

RESEARCH ARTICLE

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High Expression of *PXMP4* in Hepatocellular Carcinoma Tissues

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Abstract

Objective: To analyze the high expression of peroxisome membrane protein 4 (*PXMP4*) in hepatocellular carcinoma (HCC) and its clinical significance. **Methods:** The expression of *PXMP4* mRNA in HCC tissues and corresponding adjacent tissues was detected by Q-PCR, and the expression of *PXMP4* protein was detected by Western blot and immunohistochemistry. The correlation of *PXMP4* protein expression with clinicopathological features and prognosis of HCC was analyzed. **Results:** The expression levels of *PXMP4* mRNA and protein in HCC tissues were significantly higher than those in adjacent tissues ($P < 0.05$), and its high expression was significantly correlated with tumor differentiation, lymph node metastasis, depth of invasion and TNM stage ($P < 0.05$). Patients with high expression of *PXMP4* had a poor prognosis ($P < 0.05$). **Conclusion:** The high expression of *PXMP4* may promote the occurrence and development of HCC, and inhibition of *PXMP4* may be one of the potential molecular targets for targeted therapy of HCC.

Keywords: Hepatocellular Carcinoma- *PXMP4*- prognosis

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Introduction

The etiology of liver cancer is complex, and it is related to the long-term interaction of clinical genetics, viral hepatitis, heavy drinking and other factors [1]. Liver is an important organ of human metabolism, and its blood supply is extremely rich. Once the disease occurs, it will not only have a serious impact on the normal metabolism of the body, but also the tumor cells are prone to metastasis, resulting in organ dysfunction and reducing the overall survival rate [2]. China has a high prevalence of liver cancer [3]. At present, the radical treatment of HCC is still mainly based on surgery. Clinically, most of the patients with liver cancer are treated with comprehensive plans based on surgery, which can remove tumor cells in the body to the maximum extent and avoid postoperative recurrence and metastasis [4]. However, long-term follow-up showed that there were still some patients with poor prognosis. Most of the patients were diagnosed in the middle and late stages, and lost the chance of radical treatment. Other commonly used treatments, such as radiofrequency ablation, transarterial chemoembolization, and chemoradiotherapy, can benefit patients to a certain extent, but the long-term prognosis is still unsatisfactory [5, 3].

Peroxisomes are single-membrane-bound organelles involved in a variety of metabolic pathways [6]. *PXMP4* is a peroxisome membrane protein that is widely expressed

in cells and is one of the main components of peroxisome membrane with a molecular weight of 24kDa. *PXMP4* expression is silenced in prostate cancer mainly due to intronic CpG dinucleotide mediated DNA methylation, while *PXMP4* expression is inversely correlated with CpG island methylation value in non-small cell lung cancer (NSCLC). Moreover, *PXMP4* is negatively correlated with proliferation marker Ki-67 protein in NSCLC [7]. Our research group found that *PXMP4* can promote the proliferation, invasion and migration of colorectal cancer cells [8]. It can be inferred that *PXMP4* plays an important role in the occurrence and development of tumors, and also shows that *PXMP4* has very important clinical significance in the diagnosis and prognosis of malignant tumors. However, the role of *PXMP4* in hepatocellular carcinoma has not been reported [9].

The objective of this study was to analyze the expression of *PXMP4* in HCC and its prognostic significance.

Materials and Methods

Materials

Case report

A total of 50 HCC and corresponding paracancerous tissues from patients who underwent surgical treatment in Henan Cancer Hospital from January 2022 to December 2022 were collected. Consent was obtained from the ethics

committee of the hospital and patients were informed. There were 31 males and 19 females. 20 cases were less than 60 years old and 30 cases were more than 60 years old. Well differentiated, moderately differentiated and poorly differentiated cases were 13, 27 and 10, respectively. There were 13 cases with lymph node metastasis and 37 cases without lymph node metastasis. There were 19 cases with tumor diameter ≤ 5 cm and 31 cases with tumor diameter > 5 cm. The surgically removed tissues were isolated and fixed in formalin or cryopreserved.

Reagent

PXMP4 antibody (Novus Biologicals, USA), PVDF membrane (Millipore, USA), pre-dye Maker (Thermo, USA), *PXMP4* primer and β -Actin primer (Jierai Biological, Shanghai), Anti-gapdh rabbit monoclonal antibody, RIPA lysate, BCA protein quantification kit, ECL color development kit, Trizol, reverse transcription kit, UltraSYBR Mixture and rabbit/mouse Universal SP kit (DAB) (Kangyi).

Methods

The expression of PXMP4 mRNA was detected by quantitative real-time PCR

The samples of hepatocellular carcinoma and adjacent tissues frozen in liquid nitrogen were ground into powder and RNA was extracted by Trizol method [10]. Three μ g of RNA was used for reverse transcription to synthesize cDNA. The sequence of human LSD1 specific primer was designed by using pubmed database. The sequence of human LSD1 specific primer was as follows: forward primer 5'-ATAACATCAACAGCCAGAT-3', reverse primer R:5'-GGATGTAGCCCTTCTCTA-3'. The reference human β -actin-specific primer sequence was as follows: forward primer 5'-TCGTCATACTCCTGCTTGCTG-3' and reverse primer 5'-CGGGACCTGACTGACTACCTCA-3'. 2 μ L of cDNA synthesized by reverse transcription was used for Q-PCR, and the annealing temperature of both *PXMP4* and β -Actin was 60°C. After 40 cycles of amplification, $2^{-\Delta\Delta Ct}$ was calculated, and the value indicates the expression level of *PXMP4* mRNA.

The expression of LSD1 protein was detected by Western blot

The above frozen tissues were ground into powder, and an appropriate amount of protein lysate was added to fully dissociate. After shaking and centrifugation, the supernatant was taken as the required protein extract, and BCA kit was used for protein quantification. Protein electrophoresis and membrane transfer were carried out on 12% SDS-polypropyl amide gel. The PVDF membrane was blocked with skim milk powder for 1h, and *PXMP4* rabbit polyclonal antibody (1:1000) and the internal reference GAPDH anti-rabbit polyclonal antibody (1:2000) were incubated at 4°C overnight, and the goat anti-rabbit secondary antibody (1:2000) was incubated at room temperature for 2h. The PVDF membrane was treated with ECL and the target protein bands were detected by gel imaging system, and the gray value of protein bands was detected by Image J software [11, 12]. The expression level of the target protein was expressed

as the ratio of gray value of the target protein band to the gray value of the internal reference protein band.

The expression of PXMP4 protein was detected by immunohistochemistry

The fixed tissue was embedded in paraffin, sectioned, dissolved in wax at 60°C for 1 h, deparaffinized with xylene, hydrated with gradient ethanol, and repaired with citric acid antigen. The LSD1 protein was detected by immunohistochemical SP high-sensitivity kit. The primary anti-*PXMP4* rabbit polyclonal antibody (1:200) was incubated at room temperature. DAB was used for color development, followed by sulfoxide counterstained, dehydrated with gradient ethanol, transparent with xylene and sealed with neutral gum [13]. The *PXMP4*-positive cells showed brownish yellow granules in the nucleus. A total of 200 cells (400 \times) were randomly selected for observation under 4 high-power fields, and the staining results were judged by double-blind method. The immunohistochemical scores were analyzed by semi-quantitative method [9]. Immunohistochemical score = staining intensity score \times positive cell count score. Staining intensity score: 0, no staining, negative; 1 point, light yellow, weakly positive; 2 points, yellow or dark yellow, moderately positive; 3 points, yellowish brown or dark brown, strongly positive; Positive cell count score: 0, no positive cells; 1 point, positive cell count $\leq 25\%$; 2 points, positive cell count $> 25\%$ and $\leq 50\%$; 3 points: positive cell count $> 50\%$. The mean of four visual field immune scores was taken: 0-2 was considered negative; > 2 was defined as positive.

Statistical Analysis

SPSS21.0 was used to analyze the data. The difference between Q-PCR and Western blot quantitative results was analyzed by paired t test. Immunohistochemistry was used to detect the difference of *PXMP4* protein expression in HCC and corresponding paracancerous tissues. Paired Chi-square test was used to analyze the difference of *PXMP4* protein expression in HCC and corresponding paracancerous tissues. The correlation between *PXMP4* protein expression and clinicopathological features of HCC was analyzed by chi-square test. Fifty patients with hepatocellular carcinoma were followed up for 30 months after surgery. Survival time was defined as the date of operation to the last follow-up date or death. Uncensored data were patients who died due to hepatocellular carcinoma, and censored data were patients who were still alive after 30 months of follow-up or patients died of other causes. The survival curve was drawn by Kaplan-Meier method and the difference of survival time was tested by Log-rank test. All measurement data were expressed as ($\bar{x} \pm s$).

Results

Expression of PXMP4 in hepatocellular carcinoma and adjacent tissues

The quantitative detection of *PXMP4* mRNA and protein expression levels by Q-PCR and Western blot (Figure 1 and Table 1) showed that the expression

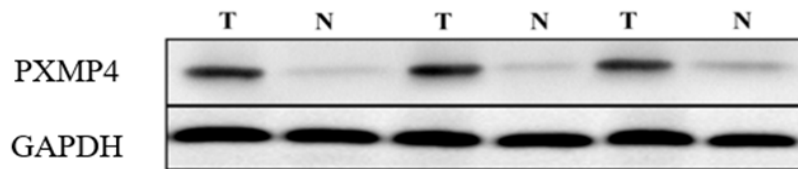


Figure 1. Detect the *PXMP4* Protein Expression in Hepatocellular Carcinoma and Its Adjacent Tissues by Western Blot

Table 1. Detect the *PXMP4* Expression in Hepatocellular Carcinoma and Its Adjacent

Groups	<i>PXMP4</i> mRNA	<i>PXMP4</i> protein
hepatocellular carcinoma	3.25 ± 1.42	0.73 ± 0.26
Adjacent cancer	1.79 ± 1.07	0.30 ± 0.16
t	5.39	8.13
P	<0.001	<0.001

levels of *PXMP4* mRNA and protein in HCC tissues were significantly higher than those in the corresponding adjacent tissues ($P < 0.001$).

The immunohistochemical test results are shown in Figure 2 and Table 2. The expression of *PXMP4* protein was mainly located in the nucleus of the tumor cells,

Table 2. Expression of *PXMP4* Protein in Hepatocellular Carcinoma and Its Adjacent Tissues

Adjacent cancer	hepatocellular carcinoma	
	<i>PXMP4</i> positive	<i>PXMP4</i> negative
<i>PXMP4</i> positive	20	0
<i>PXMP4</i> negative	10	20

which was brownish yellow granules, and some of the cytoplasm was also positive, which was light yellow. The expression was mainly negative in paracancerous tissues, while strongly positive in HCC tissues. The positive expression rate of *PXMP4* in hepatocellular carcinoma tissues (64.00%) (32/50) was significantly higher than that in the corresponding adjacent tissues (18.00%) (9/50), and the difference was statistically significant ($P < 0.05$).

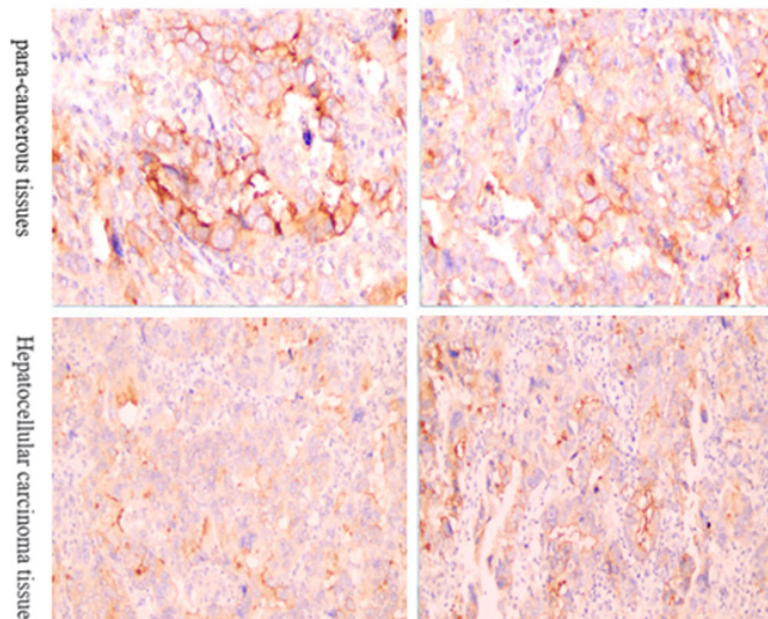


Figure 2. Detect the Expression of *PXMP4* in Rectal Adenocarcinoma by Immunohistochemistry

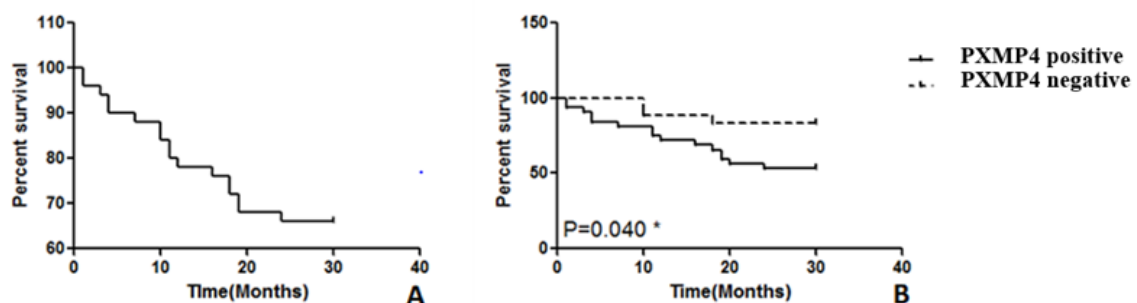


Figure 3. Survival Curve of Kaplan-Meier in Patients with Hepatocellular Carcinoma

Table 3. Correlation between *PXMP4* Protein Expression and Clinicopathological Features of Hepatocellular Carcinoma

Pathological factors	n	<i>PXMP4</i> positive	<i>PXMP4</i> negative		P
Hepatocellular carcinoma tissue	50	32(64.0%)	18(34.0%)		
Adjacent to cancer tissue	50	9(18.0%)	41(82.0%)	14.297	0
Gender					
Male	31	24(77.4%)	7(22.6%)		
Women	19	8(42.0%)	11(58.0%)	6.376	0.012
Age (years)					
≥60	30	19(63.3%)	11(36.7%)		
<60	20	13(65.0%)	7(35.0%)	0.014	0.904
Degree of differentiation					
High degree of differentiation	13	7(53.8%)	6(46.2%)		
Intermediate differentiation	27	15(55.5%)	12(44.5%)		
Low differentiation	10	10(100.0%)	0(0.0%)	7.042	0.03
Lymph node metastasis					
Positive	13	12(92.3%)	1(7.7%)		
Negative	37	20(54.0%)	17(46.0%)	6.11	0.033
Diameter of tumor					
≤5	19	10(52.6%)	9(47.4%)		
>5	31	22(71.0%)	9(29.0%)	1.719	0.19

Correlation between *PXMP4* protein expression level and clinicopathological features of hepatocellular carcinoma

Chi-square test was used to analyze the correlation between *PXMP4* protein expression and clinicopathological features of HCC (Table 3), and it was found that *PXMP4* protein expression was significantly correlated with gender, degree of tumor differentiation, and lymph node metastasis. The positive rate of *PXMP4* protein expression in male HCC tissues (77.4%) was significantly higher than that in female HCC tissues (42%, $\chi^2=6.376$, $P=0.012$). The positive rate of *PXMP4* protein expression in poorly differentiated group (100%) was significantly higher than that in well differentiated group (53.8%) and moderately differentiated group (55.5%, $\chi^2=7.042$, $P=0.03$). The positive rate of *PXMP4* in the group with lymph node metastasis (92.3%) was significantly higher than that in the group without lymph node metastasis (54%, $\chi^2=6.11$, $P=0.033$). The expression of *PXMP4* protein in HCC tissues was not significantly correlated with age ($\chi^2=0.014$, $P=0.904$) and tumor diameter ($\chi^2=1.719$, $P=0.19$).

Correlation between *PXMP4* protein expression and prognosis of patients with hepatocellular carcinoma

The overall survival rate of 50 patients with HCC at 30 months after surgery was 66.00%. The 30-month survival rate of the *PXMP4* positive expression group (56.3%) was significantly lower than that of the negative group (83.3%, Log-rank test, $\chi^2=4.202$, $P=0.040$, Figure 3).

Discussion

As a kind of organelle, peroxisome exists in the nucleus, which affects the metabolic process of cells and then affects the biological function. However, peroxisome

can affect different metabolic methods in cells or tissues due to different locations, and can be changed by changes in cellular regulation or physiological conditions [14, 15]. *PXMP4* is a ubiquitously expressed peroxisomal membrane protein that is transcriptionally regulated by peroxisome proliferator-activated receptor α , although its function remains unclear [16]. The *PXMP4* gene is located at 20q11.22 and is conserved in a variety of animals such as chimpanzees, dogs, cattle, and mice. It encodes an integral membrane protein associated with peroxisomes. Its only known binding partner is ex19, an intracellular chaperone and part of the peroxisome membrane insertion machinery. It is encoded by Nkt cell control gene Nkt1 [17-19]. Studies have shown that *PXMP4* is closely related to the repair process of oocytes [20, 18]. Moreover, previous studies have found that the expression of *PXMP4* has clinical significance in prostate cancer and non-small cell lung cancer [21], but the study of *PXMP4* in hepatocellular carcinoma has not been reported.

The results of this study showed that the expression of *PXMP4* mRNA and protein in HCC tissues was significantly higher than that in adjacent tissues. *PXMP4* was mainly located in the nucleus of hepatocellular carcinoma cells. Its high expression was significantly correlated with the degree of differentiation, lymph node metastasis, depth of invasion and TNM stage of hepatocellular carcinoma, and was negatively correlated with the survival time of patients. The study found that the expression of *PXMP4* protein and mRNA in colon cancer cells was higher than that in the control group, and the difference was statistically significant. *PXMP4* was closely related to the malignant degree and metastatic potential of colon cancer [22], which was consistent with the results in this paper.

There are still some shortcomings in this study. For example, the study on the pathogenesis of colorectal cancer is limited to in vitro experiments on related genes, and the support of animal models is lacking. Since there are many aspects involved in the development of tumors, and in terms of genes, multiple genes contribute to the development of tumors, how do these genes interact with each other? Synergistic or antagonistic? And the related molecules or signaling pathways of genes in promoting tumor proliferation all point out the direction for future research.

In conclusion, the expression of *PXMP4* is significantly increased in HCC, suggesting that *PXMP4* may be closely related to the occurrence and development of HCC, and inhibition of *PXMP4* may be one of the potential molecular targets for targeted therapy of HCC.

Author Contribution Statement

MingMing Zhao and Farra Aidah Binti Jumuddin designed and carried out the experiments. Zarina Awang and MingMing Zhao analyzed the data. MingMing Zhao wrote the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgements

Ethical Clearance

This study was approved by the Ethics Committee of the Henan Cancer Hospital.

Data Availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author.

Conflict of Interest

The authors declare no conflict of interest.

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