factor in revealing the poor overall survival in HCC patients. Seventy five patients with low Trappc4 protein expression had a significantly lower survival rate than those with high Trappc4 protein expression (73 patients) (**Figure 3**, $P < 0.01$). The difference in Kaplan-Meier survival curve between HCC patients with high and low Trappc4 expression according to immunohistochemical score was significant between the two groups ($P < 0.05$, log-rank test).

Univariate and multivariate analyses of prognostic parameters in HCC patients

To identify the variables with potential prognostic significance, univariate analysis of each clinicopathological parameter was performed for correlation with survival of HCC patients. The hazard ratio and P value for each parameter were used to predict the prognosis for HCC patients. The importance of each parameter was calculated by multivariate Cox proportional hazards model analysis. A stepwise forward-inclusion of clinicopathological parameters in the model was performed through univariate analysis, which showed that the significant prognostic factors for HCC patients included Trappc4 expression and vascular invasion (**Table 2**). However, multivariate analysis showed that the significant prognostic factors for HCC included Trappc4 expression, pathological classification, TNM stage, vascular invasion and tumor diameter (**Table 3**).

Discussion

According to last EASL-EORTC guidelines, liver cancer is the sixth most common cancer, the third cause of cancer related death, and accounts for 7% of all cancers [23]. Hepatocellular carcinoma (HCC) represents more

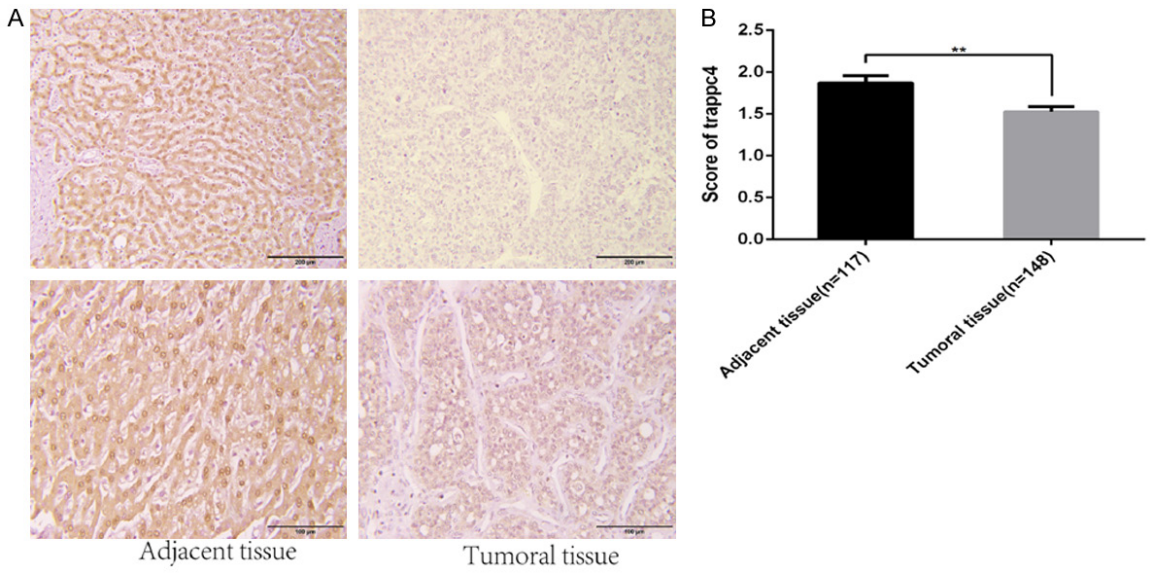

Figure 2. Immunohistochemistry of Trappc4 in HCC and adjacent tissues. A: Representative immunohistochemical staining of Trappc4 in HCC and adjacent tissues, On the left side of the picture is adjacent tissue (bar 200 μm and 100 μm). On the right side of the picture is HCC tissue (bar 200 μm and 100 μm); B: Statistical analysis of immunohistochemistry of Trappc4 in HCC and adjacent tissues. ($^{**}P < 0.01$).

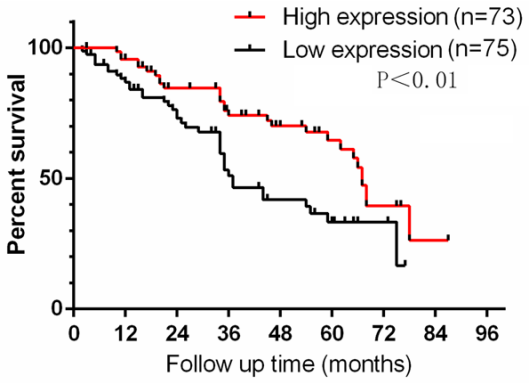

Figure 3. Kaplan-Meier plots of overall survival of 148 HCC patients, stratified by expression of Trappc4. (Lower expression, immunohistochemistry score 0 or 1; high expression, score 2 or 3) ($^{**}P < 0.01$).

than 90% of primary liver cancers and is a major global health problem [23]. At present, there is no effective method for prognosis of hepatocellular carcinoma. TRAPP complexes exist in three forms, which differ in their subunit composition, function, and subcellular localization [24]. Trappc4, one of the seven subunits of TRAPPI, can be identified in the vesicles of dendritic spines, as well as synapses, and forms clusters in dendritic spines when syndecan-2 is co-expressed in neurons [25]. Previous studies have confirmed that Trappc4 controls the Ras-Raf-MEK-ERK signal transduction cascade and it binds to mitogen-activated protein kinase kinase (MEK1) and ERK2 on the Golgi apparatus, causing enhanced phosphorylation of ERK2 by MEK1 both in vitro and *in vivo* [26]. In this study, we found the expression of Trappc4 protein was located in the cytoplasm and nucleus. From these results, we conclude that Trappc4 expression is decreased in HCC tissues, and may be associated with tumor pathology or prognosis in HCC patients.

A few markers such as hepatoma-specific γ-glutamyl transferase, oncofetal antigen glypican-3, hepatoma-specific AFP, and member 3a of Wingless-type MMTV integration site family have been developed as specific biomarkers for HCC [27-29]. Previous research has shown that secretory clusterin (sCLU) is associated with HCC progression. Abnormal sCLU expression was considered as an independent prognostic factor for HCC patients [30]. Importantly, there are studies that show that the expression level of Trappc4 associates with tumor size, lymph node invasion, distant metastasis, TNM staging, and overall survival of gastric cancer (GC) patients. These features make Trappc4 a potential biomarker in GC for risk assessment, early detection, grading, and prognosis [21]. However, in our study, we detected Trappc4

Table 2. Univariate analysis of factors associated with overall survival

Variables	Hazard ratio	95% Confidence interval	*P*
Age (years)	0.992	0.969-1.015	0.480
Gender	1.256	0.731-2.158	0.409
TNM	0.790	0.483-1.293	0.348
Histological differentiation	1.020	0.624-1.670	0.936
Vascular invasion	2.330	1.329-4.083	0.003*
	0.790	0.478-1.305	0.357

Cox proportional hazards regression model. *, $P < 0.05$.

Table 3. Multivariate analysis of factors associated with overall survival

Variables	Hazard ratio	95% Confidence interval	*P*
Age (years)	0.990	0.966-1.014	0.419
Gender	1.758	0.986-3.133	0.056
TNM	0.426	0.200-0.905	0.026*
Histological differentiation	0.921	0.428-1.979	0.833
Vascular invasion	3.447	1.859-6.394	0.000*
	0.507	0.276-0.929	0.028*

Cox proportional hazards regression model. *, $P < 0.05$.

expression in HCC tissue samples and found that paraffin-embedded adjacent tissues displayed strong staining of Trappc4 protein, but cancer tissues had less immunohistochemical staining. Furthermore, we found the expression of Trappc4 was correlated with clinicopathological parameter, such as tumor size, differentiation, TNM stage, and vascular invasion in HCC patients. All of these findings indicated Trappc4 might be involved in the pathogenesis of HCC and played critical role in the HCC development. According to the correlation study, the Trappc4 gene may be related to the invasion ability of the tumor, not necessarily related to the tumor's proliferation. It is the same as result of the previous study, which suggested that Trappc4 facilitates the invasion of gastric carcinoma [21]. The overall survival of patients with Trappc4 low expression was shorter than that in patients with high expression according to Kaplan-Meier analysis, which showed that Trappc4 was related to prognosis of HCC. We then evaluated the value of Trappc4 functioned as a predictive factor for prognosis in HCC, and found that the Trappc4 is an independent factor for the prognosis of HCC.

However, there are some limitations in our study, such as lack of data elaborating the mechanism responsible for low expression of Trappc4 in HCC. We plan to clarify the mechanism of Trappc4 involvement in the development of HCC in the future.

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Disclosure of conflict of interest

None.

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