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ORIGINAL PAPER

Diagnostic and prognostic significance of peroxiredoxin 1 expression in human hepatocellular carcinoma

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Abstract Peroxiredoxin 1 (Prdx1) is a member of the peroxiredoxin family of antioxidant enzymes and implicated in cell differentiation, proliferation, and apoptosis. The aim of the present study was to determine the expression and diagnostic and prognostic significance of Prdx1 in human hepatocellular carcinoma (HCC). Prdx1 expression was examined in 76 HCC patients and 20 healthy volunteers. The relationships between Prdx1 expression and clinicopathological features were analyzed. Receiver operating characteristics analysis was used to calculate the diagnostic accuracy of serum Prdx1, serum alpha-fetoprotein (AFP), and their combination. The prognostic impact of Prdx1 on overall survival (OS) and disease-free survival (DFS) of HCC patients was investigated. Prdx1-positive rate was significantly ($p < 0.05$) higher in HCC (77.1 %) than in adjacent non-tumorous liver tissues (18.4 %). Prdx1 immunoreactivity was positively correlated with tumor vascular endothelial growth factor expression and microvessel density. Prdx1 expression was significantly associated with tumor size, microvascular invasion, Edmondson grade, tumor capsula status, serum AFP, and tumor-node-metastasis stage. The combination of serum Prdx1 and AFP had a markedly higher area under the curve than serum Prdx1 alone. Positive Prdx1 expression was associated with unfavorable OS ($p = 0.004$) and DFS ($p = 0.001$). Multivariate analysis revealed intratumoral Prdx1 staining as an independent poor prognostic marker for OS ($p = 0.006$) and DFS ($p = 0.002$). Taken together, our data suggest that increased Prdx1 expression is associated with tumor angiogenesis and progression in HCC and serves as a promising biomarker for detection and prognosis of this malignancy.

Keywords Diagnosis · Metastasis · Peroxiredoxin 1 · Prognosis · Tumor angiogenesis

Abbreviations

AFP	α-Fetoprotein
DAB	3,3-Diaminobenzidine tetrahydrochloride
DFS	Disease-free survival
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
HBeAg	Hepatitis B e antigen
HCC	Hepatocellular carcinoma
HE	Hematoxylin and eosin
MVD	Microvessel density

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OS	Overall survival
PBS	Phosphate buffered saline
Prdx1	Peroxiredoxin 1
ROC	Receiver operating characteristics
SD	Standard deviation
TLR4	Toll-like receptor 4
TNM	Tumor-node-metastasis
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor

Introduction

Hepatocellular carcinoma (HCC) is the fifth common malignancy and the third leading cause of cancer-related deaths worldwide [1]. Despite improvements in care and therapeutic approaches, the 5-year survival rate of HCC patients is only approximately 10 % [2, 3]. One important reason for this low survival rate is that the majority of HCC patients are diagnosed at advanced stage, where curative treatments are not effective or feasible due to tumor spread. Identification of specific biomarkers, especially for early stage tumors, is of significance to improve the prognosis of HCC.

Peroxiredoxin 1 (Prdx1) is a member of the thiol-dependent peroxiredoxin family of antioxidant enzymes that control cytokine-induced peroxide levels and mediate signal transduction in mammalian cells [4]. It is implicated in regulation of numerous biological processes including cell differentiation, proliferation, and apoptosis [5]. Previous studies have reported Prdx1 to be upregulated in many types of cancer such as thyroid cancer [6], bladder cancer [7], lung cancer [8], and prostate cancer [9], suggesting its contribution to cancer development and progression. The interaction of Prdx1 with toll-like receptor 4 (TLR4) promotes prostate cancer growth through enhancement of vascular endothelial growth factor (VEGF)-dependent tumor angiogenesis [9]. However, in some other cancer types, Prdx1 may function as a tumor suppressor [10]. For example, Hoshino et al. [11] reported that the activation of Prdx1 mediates the antitumor activity of FK228, a histone deacetylase inhibitor, in esophageal cancer cells.

A recent study has demonstrated that upregulation of Prdx1 contributes to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance in liver cancer [12]. However, the clinical significance of Prdx1 in HCC is not yet clear. In this study, we examined the expression of Prdx1 in tumor tissues from 76 patients with HCC and evaluated the associations of Prdx1 tissue expression levels with clinicopathological parameters and patient survival. Additionally, we assessed the potential of serum Prdx1 in differentiating HCC patients from healthy individuals.

Materials and methods

Patients and tissue samples

This study was approved by the Human Research Ethics Committee of Anhui Medical University (Hefei, China), and written informed consent was obtained from each patient. For immunohistochemistry, tumor samples and paracarcinomatous liver tissues were collected from 76 patients with a definitive diagnosis of HCC and undergoing surgical resection at the Affiliated Provincial Hospital of Anhui Medical University between 2006 and 2009. Patients with any prior anticancer therapy or concurrent second primary cancer were excluded from this study. Demographic and clinicopathological data were retrieved from medical records and consisted of age, gender, tumor size, number of tumor nodule, tumor capsula, microvascular invasion, Edmondson grade, status of hepatitis B e antigen (HBeAg), cirrhosis, Child–Pugh grade, levels of preoperative alpha-fetoprotein (AFP), and tumor stage. Sixty-four were male and twelve were female, with a mean age of 53 ± 12 years (range 19–74 years). Tumor differentiation was defined according to the Edmondson grading system [13], and tumor stage was performed according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. Liver function was assessed using Child–Pugh classification. Follow-up data were available for all patients. Median follow-up was 24 months (range 2–72 months). For the measurement of serum Prdx1, peripheral blood samples were collected from each patient before surgery. As control, blood samples were also obtained from 20 age-matched, healthy volunteers.

Immunohistochemical staining for Prdx1, CD31, and VEGF

Tissue sections (4 μm thick) were deparaffinized with xylene, rehydrated, and subjected to microwave antigen retrieval in citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase was quenched with 3 % hydrogen peroxide for 10 min. The sections were then separately incubated with rabbit anti-human antibodies against Prdx1, VEGF (Beijing Biosynthesis Biotechnology, Beijing, China), or CD31 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After washing, sections were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 20 min. Immunoreactivity was visualized with 3,3′-diaminobenzidine substrate. Sections were counterstained with

hematoxylin, dehydrated, and mounted. Negative controls were included by omitting the primary antibody.

The tumor expression of Prdx1 and VEGF was semi-quantitatively assessed. Ten random fields per gene were selected, and the percentage of immunoreactive cells in a total of 1,000 tumor cells was determined [14]. No staining or focal or weak staining in <10 % of tumor cells was clarified as negative, moderate, or patchy immunopositivity in 10–30 % of tumor cells as "+," and strong or diffuse immunopositivity in >30 % of tumor cells as "++." To evaluate the association of Prdx1 with tumor angiogenesis, microvessel density (MVD) was calculated after immunostaining for CD31 [14]. In each tumor, at least three hotspots displaying the highest vessel density were initially identified at low-power magnification (×100), and the maximum number of microvessels was counted for each area under high-power magnification (×400). According to the median value of 68, MVD was classified as either low or high. The immunohistochemical results were evaluated by two pathologists who were blinded to clinical data, and discrepancies were resolved by consensus.

Measurement of serum Prdx1 levels by ELISA

A 5-ml venous blood sample was withdrawn from each subject and centrifuged for 10 min at 2,500 r/min at 4 °C. Serum was subsequently collected and stored at −80 °C until testing. Serum Prdx1 levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's protocol (Shanghai Yuan Ye Biological Technology Co., LTD). Briefly, 100 µl of serum samples or standards was added to a 96-well ELISA plates. After incubation for 2 h at room temperature, the wells were washed three times. Each well was added with the detection antibody and incubated for 2 h at room temperature. After washing, 100 µl of the working dilution of horseradish peroxidase-labeled streptavidin was added to each well and incubated for 20 min at room temperature. The substrate solution was then added and incubated for another 20 min. The absorbance was measured at 450 nm with a microtiter plate reader (Thermo Scientific, Waltham, MA, USA) after 100 µl of stop solution was added to each well. Each assay was performed in triplicate and repeated three times.

Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD). Significant differences in the means were determined using the Student's t test or one-way analysis of variance followed by the Tukey's test. The chi-square test and Spearman's correlation test are used to analyze the immunohistochemistry results. Receiver operating characteristics (ROC) curves were generated to determine the diagnostic performance of serum Prdx1, serum AFP, and their combination. The Kaplan–Meier method and the log-rank test were applied to determine the survival analysis. The Cox regression model was employed to determine the independent prognostic value. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the statistical package SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Immunohistochemical staining for Prdx1 and VEGF in HCC tissues

Immunohistochemistry showed that Prdx1 was mainly localized in the cytoplasm of tumor cells with varying staining intensity (Fig. 1). The positive rate of Prdx1 was significantly ($p < 0.05$) higher in HCC tissues (56/76, 77.1 %) than in adjacent non-tumorous liver tissues (14/76, 18.4 %). Cytoplasmic VEGF expression was detected in 53 of 76 HCC specimens (69.7 %; Fig. 1). Spearman's rank correlation test revealed a significant positive correlation between tissue expression of Prdx1 and VEGF in HCC tissues ($r = 0.452$, $p < 0.001$; Table 1).

Relationship between Prdx1 expression and MVD

The MVD ranged from 0 to 198/200 per field (median, 65/200 per field) in HCC tissues, as determined by CD31 staining (Fig. 2a). Tumors with positive Prdx1 expression had significantly greater MVD than Prdx1-negative counterparts (78.8 ± 39.4 vs. 33.8 ± 26.6, $p < 0.01$; Fig. 2b).

Correlation of tissue Prdx1 expression with clinicopathological parameters

We next analyzed the associations between tissue Prdx1 expression and clinicopathological parameters in HCC. As shown in Table 2, the expression level of Prdx1 was significantly associated with tumor size ($p = 0.012$), microvascular invasion ($p < 0.001$), Edmondson grade ($p = 0.004$), tumor capsula status ($p = 0.001$), serum AFP ($p = 0.008$), and TNM stage ($p < 0.001$). However, Prdx1 immunoreactivity showed no significant correlation with age, gender, HBeAg status, cirrhosis, Child–Pugh grade, and tumor nodule number.

Clinical significance of serum Prdx1 in HCC

The results of ELISA showed that HCC patients had a significantly higher level of serum Prdx1 than healthy individuals (31.3 ± 13.4 vs. 8.2 ± 6.8 ng/ml, $p < 0.01$; Fig. 3a).

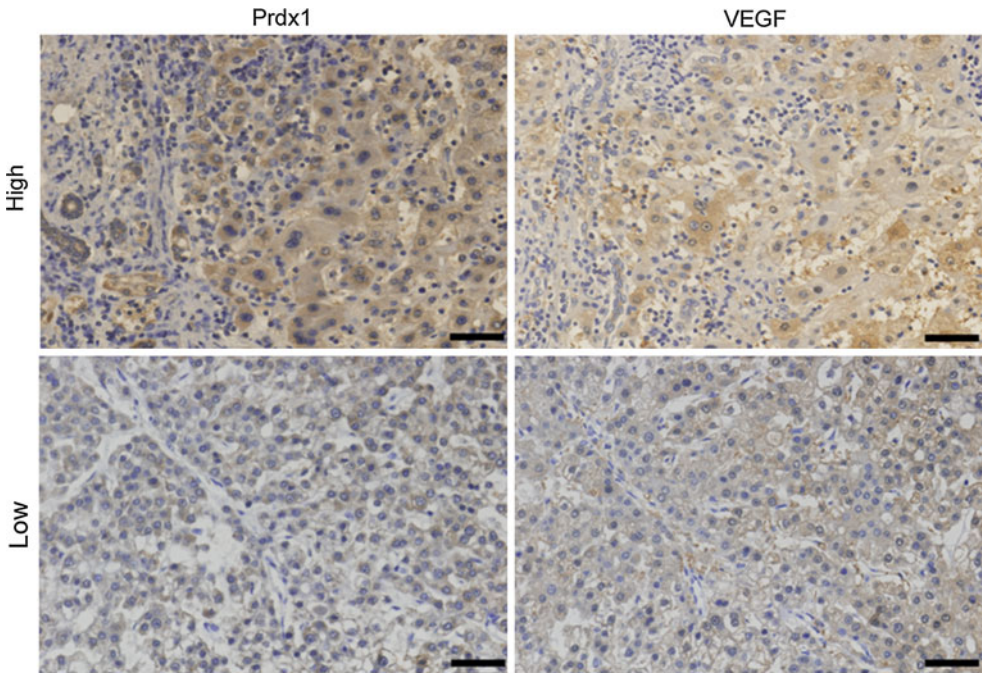

Fig. 1 Immunohistochemical staining for Prdx1 and VEGF in HCC tissues. Prdx1 and VEGF expression showed diffuse cytoplasmic staining in tumor cells. Representative sections show high and low cytoplasmic expression of Prdx1 and VEGF in tumor cells. *Bar* = 50 μM

Table 1 Expression correlation between Prdx1 and VEGF in 76 HCC cases by immunohistochemistry

Immunoreactivity	Prdx1			
	+~++	−	*r*	*p* value
VEGF				
+~++	46	7	0.452	<0.001
−	10	13		

Prdx peroxiredoxin, *VEGF* vascular endothelial growth factor

Furthermore, as shown in Table 2, there were significantly higher serum levels of Prdx1 in patients with multiple tumor nodules ($p = 0.014$), vascular invasion ($p = 0.006$), incomplete capsule ($p = 0.032$), serum positive HBeAg ($p = 0.041$), and more advanced TNM staging ($p < 0.001$). An ROC curve (Fig. 3b) was performed to evaluate the accuracy and reliability of Prdx1 for the detection of HCC. Serum Prdx1 level was effective in distinguishing HCC patients from healthy subjects (AUC = 0.817; 95 % confidence interval (CI) 0.726–0.908). Moreover, the addition of serum AFP increased the ability of serum Prdx1 to detect HCC, with an AUC of 0.822 (95 % CI 0.742–0.903).

Survival analyses

Kaplan–Meier curves (Fig. 4) were plotted to compare the overall survival (OS) and disease-free survival time (DFS) between Prdx1-positive HCC patients and Prdx1-negative

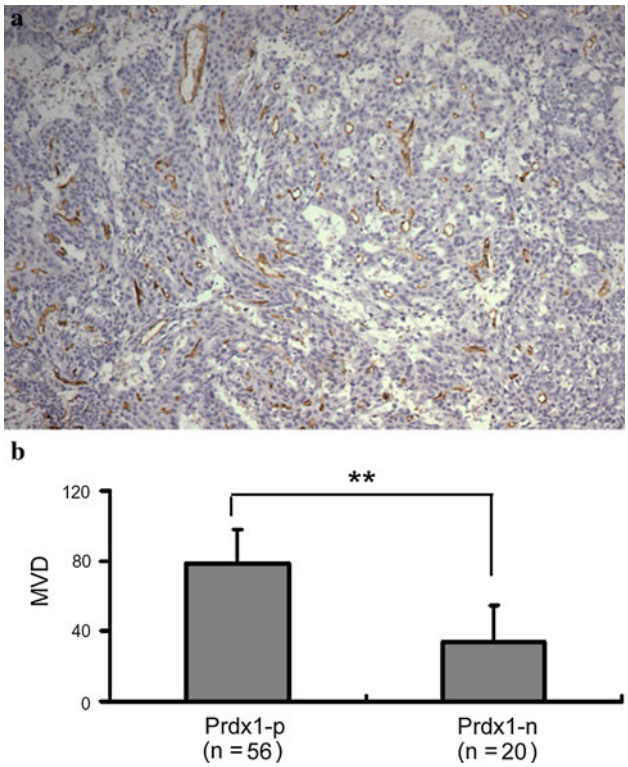

Fig. 2 Measurement of MVD in HCC tissues by CD31 staining. **a** Representative section of HCC with immunohistochemical staining of CD31. *Bar* = 50 μM. **b** Measurement of MVD by CD31 staining. Tumors with positive Prdx1 (Prdx1-p) expression had a significantly greater MVD compared to tumors with negative Prdx1 (Prdx1-n) expression. Data are expressed as the number of CD31-positive microvessels per field. $^{**}p < 0.01$

Table 2 Prdx1 expression status in relation to clinicopathological features in 76 HCC patients

Clinicopathological data	Prdx1 immunohistochemical staining			Prdx1 serum level	
	−	+∼++	*p*	Mean ± SD	*p*
Age (years)			0.86		0.419
<60	14	38		38.66 ± 8.24	
≥60	6	18		41.47 ± 15.81	
Sex			0.236		0.808
Male	19	45		39.44 ± 11.52	
Female	1	11		38.91 ± 9.46	
Tumor size (cm)			0.012		0.949
>5	7	37		39.62 ± 9.65	
≤5	13	18		39.4 ± 13.22	
Tumor nodule number			0.076		0.014
Single	20	45		37.76 ± 9.74	
Multiple	0	11		50.11 ± 13.56	
Vascular invasion			<0.001		0.006
Present	0	24		44.56 ± 10.14	
Absent	20	32		37.24 ± 10.93	
Edmondson grade			0.004		0.175
I–II	15	21		37.73 ± 10.36	
III–IV	5	35		41.19 ± 11.72	
Tumor capsula			0.001		0.032
Present	19	31		37.58 ± 12.86	
Absent	1	25		43.35 ± 5.17	
HBeAg status			1		0.041
Positive	17	47		40.35 ± 11.79	
Negative	3	9		34.98 ± 6.58	
Cirrhosis			1		0.072
Present	18	51		39.96 ± 11.53	
Absent	2	5		34.71 ± 5.39	
Child–Pugh grade			0.519		0.624
A	20	52		39.41 ± 11.29	
B	0	4		42.05 ± 9.48	
AFP (ng/ml)			0.008		0.526
>20	8	41		40.21 ± 9.91	
≤20	12	15		38.35 ± 13.26	
TNM stage			<0.001		<0.001
I–II	20	24		35.52 ± 10.38	
III–IV	0	32		45.10 ± 9.85	

Prdx peroxiredoxin, *HBeAg* hepatitis B e antigen, *AFP* alpha-fetoprotein

HCC patients. Patients with Prdx1-positive expression (23.2 months; 95 % CI 17.9–28.5) had a shorter OS compared to Prdx1-negative patients (48.7 months; 95 % CI 36.5–60.9; $p = 0.004$). Similarly, the DFS was significantly lower in patients with Prdx1-positive expression (26.1 months; 95 % CI 20.8–31.3) than in those with Prdx1-negative expression (48.0 months; 95 % CI 35.5–60.4; $p = 0.001$).

Univariate analysis indicated that tumor expression of Prdx1, sex, tumor size, vascular invasion, Edmondson grade, tumor capsula status, serum AFP, and TNM stage had significant prognostic influences on OS (Table 3). Multivariate survival analysis (Table 4) further revealed intra-tumoral Prdx1 staining as an independent poor prognostic marker for OS [hazard ratio (HR) = 2.897; 95 % CI 1.355–6.194; $p = 0.006$] and DFS (HR = 3.268;

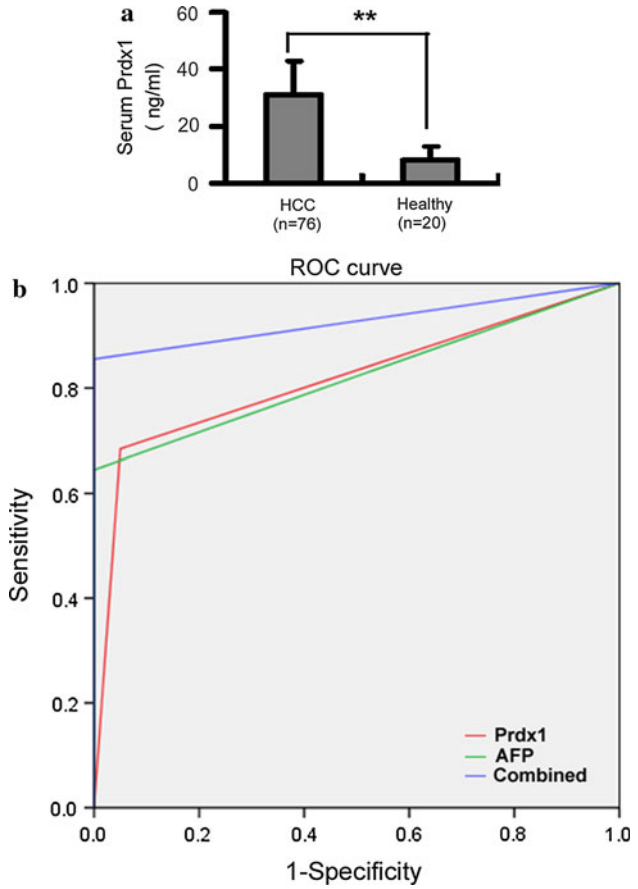

Fig. 3 Diagnostic potential of serum Prdx1 in HCC. **a** Measurement of serum Prdx1 levels in 76 HCC patients and 20 healthy individuals by ELISA. $^{**}p < 0.01$. **b** Receiver operating characteristic curves for serum Prdx1, serum AFP, and their combination in patients with HCC versus healthy controls

95 % CI 1.532–6.971; $p = 0.002$). Additionally, sex, tumor size, vascular invasion, tumor capsula status, and TNM stage were also independent prognostic factors for OS and DFS (Table 4).

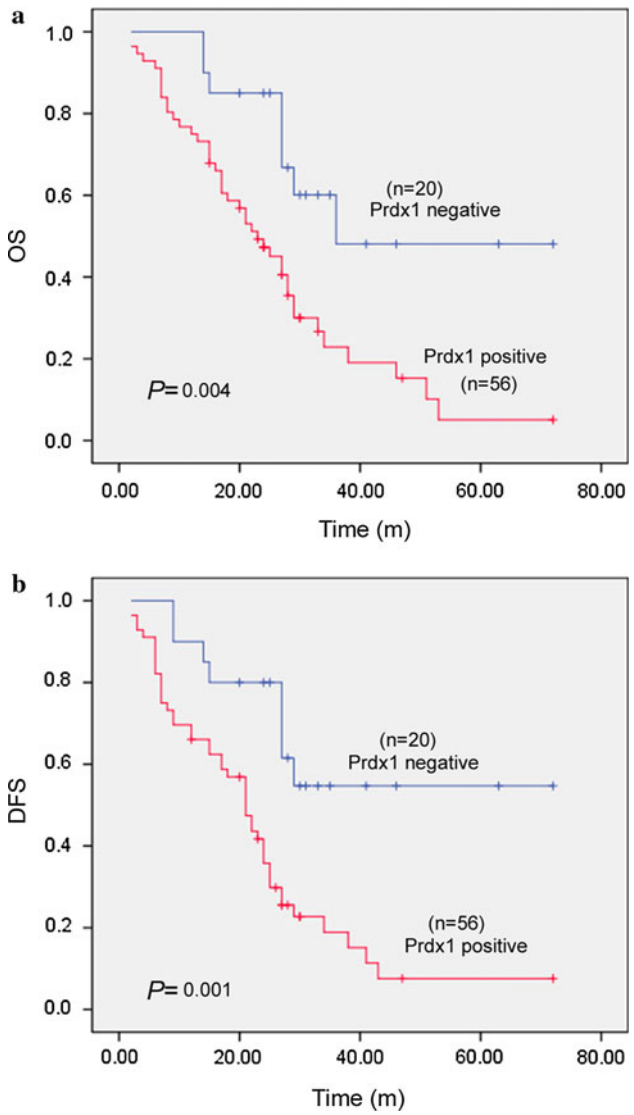

Fig. 4 Kaplan–Meier analysis of overall survival (OS) and disease-free survival (DFS) of HCC patients according to intra-tumoral Prdx1 expression. The HCC patients with positive Prdx1 expression showed significantly poorer OS (**a**) and DFS (**b**) than those with negative Prdx1 expression

Discussion

In this study, we evaluated the expression and clinical significance of Prdx1 in HCC. Upregulation of Prdx1 has been documented in many cancers, including liver cancer [6, 8, 12]. Our present data confirm the increased expression of Prdx1 in HCC relative to adjacent non-tumorous liver tissues. Moreover, we have demonstrated, to the best of our knowledge for the first time, that serum Prdx1 levels are significantly raised in HCC patients compared to healthy individuals. Identification of effective biomarkers for the detection of HCC is of clinical significance. Several non-invasive serum biomarkers such as AFP, des-gamma carboxyprothrombin, glypican-3, and osteopontin have been proposed [15]. Peroxiredoxin 3, another member of the peroxiredoxin family, has been suggested as a biomarker for HCC [16]. Serum concentrations of Prdx1 have shown diagnostic value in patients with lung cancer [17]. Thus, here we explored the potential of Prdx1 as a diagnostic marker in HCC. Our data revealed that serum Prdx1 had a similar diagnostic accuracy to serum AFP in differentiating between HCC and healthy subjects, as evidenced by comparable AUC values. Most interestingly, the combination of Prdx1 and AFP had a markedly higher AUC than each of them alone. These findings suggest that when used in combination with other biomarkers, serum Prdx1 may provide additional diagnostic power in HCC.

Table 3 Univariate analysis of factors associated with OS and DFS

Variable	OS		DFS	
	Median survival time (m)	*p*	Median survival time (m)	*p*
Prdx1		0.004		0.001
Negative	48.0		48.7	
Positive	26.1		23.2	
Age (years)		0.481		0.495
<60	33.1		31.0	
≥60	27.7		22.6	
Sex		0.007		0.027
Male	15.9		14.2	
Female	33.5		31.2	
Tumor size (cm)		0.036		0.027
>5	26.2		23.3	
≤5	39.2		37.3	
Tumor nodule number		0.063		0.059
Single	33.8		32.1	
Multiple	22.0		18.6	
Vascular invasion		0.006		0.008
Present	35.8		33.1	
Absent	20.7		18.8	
Edmondson grade		0.001		0.001
I–II	21.2		19.0	
III–IV	40.8		37.6	
Tumor capsula		<0.001		0.001
Present	37.8		35.9	
Absent	20.1		17.2	
HBeAg status		0.688		0.224
Positive	24.3		19.5	
Negative	32.2		30.5	
Cirrhosis		0.624		0.745
Present	28.3		22.4	
Absent	31.2		29.5	
Child–Pugh grade		0.194		0.082
A	32.0		30.1	
B	17.4		13.3	
AFP (ng/ml)		0.014		0.115
>20	23.9		22.5	
≤20	40.2		34.5	
TNM stage		<0.001		<0.001
I–II	40.5		38.1	
III–IV	20.1		17.1	

Prdx peroxiredoxin, *HBeAg* hepatitis B e antigen, *AFP* alpha-fetoprotein

Accumulating evidence indicates that Prdx1 plays a prominent role in tumor survival and progression [18, 19]. Du et al. [18] reported that Prdx1 is capable of suppressing proteasome inhibitor-mediated cell death in thyroid cancer cells through modulation of apoptosis signal-regulating kinase 1 activation. Ha et al. [19] showed that Prdx1 overexpression enhances transforming growth factor β1-induced epithelial–mesenchymal transition (EMT) and cell migration in cancer cells. EMT is regarded as a key event involved in tumor invasion and metastasis [20]. HCC is characterized by its high propensity for vascular invasion and metastasis. Our findings provide clinical evidence for the link between Prdx1 expression and HCC development. We found that increased hepatic and serum expression of

Table 4 Multivariate analysis of factors associated with OS and DFS

Variable	OS			DFS		
	HR	95 % CI	*p*	HR	95 % CI	*p*
Prdx1 (negative vs. positive)	2.897	1.355–6.194	0.006	3.268	1.532–6.971	0.002
Sex (male vs. female)	0.377	0.178–0.798	0.011	0.451	0.215–0.946	0.035
Tumor size, cm (≤5 vs. >5)	1.847	1.024–3.334	0.042	1.872	1.050–3.337	0.034
Vascular invasion (present vs. absent)	2.164	1.213–3.862	0.009	2.093	1.187–3.690	0.011
Edmondson grade (I–II vs. III–IV)	0.370	0.202–0.678	0.370	0.412	0.233–0.729	0.002
Tumor capsula (complete vs. none)	2.721	1.555–4.760	<0.001	2.363	1.372–4.070	0.002
TNM stage (I–II vs. III–IV)	3.067	1.739–5.410	<0.001	2.945	1.700–5.100	<0.001

Prdx peroxiredoxin, *HR* hazard ratio, *CI* confidence interval

Prdx1 was significantly associated with numerous aggressive parameters of HCC, including higher tumor size, multiple tumor nodules, microvascular invasion, advanced Edmondson grade, incomplete tumor capsula, greater serum AFP, and advanced TNM stage. These data, combined with the previous study demonstrating an involvement of Prdx1 in TRAIL resistance in HCC cells [12], highlight an important role for Prdx1 in HCC survival and metastasis.

It is widely accepted that tumor angiogenesis is a key component in tumor metastasis [21]. Our data revealed that Prdx1 expression was positively correlated with MVD in HCC, suggesting its implication in tumor angiogenesis. VEGF is a well-defined pro-angiogenic factor. Several previous studies have demonstrated that VEGF expression significantly correlates with MVD in HCC [22, 23]. Notably, our data revealed a significant positive correlation between tissue expression of Prdx1 and VEGF in HCC. These findings collectively suggest that Prdx1 may be involved in VEGF-mediated tumor angiogenesis in HCC, which provides an explanation for the significant associations between Prdx1 immunoreactivity and HCC aggressiveness. Indeed, induction of VEGF-dependent tumor angiogenesis by Prdx1 has been documented in prostate cancer [9, 24]. However, further studies are still needed to unravel the biological functions of Prdx1 in tumor angiogenesis and progression in HCC.

Several studies have shown the prognostic significance of Prdx1 expression in different malignancies [25–27]. For instance, Kim et al. [25] reported that Prdx1 expression status predicts recurrence and shorter survival in stage I non-small cell lung cancer after surgery. Likewise, Li et al. [27] demonstrated that positive Prdx1 expression serves as an independent poor prognostic marker in squamous cell/adenosquamous carcinomas and adenocarcinoma of gallbladder. Using the Kaplan–Meier analysis and log-rank test, we found that HCC patients with Prdx1-positive tumors had a significantly shorter OS and DFS than those with Prdx1-negative tumors. Furthermore, using the Cox proportional hazards regression model, we revealed positive Prdx1 expression as an independent prognostic factor in HCC. The adverse prognostic effect of Prdx1 further suggests a tumor-promoting role for this gene in HCC.

A major limitation of this single-institute study is relatively small sample size. Additionally, there is a potential selection bias inherent to any retrospective study. A prospective study with a larger cohort of patients is thus needed to confirm the present findings.

In conclusion, we have provided the first evidence that upregulation of Prdx1 is associated with tumor angiogenesis and progression in HCC. Serum Prdx1, in conjunction with serum AFP, is effective in differentiating HCC from healthy individuals. Prdx1-positive expression is an independent predictor of poor OS and DFS in HCC patients receiving curative surgery. These findings warrant further investigation into the biological relevance of increased expression of Prdx1 in HCC.

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Conflict of interest The authors declare no competing financial interests.

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