*Original Research*

# Impaired phosphate and tension homologue deleted on chromosome 10 expression and its prognostic role in radical surgery for hepatocellular carcinoma with family aggregation resulting from hepatitis B and liver cirrhosis

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## Abstract

This study aimed to retrospectively investigate the expression of the phosphate and tension homologue deleted on chromosome 10 (PTEN) protein and its prognostic role in hepatocellular carcinoma (HCC) with family aggregation resulting from hepatitis B and liver cirrhosis, which have not been established. Immunohistochemical analysis was performed to evaluate the PTEN protein expression in HCC and paired para-cancerous tissues from 79 patients with HCC caused by hepatitis B and liver cirrhosis. Of these cases, 34 represented HCC with family aggregation (HCCF group), and 45 represented HCC with no family aggregation (HCCN group). Follow-up data were collected for 3 months to 10 years and analysed for HCC recurrence, survival time and prognostic risk factors. The expression of the PTEN protein in the HCC tissue was dramatically lower in the HCCF group than in the HCCN group. The six-month, one-year and two-year overall recurrence (OR) rates of the HCCF group were significantly higher than those of the HCCN group. The one-year, two-year and five-year overall survival (OS) rates of the HCCF group were lower than those of the HCCN group. Impaired PTEN protein expression was an independent prognostic risk factor that was significantly correlated with OR and OS in HCC patients. Dramatically impaired PTEN protein expression in HCC patients with family aggregation resulting from hepatitis B and liver cirrhosis was correlated with OR and OS, and impaired PTEN expression was an independent risk factor for prognosis after radical surgery.

**Keywords:** phosphate and tension homologue deleted on chromosome 10, hepatocellular carcinoma with family aggregation, prognosis, liver cirrhosis

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## Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant cancers, and its mortality rate ranks third among all cancers worldwide. There are 360,000 new cases of HCC and 350,000 deaths every year in China.[1]

Chronic hepatic B virus (HBV) infection is the major risk factor for HCC in China. An epidemiological survey confirmed that HCC has a strong familial component. Yu *et al.*[2] reported that approximately 55.7% of the family members in a hepatitis B aggregated family eventually developed HCC. Gao *et al.*[3] reported that the HBV infection rate was 41.39% in 202 HCC aggregated families. Despite recent advances in treatment, surgical resection is still the most important treatment for HCC, but tumour recurrence and metastasis remain major obstacles to long-term survival.[4–6] The recurrence rates attributed to distant metastasis and intrahepatic recurrence after radical surgery are 65% and 43%, respectively.[7]

The phosphate and tension homologue deleted on chromosome 10 (PTEN) gene is located on 10q23.3 and is a member of the protein tyrosine phosphatase (*PTP*) gene

family. The PTEN protein was initially identified as a cytoplasmic phosphatase that participates in key signal transduction pathways, and it is involved in the growth, adhesion, invasion and apoptosis of tumour cells.[8–11] Abnormal PTEN protein expression has been detected in a variety of malignant tumours, including HCC[12,13]; however, there are no data on HCC with family aggregation resulting from chronic viral hepatitis B and liver cirrhosis. The present study aimed to retrospectively investigate PTEN protein expression in tissues from patients with HCC with family aggregation resulting from hepatitis B and liver cirrhosis and its correlation with the prognosis of HCC after radical surgery.

## Materials and methods

### Patient selection and data collection

A total of 79 HCC patients who underwent radical resection at the Third Affiliated Hospital of Sun Yat-sen University between January 2001 and January 2010 were enrolled in the study. All patients had HCC resulting from hepatitis B and liver cirrhosis, patients with other possible aetiology were excluded, and the diagnosis of HCC was made according to the guidelines of the American Liver Disease Studies Association. There were 34 cases in the HCC with family aggregation group (HCCF group) and 45 cases in the HCC with no family aggregation group (HCCN group). Normal liver tissues were collected from 40 patients with benign disease as controls. The indication criteria for radical surgery was according to the Chinese expert consensus for HCC as followings: patients with Child-pugh A or B, who's hepatic functional reserve is acceptable, single nodule or the number of multiple nodules is less than three, and all nodules locate in the same segment or lobe of the liver. This study was approved by the Institutional Review Board of the Key Lab of Liver Disease Research in Guangdong Province. All Patients have signed an informed consent.

The definition of HCC with family aggregation[3] was a case in which two or more patients in one family first- or second-degree relatives were diagnosed with HCC. This study included 34 cases of HCC with family aggregation from 13 families. There were six families with three or more cases of HCC.

The clinical characteristics of each patient, including age, gender, serous alpha-fetoprotein (AFP) level, preoperative tumour size, tumour number, intrahepatic vascular invasion according to abdominal computed tomography (CT) or magnetic resonance imaging, the familial history of HCC and HBV DNA load, were recorded. Pathological findings, including tumour differentiation, tumour encapsulation, intrahepatic vascular invasion, tumour size and tumour number were also recorded. The tumour differentiation status was graded according to the method of Edmondson and Steiner.[14] The tumours were classified into two groups: well differentiated (grades I and II) and poorly differentiated (grades III and IV).

After radical surgery, all patients were regularly followed up. Monitoring via abdominal ultrasound was performed every 2–3 months in the first two years after the operation (with abdominal and chest CT performed if necessary) and every 5–6 months thereafter. Serous AFP was measured every month in the first year and every 3–6 months for the next 2–5 years.

### Immunohistochemical and image analysis

HCC samples and paired para-cancerous tissues of 79 patients with HCC resulting from hepatitis B and liver cirrhosis were analysed with immunohistochemistry and relative optical density (OD) calculations, along with control liver tissues. Briefly, formalin-fixed and paraffin-embedded sections (4-μm thick) were cut and mounted on aminopropyltriethoxysilane-treated slides. Slides were routinely deparaffinized with xylene and rehydrated in ethanol washes. Nonenzymatic antigen retrieval was performed by microwave heat treatment three times for 7 min each in 0.01 mol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity and non-specific background staining were blocked by incubating the slides in methanol containing 0.3% $H_2O_2$ for 30 min. The slides were subsequently washed with phosphate-buffered saline (PBS) for 15 min and then incubated with anti-PTEN protein rabbit polyclonal antibody (Invitrogen, Camarillo, CA, USA) for 1 h at 37°C. The working dilution of the primary antibody was 1:50. The sections were rinsed with PBS for 15 min and then incubated with ENVISION+ rabbit/horseradish peroxidase for 45 min. After washing with PBS for 15 min, the final products were visualized using the 3-amino-9-ethylcarbazole substrate system, and the sections were counterstained with Mayer's haematoxylin for 20 s before mounting. Both positive and negative controls were performed for each section. Normal rabbit serum immunoglobulin G (IgG) was used in place of the primary antibody as a negative control. All experiments were performed in duplicate.[15]

The stained sections were observed under a light microscope (×400; Olympus, Japan), and digital images were analysed with the HPIAS-2000 multimedia coloured pathology image analysis system. To determine the OD and the percentage of PTEN-positive protein staining area, 10 complete sections without overlapping fields were randomly selected for quantitative analysis. Each section was evaluated by three different researchers, and the mean value of each slice was calculated. The OD values were divided into four levels: level 1 ($OD < 0.1$), level 2 ($0.1 \leq OD < 0.2$), level 3 ($0.2 \leq OD < 0.3$) and level 4 ($OD \geq 0.3$).

### Statistical analysis

The data are presented as the means ± SD. Student's *t*-test and Pearson's chi-square ($\chi^2$) test were used for statistical analyses. The logistic regression model was used to identify independent risk factors. The survival rate of the HCC patients was analysed with Kaplan-Meier survival curves and the log-rank (Mantel-Cox) statistical method. A multivariate Cox regression model was used to identify the risk factors for disease-free survival. Correlation analysis was used to determine the correlation between PTEN OD and the prognosis of HCC patients. SPSS 19.0 for Windows was

used to conduct the statistical analyses. A value of $P < 0.05$ was considered statistically significant.

## Results

### Clinical characteristics

Of the patients studied, 63 (80.4%) were male and 16 (19.6%) were female; the male/female ratio was thus 4.9:1. The mean age of the patients was $49 \pm 5.2$ years. There were no significant differences in patient clinical characteristics between the HCCF and HCCN groups ($P > 0.05$), including age, gender, liver function (Child-Pugh score), tumour differentiation, tumour size, tumour number, micro-portal vein invasion, serous AFP value or HBV DNA lo (Table 1).

### PTEN protein expression in HCC tissues from the HCCF and HCCN groups

The PTEN protein was expressed mainly in the cytoplasm, appearing as diffuse tan or brown particles (Figure 1a–e). In the HCCF and HCCN groups, the OD values of the PTEN protein in HCC tissues were $0.1014 \pm 0.0572$ and $0.1732 \pm 0.0563$, respectively, which was significantly lower than those in the paired para-cancerous tissues of HCCF and HCCN groups and normal liver tissues ($0.2051 \pm 0.0321$, $0.3542 \pm 0.0358$ and $0.4213 \pm 0.6524$, respectively, $P < 0.05$). The OD values of the PTEN protein in the HCC tissues and paired para-cancerous tissues of the HCCF group were significantly lower than those in the HCCN group ($P < 0.05$; Figure 1f). However, there was no significant difference of PTEN expression among the 34 cases of HCCF group ($P > 0.05$).

### HCC recurrence and HCC patient survival during follow-up

All HCC patients were followed up for 3 months to 10 years (median 48.5 months). The six-month, one-year and two-year overall recurrence (OR) rates of the HCCF group were 15.1%, 22.3% and 40.1%, respectively, significantly higher than the rates in the HCCN group (10.3%, 17.4% and 25.6%, respectively, $P = 0.008$). The one-year, two-year and five-year overall survival (OS) rates of the HCCF group were 57%, 46% and 31.8%, respectively, much lower than the rates of the HCCN group (85.3%, 75.2% and 61.5%, respectively, $P = 0.02$; Figure 2).

### Risk factors for HCC recurrence and HCC patient survival after radical surgery

Univariate analysis was used to identify the risk factors contributing to OR and OS, which are presented in Table 2. In total, five factors, Child-Pugh score, tumour differentiation, intrahepatic vascular invasion, HBV DNA load >100 IU/mL and impaired PTEN protein expression, were significant risk factors for poorer prognosis. There were 13 HCC patients (16.5%) with a level 1 OD value, 33 patients (41.8%) with level 2, 20 patients (25.3%) with level 3 and 13 patients (16.4%) with level 4. The two-year OR rate was 81.4% in patients with a level 1 OD value, 39.3% in patients with level 2, 25.6% in patients with level 3 and 9.61% in patients with level 4. The five-year OS rate was 11.2% in

**Table 1** Difference of clinical characteristics of patients in HCCF and HCCN groups

| Variables | | HCCF group | HCCN group | $t$ or $\chi^2$ value | $P$ value |
|---|---|---|---|---|---|
| Age | >60 years | 11 | 19 | 3.174 | 0.112 |
| | ≤60 years | 23 | 26 | | |
| Gender | Male | 28 | 36 | 0.623 | 0.751 |
| | female | 6 | 9 | | |
| Child-Pugh score | Class A | 15 | 20 | 0.876 | 0.754 |
| | Class B | 19 | 25 | | |
| Tumour differentiation | I–II | 23 | 30 | 0.435 | 0.754 |
| | III–IV | 11 | 15 | | |
| Tumour encapsulation | Complete | 19 | 25 | 0.912 | 0.364 |
| | Incomplete | 15 | 20 | | |
| Tumour | 1 | 21 | 26 | 0.130 | 0.937 |
| | 2 | 9 | 13 | | |
| | 3 | 4 | 6 | | |
| Tumour size | >5 cm | 9 | 12 | 0.862 | 0.546 |
| | ≤5 cm | 25 | 33 | | |
| Intrahepatic vascular invasion | Yes | 4 | 5 | 0.798 | 0.387 |
| | No | 30 | 40 | | |
| AFP | > 20 ng/mL | 29 | 38 | 0.413 | 0.751 |
| | ≤20 ng/mL | 5 | 7 | | |
| HBV-DNA load | >100 IU/mlmL | 9 | 11 | 0.732 | 0.731 |
| | ≤100 IU/mlmL | 25 | 34 | | |

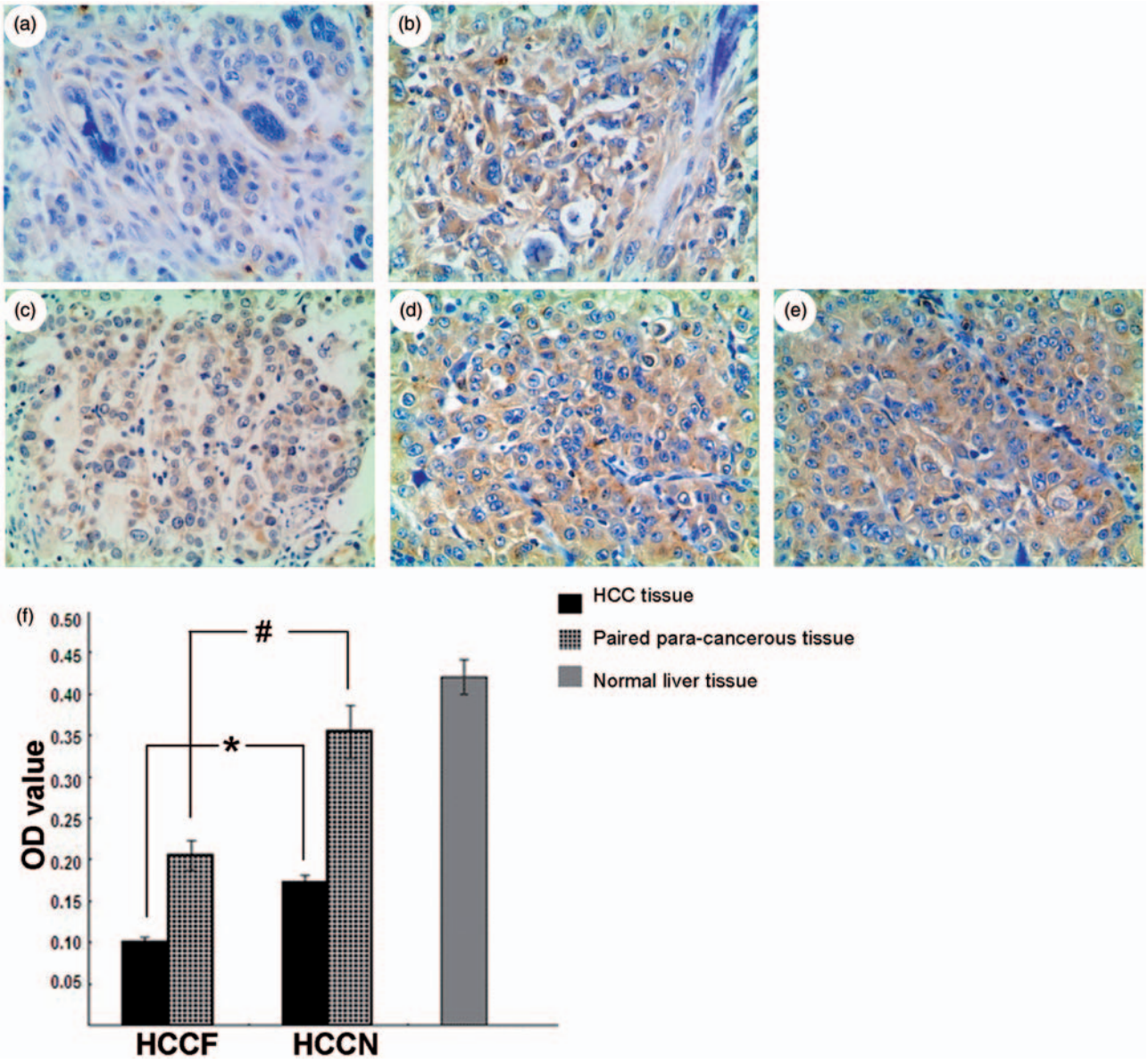


**Figure 1** PTEN protein expression in HCCF, HCCN and normal liver tissues. The PTEN protein was expressed mainly in the cytoplasm, appearing as diffuse tan or brown particles in immunohistological sections (×400). HCC tissues from the HCCF group are shown in (a), paired para-cancerous tissues from the HCCF group in (b), HCC from the HCCN group in (c), paired para-cancerous tissues of the HCCN group in (d) and normal liver tissues in (e). The OD values of PTEN protein expression in the HCC and paired para-cancerous tissues from the HCCF group were significantly lower than those of the HCCN group (* and # indicate $P < 0.05$) (f). (A color version of this figure is available in the online journal)

patients with a level 1 OD value, 40.3% in patients with level 2, 71.2% in patients with level 3 and 83.4% in patients with level 4.

### Impaired PTEN protein expression is an independent prognostic risk factor

Cox multivariate regression analysis was performed for all five significant prognostic risk factors. The data revealed that intrahepatic vascular invasion, tumour differentiation and OD values of impaired PTEN protein expression were independent prognostic factors for both two-year OR and five-year OS (Table 3). Additionally, the Cox proportional hazards regression model analysis of intrahepatic vascular invasion, tumour differentiation and impaired PTEN protein expression revealed that the Wald $\chi^2$ of impaired PTEN protein expression (OD values) was 19.38, higher than that of the other two variables (Table 4).

Subsequently, the correlation between the OD values of impaired PTEN protein expression and surgery outcomes were analysed. The results revealed that both the recurrence rates and survival rates were significantly correlated with the OD value of PTEN protein expression (Figure 3).

The Kaplan-Meier curves for HCC patients with different OD values of PTEN protein expression revealed a

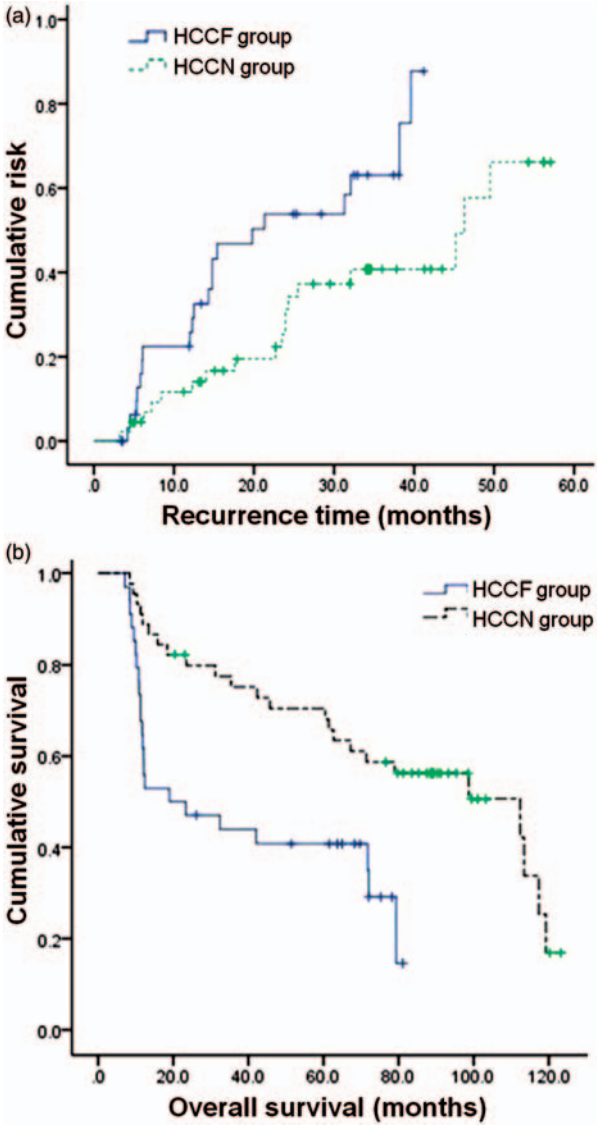


**Figure 2** The overall recurrence and survival rates of the HCCF and HCCN patients. The cumulative recurrence and survival rates were significantly different between patients in the HCCF and HCCN groups. According to the Kaplan-Meier survival curves, the six-month, one-year and two-year recurrence rates in the HCCF group were 15.1%, 22.3% and 40.1%, respectively, significantly higher than those in the HCCN group (10.3%, 17.4% and 25.6%, respectively; log-rank test, $P = 0.008$) (a). The one-, two- and five-year survival rates of the HCCF group were 57%, 46% and 40.3%, respectively, lower than those of the HCCN group (85.3%, 75.2% and 61.5%, respectively, log-rank test, $P = 0.02$) (b). (A color version of this figure is available in the online journal)

significant difference in the cumulative recurrence rates among patients with different levels of OD values (log-rank test, $P = 0.039$ for level 1, $P = 0.043$ for level 2, $P = 0.047$ for level 3, $P = 0.023$ for level 4; Figure 4a). The two-year recurrence rates for patients with different OD values were 81.4% (level 1), 39.3% (level 2), 25.6% (level 3) and 9.61% (level 4; $P < 0.05$). The five-year OS rates for patients with different OD values were 11.2% (level 1), 40.3% (level 2), 71.2% (level 3) and 83.4% (level 4; $P < 0.05$). There was a significant difference in cumulative survival among patients with different levels of OD values (log-rank test, $P = 0.018$ for level 1, $P = 0.01$ for level 2, $P = 0.007$ for level 3 and $P = 0.030$ for level 4; Figure 4b).

Taken together, these findings indicated that dramatically impaired PTEN protein expression was an independent prognostic risk factor for HCC with family aggregation resulting from hepatitis B and liver cirrhosis.

## Discussion

HCC is one of the most prevalent cancers worldwide. As in many cancers, variants in the genes involved in various stages of hepatocarcinogenesis may determine susceptibility to HCC development.[16]

The *PTEN* gene, located at 10q23.3, is a member of the *PTP* gene family. Abnormal PTEN protein expression has been detected in a variety of human tumours. The *PTEN* gene, one of the most common tumour suppressor genes aside from P53, plays an important role in tumourigenesis. Research has indicated that PTEN can block cell cycle progression and inhibit cell proliferation and growth by interfering with the Akt/PKB pathways.[17] Mutations in or loss of the *PTEN* gene can lead directly to excessive cell proliferation and decreased apoptosis.[18,19] At the same time, PTEN can induce apoptosis and block the cell cycle by inhibiting the accumulation of cyclin D1, and inducing the expression of p21, a cyclin-dependent kinase inhibitor.[20,21] PTEN gene loss and mutations have been detected in a variety of malignant tumours in humans, including malignant glioma and prostate and breast cancer.[22–24]

The absence or reduction of *PTEN* gene expression is closely related to hepatocarcinogenesis. Hu *et al.*[25] performed a semi-quantitative analysis of PTEN mRNA in HCC samples, paired para-liver tissues and normal liver tissues and showed that the PTEN mRNA expression level in HCC tissue was significantly lower than that in paired para-liver tissues and normal liver tissues; however, the relationship between PTEN and HCC with family aggregation resulting from chronic viral hepatitis B and liver cirrhosis remains unclear.

Chronic HBV infection is the major risk factor for HCC in China. An epidemiological survey confirmed that HCC has a strong familial component.[2,3] Gao Yanhui *et al.* reported that HBV infection was likely one of the main reasons for HCC with family aggregation in Southern China.[3] The present study revealed that the OD values of PTEN protein expression in HCC tissues and paired para-cancerous tissues in the HCCF group were significantly lower than those in the HCCN group ($P < 0.05$), which indicated that impaired PTEN protein expression was significantly correlated with HCC with family aggregation resulting from chronic hepatitis B and liver cirrhosis. According to the above results, there may be some connection between chronic viral hepatitis B and liver cirrhosis and markedly impaired PTEN protein expression, but this relationship requires further investigation. PTEN protein expression was also correlated to

**Table 2** Univariate analysis of factors contributing to OR and OS rates of HCC patients

| Variables | | 2 years OR | *P* value | 5 years OS | *P* value |
|---|---|---|---|---|---|
| Age | >60 years | 24.7% | 0.53 | 62.3% | 0.217 |
| | ≤60 years | 32.1% | | 71.2% | |
| Gender | Male | 21.3% | 0.41 | 51.0% | 0.312 |
| | Female | 19.7% | | 61.3% | |
| Child-Pugh score | Class A | 19.1% | 0.043 | 71.2% | 0.034 |
| | Class B | 56.8% | | 62.7.3% | |
| Tumour differentiation | I–II | 19.5% | <0.01 | 73.5% | <0.01 |
| | III–IV | 43.5% | | 42.3% | |
| Tumour encapsulation | Complete | 21.3% | 0.32 | 61.2% | 0.87 |
| | Incomplete | 32.1% | | 51.3% | |
| Tumour size | >5cm | 50.3% | 0.053 | 51.2% | 0.062 |
| | ≤5cm | 40.2% | | 56.3% | |
| Intrahepatic Vascular invasion | Yes | 45.7% | <0.01 | 16.4% | <0.01 |
| | No | 15.4% | | 78.1% | |
| AFP | > 20 ng/mL | 41.2% | <0.01 | 51.6% | 0.723 |
| | ≤20 ng/mL | 29.3% | | 53.1% | |
| HBV-DNA load | >100 IU/mlmL | 39.4% | <0.01 | 34.1% | 0.02 |
| | ≤100 IU/mlmL | 18.9% | | 60.8% | |
| OD value of expression of PTEN protein | Level 1 | 81.4% | <0.01 | 11.2% | <0.01 |
| | Level 2 | 39.4% | | 40.3% | |
| | Level 3 | 25.6% | | 71.2% | |
| | Level 4 | 9.61% | | 83.4% | |

**Table 3** Multivariate analysis of factors contributing to OR and OS rates of HCC patients

| Variables | *P* value of multivariate analysis for OR | HR (95%CI) | *P* value of multivariate analysis for OS | HR (95%CI) |
|---|---|---|---|---|
| Child-Pugh score | >0.05 (NS) | | >0.05 (NS) | |
| Tumour differentiation | <0.01 | 5.34 (3.172–7.324) | <0.01 | 4.33 (2.037–4.708) |
| Intrahepatic vascular invasion | <0.01 | 4.78 (3.178–6.907) | <0.01 | 3.98 (2.098–5.179) |
| HBV-DNA load | >0.05 (NS) | | >0.05 (NS) | |
| OD value of expression of PTEN protein | <0.01 | 3.98 (1.432–5.347) | <0.01 | 4.33 (1.178–6.782) |

CI: confidence interval; HR: hazard ratio; NS: not significant.

**Table 4** Cox proportional hazards regression model analysis for significantly prognostic variables for HCC patients

| Variables | Wald $\chi^2$ | *P* | RR | RR(95% CI) |
|---|---|---|---|---|
| OD value of PTEN expression | 19.38 | <0.01 | 5.37 | 2.54–11.34 |
| Tumour differentiation | 5.49 | 0.032 | 2.92 | 1.19–7.24 |
| Intrahepatic vascular invasion | 4.25 | 0.047 | 2.61 | 1.08–6.56 |

CI: confidence interval; RR: relative risk (hazard ratio).

prognosis of other malignancy other than HCC. Accumulating evidences have been revealed recently. Impaired PTEN protein expression was correlated to poor prognosis of solid tumours, e.g. cervical cancer, pancreatic ductal adenocarcinoma, salivary gland cancer, prostatic intraepithelial neoplasia, colorectal cancer and so on,[26–30] however, it was not significant for PTEN protein expression to be a prognostic factor in malignant pleural mesothelioma.[31]

PTEN can inhibit cell proliferation and promote cell apoptosis by downregulating the activity of PI3K/Akt induced by PTEN, ultimately affecting HCC progression in HBV-related HCC.[32] The present study revealed that PTEN protein expression was impaired in HCC resulting from chronic hepatitis B and liver cirrhosis, and it was dramatically and significantly impaired in HCC with family aggregation resulting from chronic hepatitis B and liver cirrhosis. However, there was no significant difference of PTEN expression among the families of HCCF group, which indicated possible epigenetic mechanisms rather

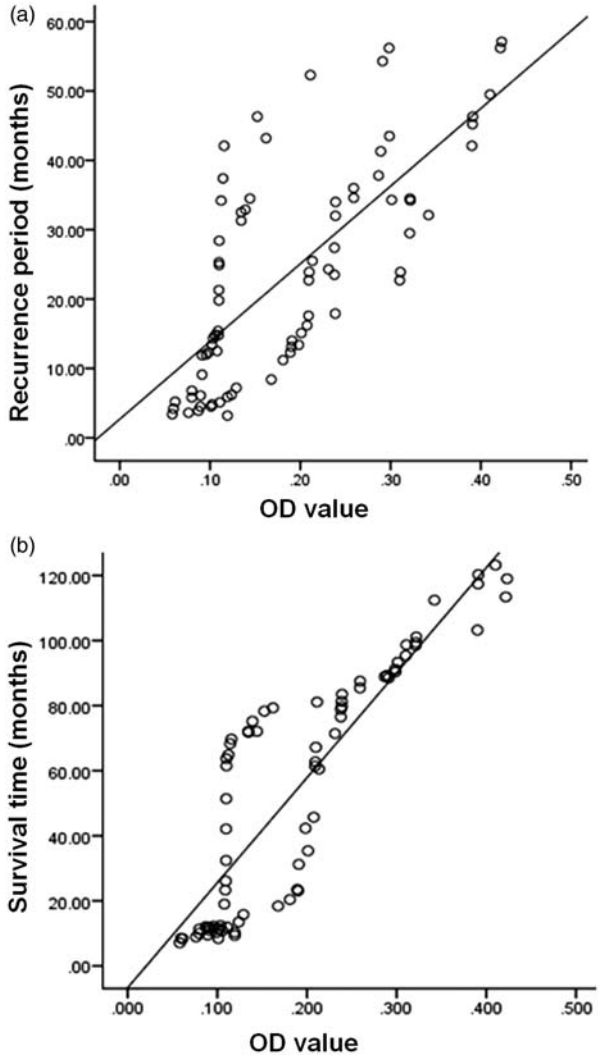


**Figure 3** The correlation between PTEN protein expression (OD value) and prognosis in HCC patients. The time to recurrence was significantly correlated with the OD value ($R = 0.524$, $P = 0.013$) (a). The survival time was also significantly correlated with the OD value ($R = 0.753$, $P = 0.008$) (b)

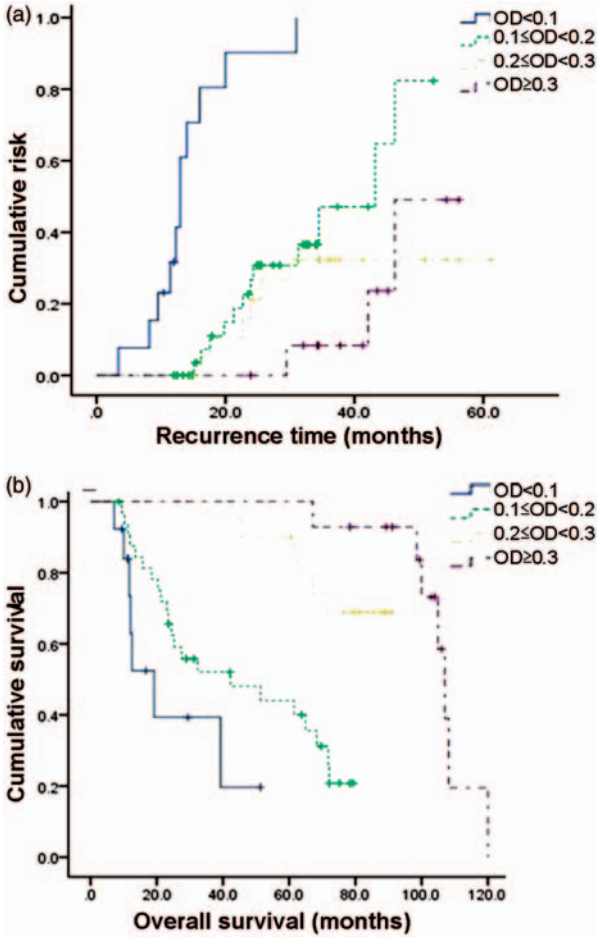


**Figure 4** The cumulative recurrence and survival rates of HCC patients with different OD values of PTEN protein expression. The results of Kaplan-Meier curves revealed a significant difference in the cumulative recurrence and survival rates of patients with different levels of PTEN OD values. The two-year recurrence rates were 81.4%, 39.3%, 25.6% and 9.61% for patients with level 1 ($OD < 0.1$), level 2 ($0.1 \leq OD < 0.2$), level 3 ($0.2 \leq OD < 0.3$) and level 4 ($OD \geq 0.3$) staining, respectively ($P < 0.05$) (a), and the five-year overall survival rates were 11.2%, 40.3%, 71.2% and 83.4% for patients with level 1, level 2, level 3 and level 4 staining, respectively ($P < 0.05$) (b). (A color version of this figure is available in the online journal)

than genetic mechanisms for the impairment of PTEN expression. Furthermore, impaired PTEN expression was significantly correlated with worse OR and survival according to the results of Cox multivariate regression analysis and Cox proportional hazard regression model analysis; low OD values of PTEN protein expression were associated with significantly worse OR and OS. The present study revealed that PTEN protein expression plays a valuable prognostic role in radical surgery for HCC with family aggregation resulting from chronic viral hepatitis B and liver cirrhosis.

## Conclusions

PTEN protein expression was dramatically and significantly impaired in HCC with family aggregation resulting from chronic viral hepatitis B and liver cirrhosis, and impaired PTEN protein expression in HCC was an independent risk factor for surgical outcome of HCC patients. The relationship between chronic viral hepatitis B and PTEN protein expression in HCC requires further investigation.

**Authors contribution:** YZ, JY and MD conceived and designed the experiments. RX commented on the manuscript. YZ, JY, KH, ZY and YZ performed the experiments. YZ and RX analysed the data. YZ and MD contributed reagents/materials/analysis tools. YZ and JY wrote the paper.

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