

## Original Article

# Peroxiredoxin 4 promotes embryonal hepatoblastoma cell migration but induces fetal cell differentiation

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Received February 4, 2020; Accepted May 28, 2020; Epub June 15, 2020; Published June 30, 2020

**Abstract:** Hepatoblastoma (HB) is the leading primary hepatic malignancy in children and likely emerges due to failure of hepatic progenitor cells to properly differentiate. The peroxiredoxin (PRDX) family is frequently linked to cancer. In our previous study, we demonstrated that expression of the only secreted family member, PRDX4, was correlated with hepatocellular carcinoma. The aim of this new study was to investigate PRDX4's role in HB. We collected 87 HB specimens and performed PRDX4 immunohistochemistry staining. Clinical analysis was conducted and the effect of PRDX4 overexpression on two HB cell lines (Huh6 and HepG2) was also examined. Clinical data revealed elevated PRDX4 expression in embryonal component was correlated with advanced stage (IV) and metastasis. In comparison, increased PRDX4 expression in fetal component was associated with well differentiation. In vitro experiments showed PRDX4 overexpression enhanced migration in embryonal-like HB cells (Huh6), which was accompanied by epithelial-mesenchymal transition (EMT). By contrast, PRDX4 overexpression inhibited proliferation, decreased stemness markers, and increased hepatic markers in fetal-like HB cells (HepG2), which indicated induction of tumor cell differentiation. In conclusion, PRDX4 promotes embryonal hepatoblastoma cell migration but induces fetal cell differentiation. It can be adopted as an important marker for HB prognosis and a potential treatment target.

**Keywords:** PRDX4, hepatoblastoma, migration, epithelial-mesenchymal transition, differentiation

## Introduction

Hepatoblastoma (HB), which originates in hepatic primordial embryonic cells, is the most common childhood hepatic malignancy, often diagnosed in the first three years of life [1-3]. It is thought to result from failure of liver stem cells to differentiate into hepatocytes [4]. Malignant transformation of normal hepatic stem/progenitor cell during embryogenesis gives rise to HB [5]. Histologically, it is classified

as epithelial type (pure fetal subtype, mixed embryonal/fetal subtype, small-cell undifferentiated subtype, and macrotrabecular subtype), mixed epithelial and mesenchymal type (with or without teratoid features), and other unspecified types [6]. Intrahepatic tumor extension and distant metastasis are two critical factors for poor prognosis [7, 8]. Standard treatment for HB is chemotherapy combined with tumor resection. Although 3-year event-free survival (EFS) after treatment is >80% for standard-risk

cases [9], it is ~60% for high-risk HB, even after intensive chemotherapy and liver surgery [10, 11]. Thus new diagnostic markers are needed to better predict prognosis, as are novel drugs to improve treatment response.

Peroxiredoxins (PRDXs) are a ubiquitous family of antioxidant enzymes expressed in mammalian cells [12] and are frequently associated with cancer initiation and progression [13-16]. As the only secreted member of the PRDX family [17, 18], PRDX4 is also implicated in many cancers [19-23] and plays specific role depending on cancer type. In oral cavity squamous cell carcinoma, high PRDX4 expression is associated with worse disease-specific survival [21]. By contrast, increased PRDX4 expression in gastric cancer is correlated with prolonged survival [16]. Previously, we reported that PRDX4 weak expression combined with high MIB-1 index predicted poor prognosis in stage I lung adenocarcinoma [24]. Moreover, recently we found that PRDX4 overexpression blocked initiation of hepatocellular carcinoma (HCC) but played a dual role in HCC progression [25].

Given that HB is the most common primary hepatic malignancy in young children [26], here we examined PRDX4 expression in HB samples and investigated the relationship between PRDX4 expression and tumor progression. We analyzed potential correlations between PRDX4 expression and clinical features, especially tumor stage and distant metastasis. Furthermore, we overexpressed PRDX4 in two different HB lines (HepG2 and Huh6) and examined tumor cells' biologic behavior's changes. The present study demonstrates PRDX4 plays complex role in hepatoblastoma. In detail, PRDX4 high expression promotes embryonal HB cell migration and is associated with advanced tumor stage and metastasis. Meanwhile, PRDX4 overexpression induces fetal HB cell differentiation and is linked with a better differentiated histological type.

### Materials and methods

#### *Human hepatoblastoma tissues and cell lines*

We collected 87 HB specimens from 5 medical centers in Japan. All samples were formalin-fixed and paraffin-embedded. All subjects or their parents gave their informed consent for inclusion. Ethical approval was obtained from

ethics committee of Kanazawa Medical University (No. I367). Associated clinical information, including demographic features and clinical characteristics, was also collected. Two HB cell lines (HepG2 and Huh6) were employed for in vitro studies. HepG2 was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB, Osaka, Japan) and has been confirmed as a HB cell line [27]. Huh6 was obtained from RIKEN BRC Japan. The two cell lines have been defined as fetal-like HB cell (HepG2) and embryonal-like HB cell (Huh6) respectively in terms of genetic mutation and metabolism differences according to Stefania Crippa's study [28].

#### *Pathology and immunohistochemical staining*

Haematoxylin and eosin (H&E) staining was conducted to confirm HB diagnosis. We adopted an international pediatric liver tumor consensus classification as the histological classification guideline [29]. The antibody used for human peroxiredoxin-4 (hPRDX4) immunohistochemical (IHC) staining was from Thermo Fisher (PA3-753, dilution 1:1000). The IHC procedure included: 1) deparaffinization and rehydration; 2) 0.5% hydrogen peroxide blocking for 15 minutes at room temperature; 3) antigen retrieval in trypsin solution; 4) primary antibody staining overnight at 4°C; 5) Secondary antibody (Histofine Simple Stain MAX-PO424152) staining for 30 minutes at room temperature; and 6) 3, 3' diaminobenzidine (DAB) imaging and haematoxylin counterstaining. H&E and IHC staining images were captured and analyzed quantitatively using NanoZoomer Digital Pathology Virtual Slide-Viewer software (Hamamatsu Photonics Corp., Hamamatsu, Japan). Both histological classification and analysis of the extent of PRDX4 expression were performed independently by two experienced pathologists (S.Y. and A.S.).

#### *Cell culture and transfection*

HepG2 and Huh6 lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Cells at 60% confluence were transfected with empty pCMV-Tag-2b vector (Invitrogen) or a PRDX4 expression plasmid. Opti-MEM and Lipofectamine 2000 (Thermo Fisher) were employed for transfection.

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### *Cell counting Kit-8 proliferation assay*

Cells were harvested on day 3 after transfection and then seeded in 96-well plates at a density of 2000 cells per well. Six replicate wells were used for each group. Cell viability was measured at 0, 24, 48, and 72 hours after seeding using cell counting Kit-8 (CCK8, Dojindo Molecular Technologies) according to manufacturer's instructions.

### *Cell migration assay*

Cells on day 3 after transfection were used for migration assay. A suspension of  $15 \times 10^4$  transfected cells was applied to 8-mm pore inserts (Corning Incorporated), with serum-free media in the upper chamber and 30% fetal bovine serum in the lower chamber. After 48 hours of culture, the migrated cells were fixed by methanol and stained with 4', 6-diamidino-2-phenylindole (DAPI). The assay was repeated in triplicate. Migrated cells were counted in pictures of 40 folds field by Image J software.

### *Western blotting*

Protein extracts (10-20 ug) isolated from cell pellets were loaded onto SDS-PAGE gels (Bio-Rad), and after electrophoresis transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% skim milk and probed with corresponding antibodies. The following antibodies and dilutions were used: PRDX4 (PA3-753, Thermo Fisher, 1:1000), AFP (sc-130302, Santa Cruz, 1:200), EpCAM (ab71916, abcam, 1:1000), CD44 (ab51037, abcam, 1:5000), Oct4 (PA5-27438, 1:5000), Vimentin (5741, Cell Signaling, 1:1000), E-cadherin (3195, Cell Signaling, 1:1000), N-cadherin (13116, Cell Signaling, 1:1000), p-p38 (4511s, Cell Signaling, 1:1000), p-SAPK/JNK (4668s, Cell Signaling, 1:1000), p-Erk (4370s, Cell Signaling, 1:1000), PCNA (sc-56, Santa Cruz, 1:200), PEPCK (sc-271029, Santa Cruz, 1:100), CYP3A4 (sc-53850, Santa Cruz, 1:200),  $\beta$ -actin (O11-24554, Fujifilm, 1:1000). The quantitation of band was conducted by Image J software.

### *Statistical analysis*

Categorical variables were compared using Chi-Squared test or Fisher's exact test. Con-

tinuous variables were expressed as means  $\pm$  SD, and a two-tailed unpaired t-test was used for comparison. All statistical analyses were performed using the SPSS statistical software package, version 16.0. A two-sided *p* value less than 0.05 was considered statistically significant.

## Results

### *Correlation between PRDX4 expression and clinical characteristics*

#### *Clinical characteristics of the study population:*

A total of 87 HB cases were included in our study. The oldest age at diagnosis was 13 years and the youngest was <3 months. Most cases (66/87, 75.86%) were diagnosed in their first 3 years, in line with other studies [30]. Some preponderance was found for male sex (male 66.67% versus female 33.33%). Only two cases showed a serum alpha-fetoprotein (AFP) levels <1000 ng/ml at diagnosis. 32 cases contained only fetal component in histology, while 37 cases consisted of both embryonal and other components. Metastasis occurred in 26.44% of the whole cases and pretreatment extent of disease (PRETEXT) stage IV accounted for 25.93% of all cases. All clinical features are shown in **Table 1**. The 5-year overall survival (OS) was 92.94% in our study, whereas 5-year event-free survival (EFS) decreased to 77.65%.

#### *Increased PRDX4 expression in embryonal component is linked with metastasis and advanced PRETEXT stage, but is associated with better differentiation in fetal component:*

We firstly analyzed HB cases without considering histological pattern, aiming to reveal the possible correlation between PRDX4 expression and prognosis. Survival analysis shows no significant difference in event free survival rate or overall survival rate between strong and weak PRDX4 expression groups (data not shown). Examples of strong and weak PRDX4 expression cases were shown in **Figure S1**. Then we focused on PRDX4's expression in individual HB component. In embryonal component, we found that PRDX4 expression was higher in cases with metastasis than those without metastasis ( $35.56 \pm 14.67$  versus  $21.14 \pm 14.76$ ,  $P = 0.015$ ) and also stronger in PRETEXT stage IV samples than other stage samples ( $34.09 \pm 16.56$

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**Table 1.** Clinical characteristics of hepatoblastoma subjects (n = 87)

Clinical characteristics at diagnosis	Study population n (%)
Sex	
Female	29 (33.33%)
Male	58 (66.67%)
Age (years)	
0-3	66 (75.86%)
3-7	15 (17.24%)
≥8	6 (6.90%)
Maximal tumor diameter (cm)	
<10	34 (40.00%)
≥10	51 (60.00%)
Serum AFP (ng/ml)	
≤1000	2 (2.38%)
1001-1000000	62 (73.81%)
>1000000	20 (23.81%)
PRETEXT stage	
I-III	60 (74.07%)
IV	21 (25.93%)
Metastasis	
Absent	64 (73.56%)
Present	23 (26.44%)
VPEFR	
Absent	32 (42.11%)
Present	44 (57.89%)
Histological category	
Pure fetal	32 (36.78%)
HB with embryonal component	37 (42.53%)
Others	18 (20.69%)
Recurrence within 5 years	
Absent	66 (77.65%)
Present	19 (22.35%)
Death within 5 years	
Absent	79 (92.94%)
Present	6 (7.06%)

AFP: alpha-fetoprotein; PRETEXT: pretreatment extent of disease; VPEFR: involvement of Vena cava or involvement of Portal vein or contiguous Extrahepatic intra-abdominal tumor extension or multiFocal liver tumor or tumor Rupture at diagnosis; HB: hepatoblastoma.

versus  $20.65 \pm 13.98$ ,  $P = 0.016$ ) (**Figure 1**). Furthermore, PRDX4 high expression group had a significant higher proportion of metastasis cases than low expression group when we adopted the median PRDX4 score (= 25) as the cut-off point (**Table 2**). In fetal component, PRDX4 expression was higher

in well-differentiated fetal than other fetal ( $50.00 \pm 19.01$  versus  $29.66 \pm 19.65$ ,  $P < 0.001$ ) (**Figure 2**). In pure fetal HB cases, no significant difference was found in PRDX4 expression between stage IV and other stages cases, or between metastasis and no metastasis cases. Data were shown in supplementary materials.

### *In vitro analysis of PRDX4 overexpression in two HB cell lines (Huh6 and HepG2)*

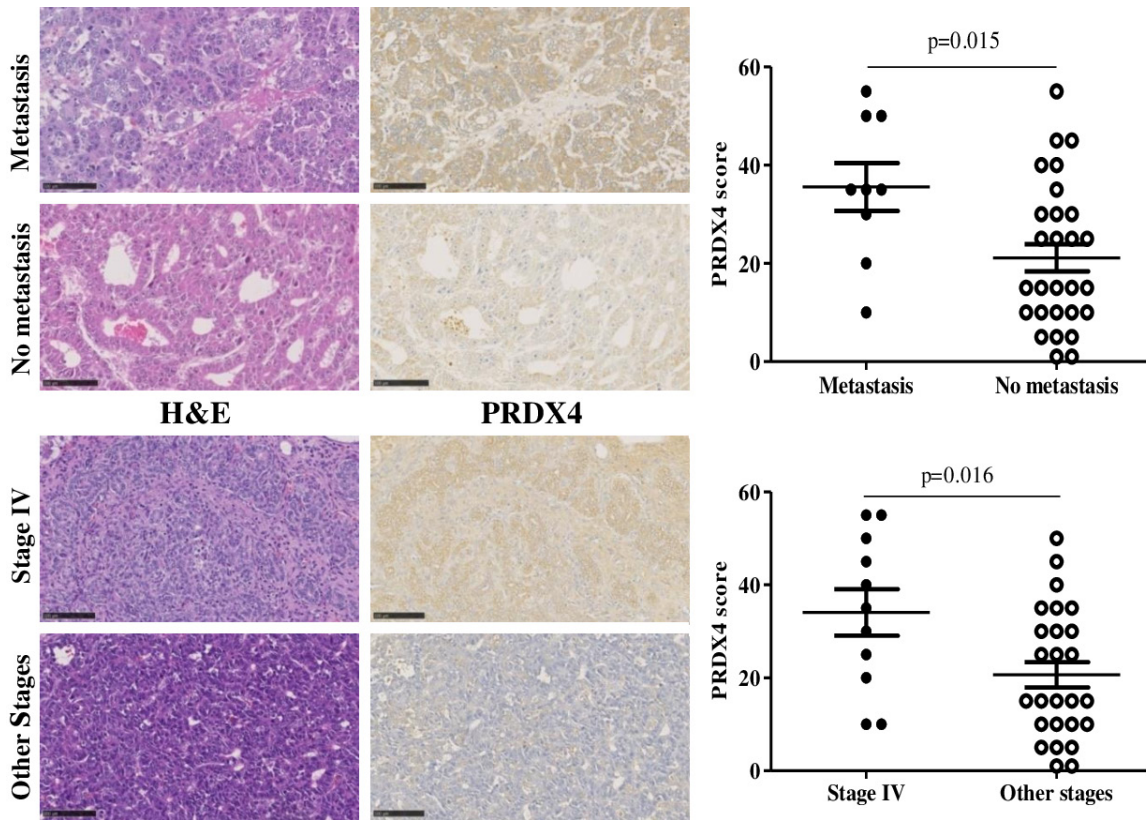
We employed two HB cell lines (Huh6 and HepG2) to examine the role of PRDX4 in vitro. Huh6 represents a less-differentiated embryonal-like HB cell, whereas HepG2 represents a more-differentiated fetal-like HB cell [28]. The basic expression and overexpression of PRDX4 in these two cell lines were shown in **Figure S2**.

*PRDX4 overexpression promotes migration and induces epithelial-mesenchymal transition (EMT) in embryonal-like HB cell (Huh6):* Transwell assay shows that the migration ability of Huh6 cell was enhanced by around 200% after PRDX4 overexpression. Epithelial marker E-cadherin was decreased, whereas mesenchymal markers like N-cadherin and Vimentin were increased (**Figure 3**), indicating the happening of EMT in embryonal-like HB cell (Huh6) following PRDX4 overexpression.

*PRDX4 overexpression inhibits stemness in fetal-like HB cell (HepG2):* In light of the clinical finding that PRDX4 expression was higher in well-differentiated fetal component than other fetal components, we examined whether PRDX4 was also associated with tumor stemness and differentiation in vitro. Following PRDX4 overexpression in HepG2, proliferation was inhibited by around 20%; stemness markers (AFP, EpCAM, OCT4, CD44) were decreased and differentiation markers (PEPCK and CYP3A4) were increased (**Figure 4**).

*PRDX4 overexpression exerts different effects on phosphorylated mitogen-activated protein kinases (p-MAPK) in embryonal-like and fetal-like HB cells:* Western blot analysis shows that conspicuous changes of p-MAPK happened after PRDX4 overexpression in both HB cell lines. For p-p38 and p-Erk, PRDX4 overexpression suppressed their expression in both cell lines, though the inhibitory effect was

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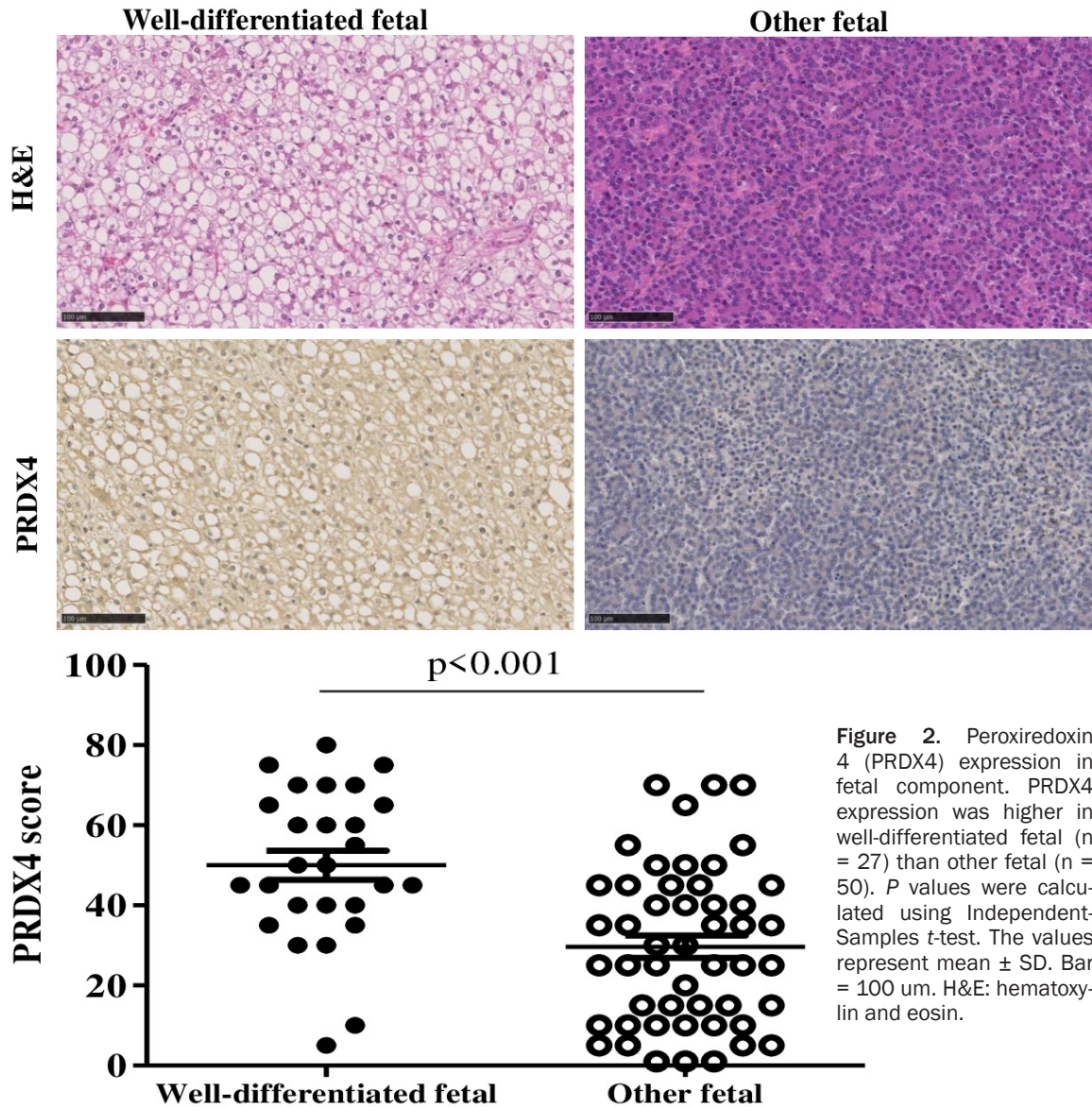


**Figure 1.** Peroxiredoxin 4 (PRDX4) expression in embryonal component. PRDX4 expression was stronger in metastasis cases ( $n = 9$ ) than no metastasis cases ( $n = 28$ ) and also higher in stage IV cases ( $n = 11$ ) than other stage cases ( $n = 26$ ).  $P$  values were calculated using Independent-Samples  $t$ -test. The values represent mean  $\pm$  SD. Bar = 100  $\mu$ m. H&E: hematoxylin and eosin.

**Table 2.** Comparison of clinical characteristics between PRDX4 high and low expression group in hepatoblastoma with embryonal component

Clinical characteristics	Low expression group	High expression group	$p$ value
Sex			0.19
Female	10	4	
Male	11	12	
Age (years)			1.00
<3	18	13	
$\geq 3$	3	3	
Maximal tumor diameter (cm)			0.09
<10	10	3	
$\geq 10$	11	13	
Serum AFP (ng/ml)	$7.48 \times 10^5 \pm 8.98 \times 10^5$	$7.93 \times 10^5 \pm 6.31 \times 10^5$	0.87
PRETEXT stage IV			0.15
Present	4	7	
Absent	17	9	
Metastasis			0.02*
Present	2	7	
Absent	19	9	

\* $P < 0.05$ ; AFP: alpha-fetoprotein; PRETEXT: pretreatment extent of disease. Fisher's exact test was used for analysis of categorical variables. Continuous variable's analysis was conducted by Independent-Samples  $t$ -test.



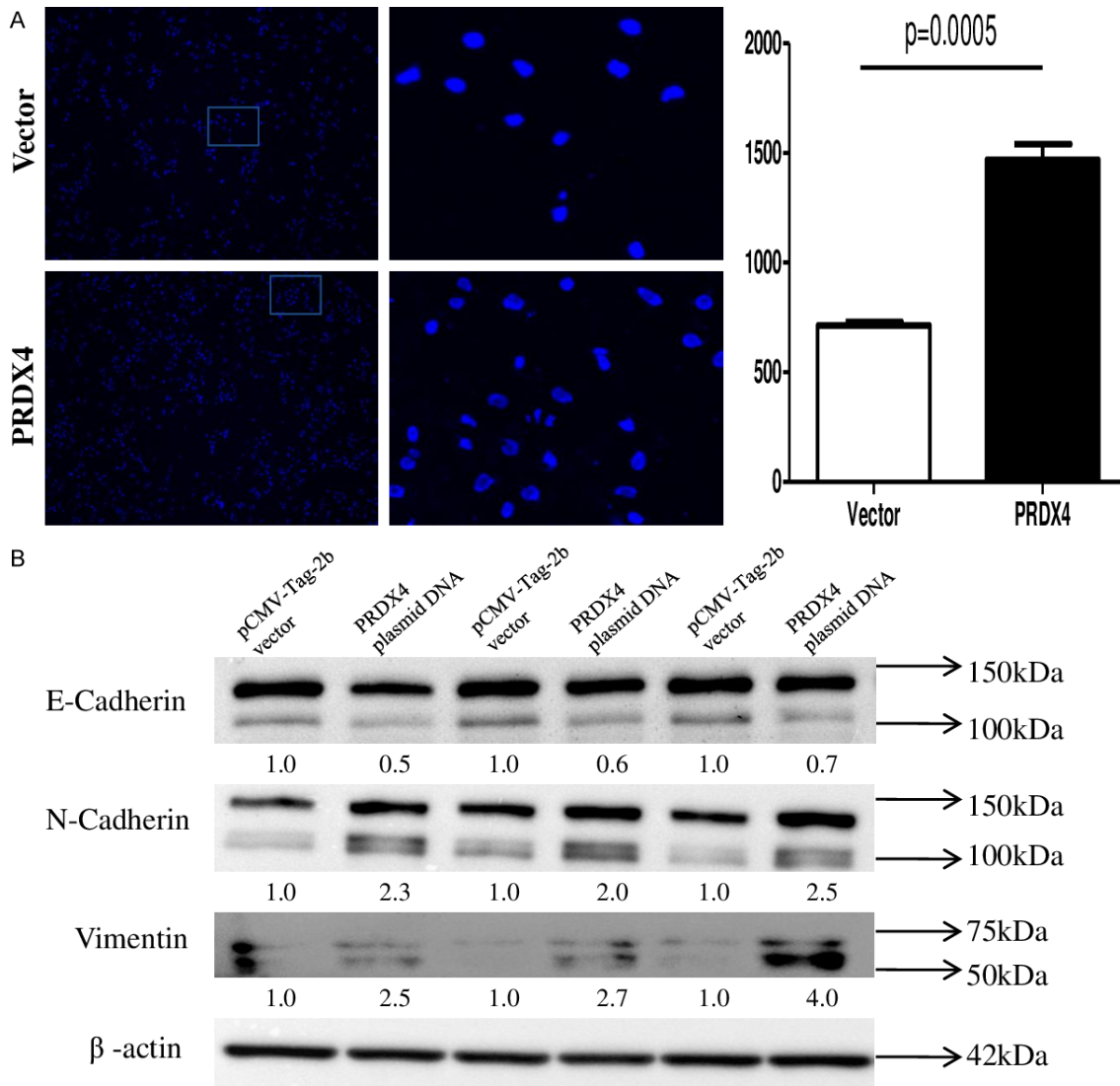
**Figure 2.** Peroxiredoxin 4 (PRDX4) expression in fetal component. PRDX4 expression was higher in well-differentiated fetal (n = 27) than other fetal (n = 50). P values were calculated using Independent-Samples t-test. The values represent mean  $\pm$  SD. Bar = 100  $\mu$ m. H&E: hematoxylin and eosin.

more obvious in Huh6. For p-JNK, PRDX4 enhanced its expression in Huh6, whereas opposite effect was observed in HepG2 (Figure 5).

**Discussion**

Although HB is the most common childhood hepatic malignancy, its rare incidence (around 1/1000000) [31, 32] hinders its further investigation. High risk HB still has a poor prognosis even after intensive chemotherapy and surgery. Clinical factors associated with prognosis include PRETEXT stage, metastasis, initial AFP level, VPEFR and age [7, 8, 33], whereas histology is still in debate. Among these factors,

PRETEXT stage IV and presence of metastasis are two leading factors for poor prognosis, with relative risk of 4.8 and 3.8 respectively [33]. PRDX4, a primary antioxidant, has been demonstrated to be involved in many tumors. Mounting evidences have indicated its role in tumor invasion and metastasis. In breast carcinoma [34], colon cancer [35], and oral cavity squamous cell carcinoma [21], elevated PRDX4 was correlated with distant metastasis. In prostate cancer, high expression of PRDX4 was associated with increase in tumor stage [20]. Our recent study in hepatocellular carcinoma revealed low PRDX4 expression group exhibited an increased incidence of portal and hepatic vein inva-



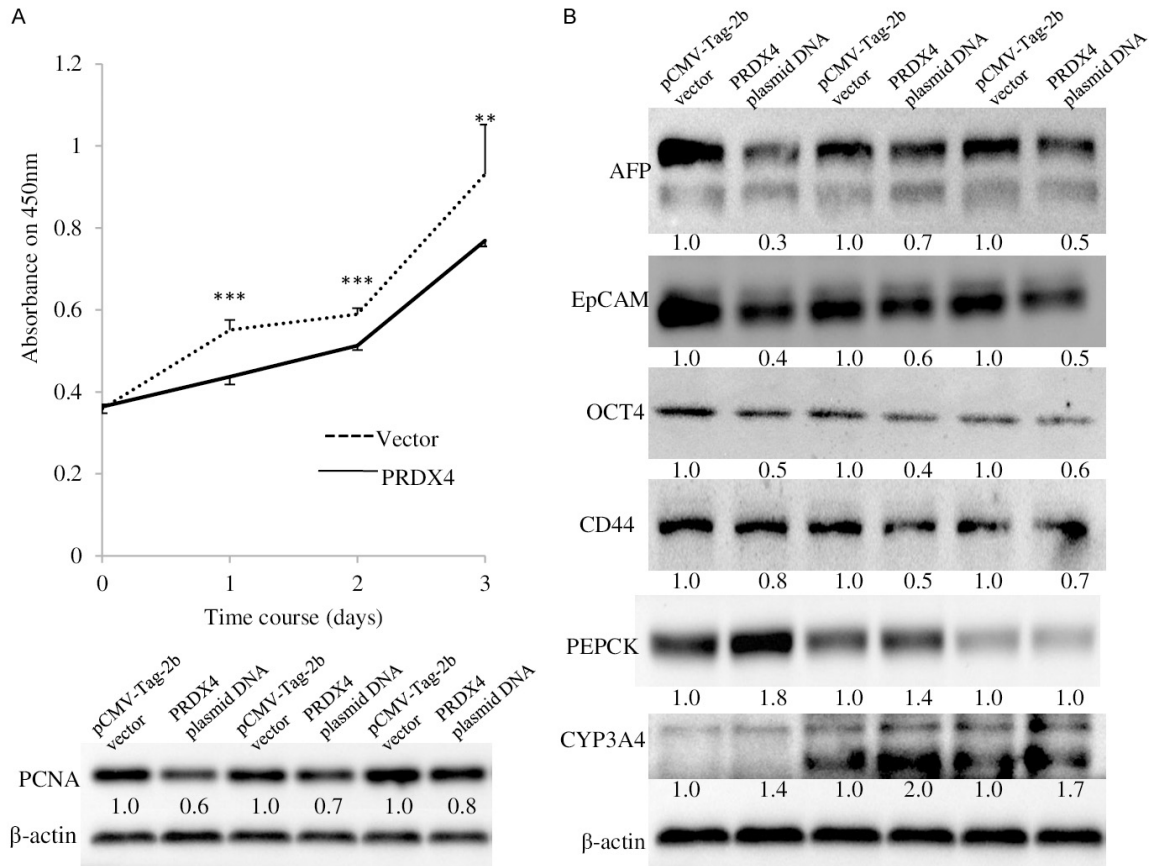
**Figure 3.** Peroxiredoxin 4 (PRDX4) overexpression promotes Huh6 migration and induces epithelial-mesenchymal transition. A. PRDX4 enhanced Huh6 migration by around 200% compared with empty pCMV-Tag-2b vector control. B. PRDX4 decreased E-Cadherin expression and increased N-Cadherin and Vimentin expressions in Huh6 as compared with empty pCMV-Tag-2b vector control.

sion, indicating the complex role of PRDX4 in tumor migration and invasion [25]. In the present study, we found elevated PRDX4 expression in embryonal component was associated with advanced stage and metastasis. In vitro study confirmed PRDX4 can promote embryonal-like HB cell migration, accompanied by epithelial-mesenchymal transition. However, no correlation was found between PRDX4 expression and tumor cell migration in fetal-like HB. By contrast, elevated PRDX4 was linked with a well-differentiated fetal pattern, which was associated with a better prognosis in many studies [6, 36-39]. Following in vitro study also shows PRDX4 overexpression can suppress

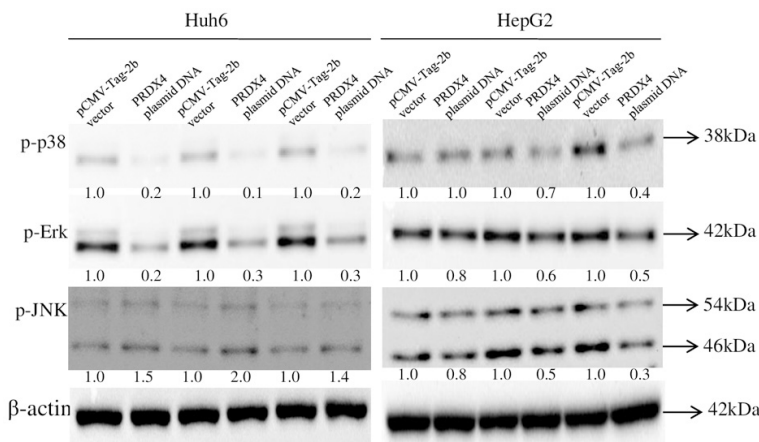
fetal-like HB cell stemness, evidenced by inhibited proliferation, decreased expression of stem cell markers, and increased expression of hepatocyte maturation markers.

It seems hard to explain the different relationships between PRDX4 and tumor behaviors in two HB cell lines. One possible explanation is the heterogeneity and complexity of hepatoblastoma. As the two most common epithelial components, fetal and embryonal HB differs in many aspects, including morphology, degree of differentiation [40, 41], and metabolism [28]. Those differences may lead to different tumor biologic behaviors, treatment response,

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**Figure 4.** Peroxisredoxin 4 (PRDX4) overexpression inhibits HepG2 stemness. A. PRDX4 inhibited HepG2 proliferation by around 30% compared with empty pCMV-Tag-2b vector control. PCNA: proliferating cell nuclear antigen. B. PRDX4 decreased expressions of stemness markers (AFP, EpCAM, OCT4, and CD44) and increased expressions of differentiation markers (PEPCK and CYP3A4) in HepG2 as compared with empty pCMV-Tag-2b vector control.



**Figure 5.** Comparison of phosphorylated mitogen-activated protein kinases (MAPK) expressions between Huh6 and HepG2 after peroxiredoxin 4 (PRDX4) overexpression. For p-p38 and p-Erk, PRDX4 strongly inhibits their expressions in Huh6 and moderately inhibits their expressions in HepG2. For p-SAPK/JNK, PRDX4 increases its expression in Huh6, while decreases its expression in HepG2.

and prognosis. For example, the well-differentiated pure fetal cases with low mitotic activity can even be surgically cured without chemotherapy [42, 43]. But for HB with embryonal component, chemotherapy is necessary. In our four pulmonary metastasis samples, every sample consists of embryonal component, whereas only one case includes fetal component (Figure S3). Moreover, pure fetal cases have a smaller opportunity of developing to advanced stage (PRETEXT stage IV) than those with embryonal component cases (Table S1). The intrinsic difference may partly explain



the different roles of PRDX4 in embryonal and fetal HB. Another explanation of this discrepancy may be the different changes of p-MAPK after PRDX4 overexpression in embryonal-like and fetal-like HB cells. Mitogen-activated protein kinase is a family which is associated with cell differentiation, proliferation, migration, and apoptosis [44-46]. Alteration of PRDX4 expression has been found to alter MAPKs expression in lung tumor cell [23]. In the present study, PRDX4 overexpression strongly suppressed the expression of p-p38 and p-Erk in embryonal-like HB cell, whereas mild suppression was observed in fetal-like HB. With respect to p-JNK, the effect of PRDX4 overexpression was totally different. p-JNK was increased in embryonal-like HB cell after PRDX4 overexpression, whereas decreased expression was induced in fetal-like HB cell. JNK pathway was frequently associated with cell migration [46, 47]. As the key player in this pathway, Elevated p-JNK after PRDX4 overexpression may contribute to the enhanced migration ability of embryonal-like HB cell, while full spectrum inhibition may explain the different effect in fetal-like HB cell.

Overall, PRDX4 plays different roles in different HB components. It can induce EMT and promote tumor cell migration in embryonal HB. In clinic, special attention should be paid to those HB subjects who have a high PRDX4 expression in embryonal component. The scan for metastasis should be more comprehensive and the corresponding adjustment in treatment may be needed. PRDX4 may be a potential treatment target. In comparison, high PRDX4 expression was associated with differentiation in fetal HB cells. PRDX4 strong expression may suggest a better prognosis in pure fetal subjects.

Our study has some limitations. The first is the relative small number of HB cases. This sample size makes multivariable analysis (Cox Regression) difficult to perform. Secondly, our study included only Japanese patients, and one should be cautious in applying conclusions to other populations. Thirdly, the mechanism underlying these findings was not fully investigated. Further studies are needed to elucidate the detailed process.

In conclusion, PRDX4 promotes embryonal hepatoblastoma cell migration but induces

fetal cell differentiation. It can be adopted as an important marker for HB prognosis and a potential treatment target.

### Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research 19K16783 to X.G., 16K08750 and 20K07454 to S.Y. and 17K10803 to H.U. from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan; and by a Grant for Promoted Research from Kanazawa Medical University (S2018-6) (to X.G.). We would like to thank Prof. Akihide Tanimoto and Prof. Hirohisa Yano for their support; we thank Yuka Hiramatsu and Manabu Yamashita for their expert technical assistance; we also thank the patients for taking part in this study.

### Disclosure of conflict of interest

None.

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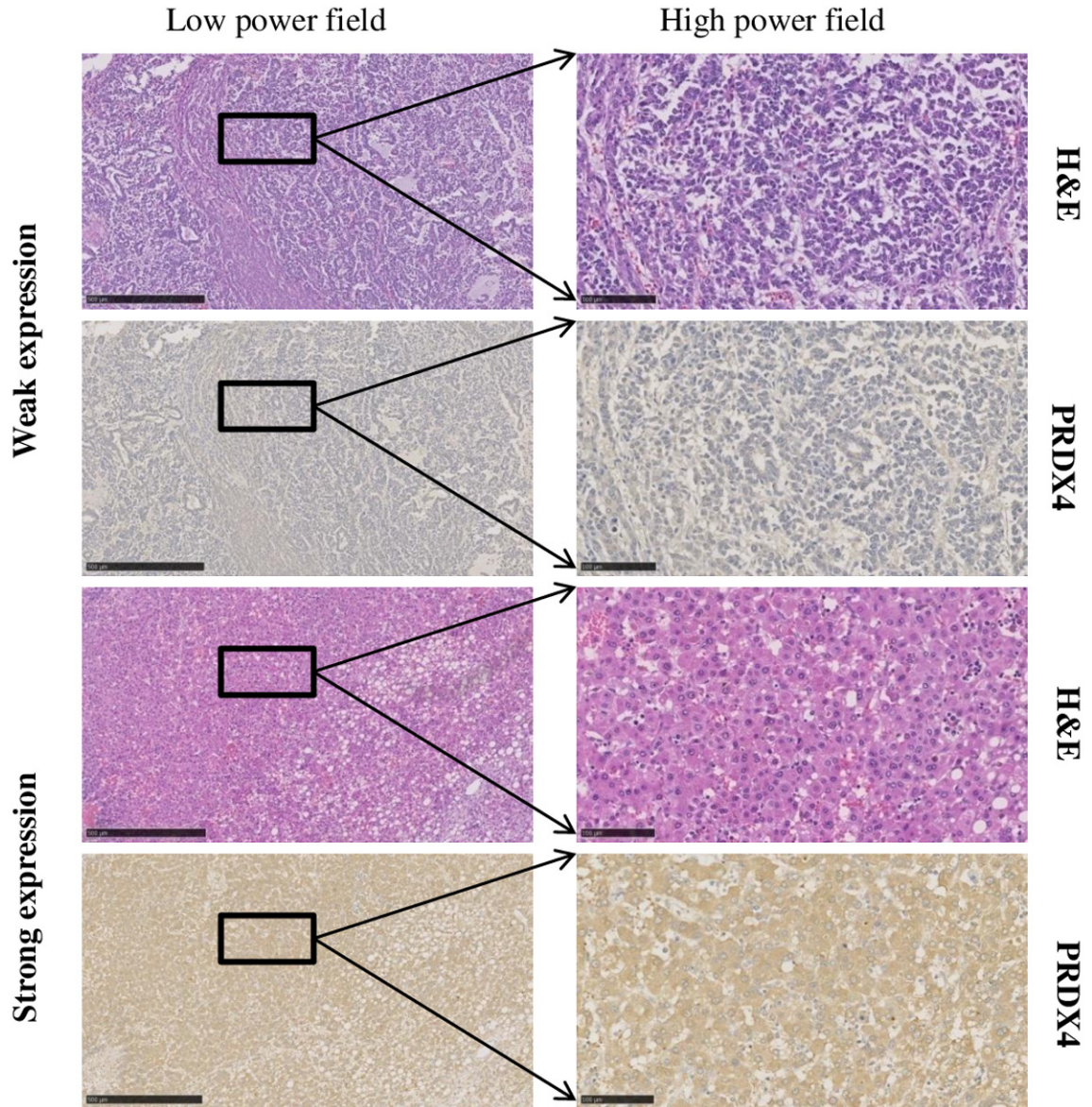
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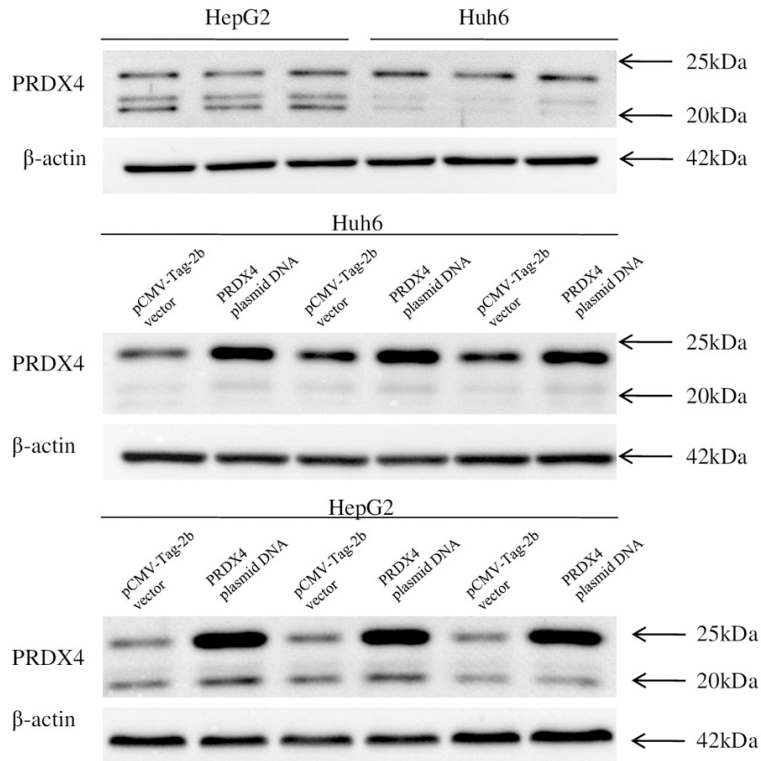
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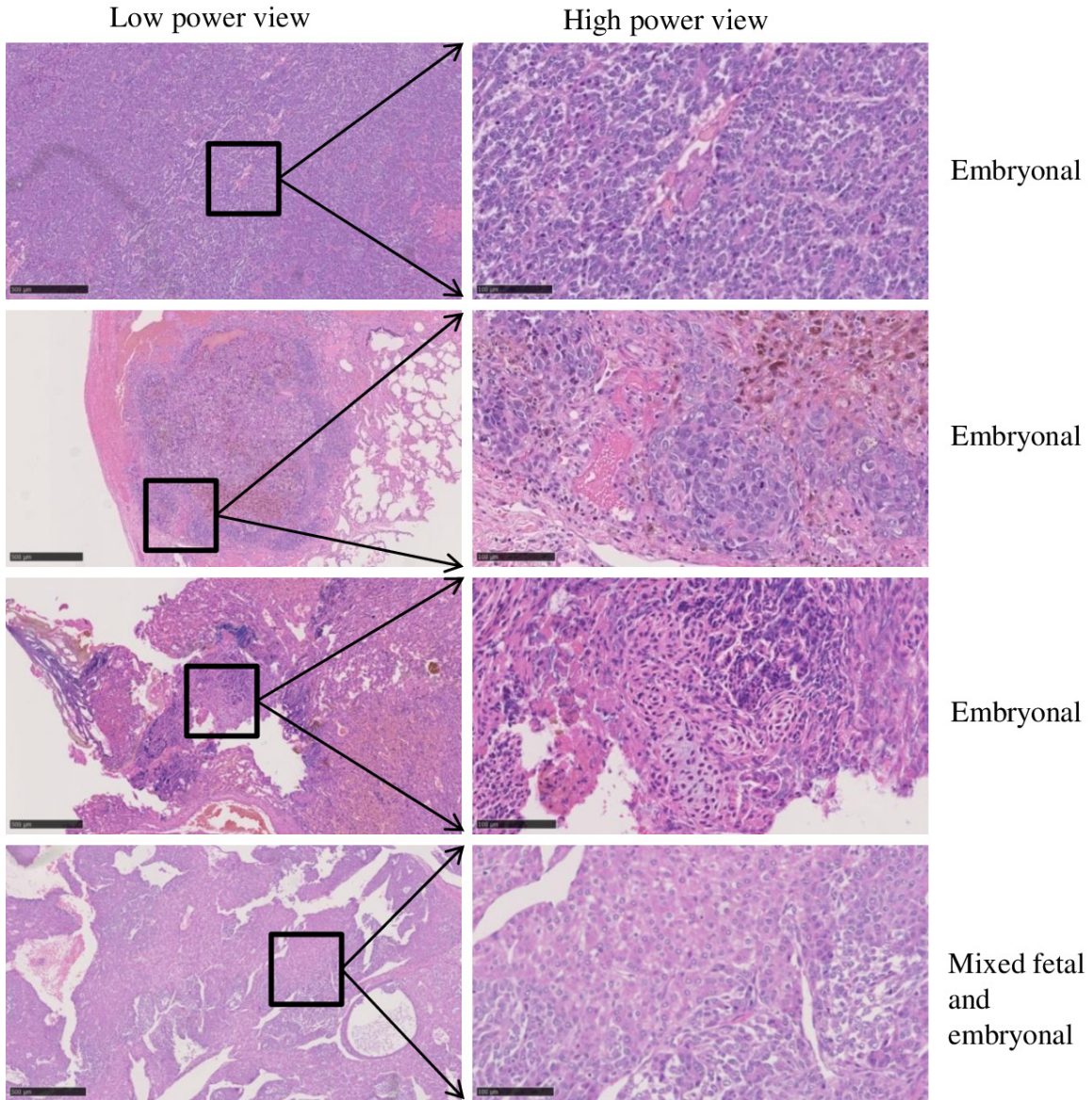
**Figure S1.** Representative immunohistological staining pictures of peroxiredoxin 4 (PRDX4) and corresponding hematoxylin and eosin (H&E) staining in hepatoblastoma samples.

## Peroxiredoxin 4 in hepatoblastoma



**Figure S2.** Peroxiredoxin 4 (PRDX4) basic expression and overexpression in two hepatoblastoma cell lines (HepG2 and Huh6). PRDX4 expression was increased by more than 3 folds by *PRDX4* plasmid DNA compared with empty pCMV-Tag-2b vector control.

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**Figure S3.** Representative pictures of hematoxylin and eosin staining in pulmonary metastasis samples. All four samples contained embryonal component and only one contained fetal component.

**Table S1.** Incidence of metastasis and PRETEXT stage IV in different hepatoblastoma (HB)

	Pure fetal HB	HB with embryonal component (including 4 metastasis cases)	<i>p</i> value
Metastasis	7/32 (21.88%)	13/41 (31.71%)	0.35
PRETEXT IV	3/27 (11.11%)	14/41 (34.15%)	0.05*

\* $P \leq 0.05$ ; PRETEXT: pretreatment extent of disease. Chi-Squared test with continuity correction was used for analysis.