

**The differential expression of perilipin-2 in hepatoblastoma and its association
with the prognosis**

Running title: perilipin-2 in hepatoblastoma

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Conflicts of Interest

The authors declare no conflicts of interest in association with the present study.

Abstract

Perilipin-2, a lipid droplet (LD) coating protein, has been found to be involved in cancer progression. However, its role in hepatoblastoma (HB) is undefined. We collected 87 HB samples and the corresponding clinical data. Immunohistochemistry (IHC) staining was performed to detect perilipin-2 and the association of the perilipin-2 expression with clinical characteristics and ~~the~~ prognosis was analyzed. The expression of perilipin-2 was increased in fetal HB components in comparison to embryonal HB components. The predominant staining pattern was vesicular in fetal HB cells, while it was granular in embryonal HB cells. Furthermore, ~~the~~ strong expression of perilipin-2 was associated with the histopathological type of fetal predominant HB. Although event-free survival (EFS) did not differ to a statistically significant extent between the strong and weak expression groups in a univariate survival analysis, a multivariate survival analysis revealed ~~the~~that EFS was significantly improved in the strong perilipin-2 expression group. In conclusion, perilipin-2 is differentially expressed in HB and the strong expression of perilipin-2 predicts a better prognosis.

Key words: perilipin-2, hepatoblastoma, differential expression, prognosis

Introduction

Perilipin-2, also known as adipophilin or adipose differentiation-related protein (ADRP), is a lipid droplet (LD)-associated protein which coats the neutral lipid core and regulates the lipid metabolism in many cells. It is ubiquitously expressed and specifically targets the LD surface (1-4). Due to the close association with LD, it has been proposed that perilipin-2 can be adopted as a general marker for LD in non-adipogenic cells (5, 6). As the ~~most rich~~^{richest} perilipin expression is in liver (7, 8), ~~the~~ perilipin-2 expression is directly correlated with the number and size of hepatic LDs (9-11) and thus has been viewed as a reliable marker of hepatic LDs (12). In the human liver under physiological conditions, hepatocytes were found to be weakly positive for perilipin-2 because LDs were few in number and small in size (13). In comparison, the expression of perilipin-2 was obviously upregulated and strong in hepatocyte steatosis (6, 7, 13), regardless of whether the condition was due to alcoholic or non-alcoholic fatty liver. It has been widely accepted that LD plays important functions in multiple cancer hallmarks (14) and perilipin-2, a LD-coating protein, is associated with cancer progression. In breast cancer, the elevated expression of perilipin-2 was associated with tumor malignancy (15, 16) and a poor prognosis (17, 18). In lung adenocarcinoma, perilipin-2-positive staining was linked to tumor progression (19) and predicted shortened survival (19, 20). This similar relationship was also revealed in pancreatic ductal adenocarcinoma (21). In contrast, the increased expression of perilipin-2 in clear cell renal cell carcinoma was associated with decreased tumor invasion and a better prognosis (22-24). As a lipid

metabolism-regulating and LD marker protein in the liver, it is reasonable to hypothesize that perilipin-2 is involved in liver cancer.

Hepatoblastoma (HB) is the predominant liver cancer in young children (25-27), with an annual incidence of approximately 1/1000000 (28, 29). Although the occurrence of HB is rare, it ranks 3rd among abdominal malignancies in children and the incidence is increasing year-by-year (27, 29, 30). HB tissues are mainly composed of four histological components: fetal, embryonal, small-cell undifferentiated/SCU, and mesenchymal components (31), with different formulations in individual HB specimens. Fetal and embryonal components are the two most common components in HB; mesenchymal components are contained in 20-30% of HB tissues, while SCU components are found in relatively few HB samples (31). From SCU to fetal HB, the tumor cells in different epithelial HB components resemble certain hepatocyte precursors and recapitulate different stages of liver development (31-34). It has been widely accepted that the failure of hepatic progenitor cells to properly differentiate into hepatocytes results in HB (35, 36). Although event-free survival (EFS) in standard-risk or classical HB groups is >80% within 3 years (37), it is decreased to around 60% in high-risk or aggressive HB (38, 39). The search for novel markers to assist in the diagnosis and better ~~predict the~~ prognosis is still ongoing. In our recent study about HB, we found that peroxiredoxin-4 (PRDX4), an antioxidant that regulated lipid metabolism under oxidative stress in non-alcoholic steatohepatitis (40) and atherosclerosis (41), was associated with the progression of HB (42). As a lipid metabolism-associated protein, perilipin-2 may mediate PRDX4's regulation of lipid

metabolism and thus be involved in the progression of HB because of the pervasive alteration of the lipid metabolism in cancer.

In the present study, we examined the expression of perilipin-2 in HB by IHC staining. The relationship between the expression of perilipin-2 and clinical characteristics, especially the prognosis, was analyzed.

Materials and Methods

Human HB samples

A total of 87 HB specimens and their associated clinical data were collected from 5 medical centers in Japan. Samples were formalin-fixed and paraffin-embedded. Informed consent for inclusion in the present study was obtained from the patients or their guardians. The study was approved by the Ethics Committee of Kanazawa Medical University (NO. I367). Among the 87 HB cases, most of them (82/87, 94.25%) received the cisplatin-based chemotherapy and the specimens were collected after the initial chemotherapy; only 5 samples were collected without initial chemotherapy.

Perilipin-2 IHC staining

Formalin-fixed and paraffin-embedded sections (thickness: 3 μ m) were used for staining. Hematoxylin and eosin (H&E) staining was first conducted to determine the pathological pattern of HB tumor tissues and the representative section that contained the greatest variety of HB components was selected for perilipin-2 IHC staining. The whole process was conducted using a Leica Bond-Max autostainer (Leica, Buffalo Grove, USA) and a Bond Polymer Refine Detection kit. After deparaffinization and

rehydration, tissue sections went through a heat-induced (121°C) epitope retrieval process in Bond Epitope Retrieval Solution 1 (Citrate-based pH 6.0 epitope retrieval buffer) for 20 minutes. AP125 (Progen Biotechnik, Heidelberg, Germany), diluted to 1:100, was used as the primary antibody for perilipin-2. Signals were visualized by Dako REAL EnVision detection system and Peroxidase/DAB+ (Dako Cytomation), according to [the](#) manufacturer's instructions. The images of H&E staining and perilipin-2 IHC staining were captured by the NanoZoomer Digital Pathology Virtual Slide Viewer software program (Hamamatsu Photonics Corp., Hamamatsu, Japan).

Evaluation of perilipin-2 IHC staining

For each specimen, the histological components and their relative proportions were evaluated at first by H&E staining. Five representative high-power field images for each ingredient were selected for evaluation of perilipin-2 IHC staining. The final immunoreactivity score for each specimen was calculated by combining each ingredient's score and its relative proportion. All histological and IHC staining slides were evaluated by two independent pathologists (Akihiro Shioya and Xin Guo) in the Department of Pathology. The histological classification, immunoreactivity score (the proportion of positive cells compared to the total HB cells), and staining pattern (vesicular or granular) of each perilipin-2 IHC staining slide were evaluated carefully and independently using a blind protocol design (pathologists blinded to the clinicopathological data). Agreement was initially reached between them in 75% of all cases. Another 20% reached a consensus after discussion. For the remaining disagreement (5%), a third board-certified pathologist (Sohsuke Yamada) in our

department joined the discussion and the consensus was finally reached in these cases.

Definition of weak and strong expression of perilipin-2 in survival analysis

To clarify the relationship between perilipin-2 expression and prognosis (EFS), receiver operating characteristic (ROC) curve was produced between them. The maximal Youden's index was reached when the cut-off value was 12.5% for perilipin-2 expression. Based on this cut-off point, patients were divided into strong ($\geq 12.5\%$) and weak ($< 12.5\%$) expression groups during survival analysis.

Statistical analysis

Continuous variables were expressed as the mean \pm standard deviation (SD). Comparisons were performed using a two-tailed independent Welch's *t*-test or a non-parametric statistical analysis. A paired-samples *t*-test was used for the analysis of paired data. Categorical data were compared by using either Pearson's chi-squared test or Fisher's exact test. A Kaplan-Meier analysis was used as a univariate survival analysis and a multivariate analysis was performed using Cox regression. All statistical analyses were performed using the SPSS statistical software package, version 16.0. Two-sided *P* values of < 0.05 were considered to indicate statistical significance.

Results

The clinical characteristics of HB cases and their perilipin-2 expression

Eighty-seven HB samples and their corresponding clinical information were collected.

Males outnumbered females and children of < 3 years of age accounted for most of the cases. In the study population, 25.93% of cases were classified as pretreatment extent

of disease stage IV (PRETEXT IV), while metastasis was detected in 26.44% of cases.

Only two cases showed serum alpha-fetoprotein (AFP) levels of <1000 ng/ml at the time of the diagnosis. Recurrence was detected in 22.35% of the total cases within 5 years after the diagnosis, while the mortality rate decreased to 7.06% in the same period. Regarding the histological findings, fetal and embryonal HB components were the two most common components, being detected in 79 of 87 (90.80%) and 41 of 87 (47.13%) samples respectively. An SCU component was found in only 2 HB samples.

With respect to the perilipin-2 expression, the staining score (%) ranged from 0 to 90. The mean was 15.11% and the median was 7%. Positive staining patterns were mainly classified as vesicular and granular. Representative images of perilipin-2 staining in different HB components are presented in **Figure 1**. The pPerilipin-2 expression was compared between groups categorized by clinical characteristics. The perilipin-2 expression did not differ to a statistically significant extent between the groups, with the exception of the different histological patterns. The pPerilipin-2 expression was increased in HB samples of the fetal predominant pattern, which was defined by the fetal component accounting for >60% of the tumor area. The clinical characteristics of HB cases and their perilipin-2 expression are detailed in **Table 1**.

The differential expression of perilipin-2 in fetal and embryonal HB components

We compared the perilipin-2 expression in fetal and embryonal components as they are the two most common HB components. An analysis showed that the perilipin-2 expression (%) was significantly higher in fetal HB components than in embryonal components (fetal vs. embryonal: 18.76 ± 20.74 vs. 5.41 ± 6.92 , $p < 0.001$) (**Figure 2**).

As 37 HB cases in our study contained both fetal and embryonal components (mixed fetal and embryonal HB), a paired-samples *t*-test was employed for the comparison of these HB cases. The result also revealed that the expression of perilipin-2 (%) was significantly increased in the fetal components (fetal vs. embryonal: 14.70 ± 17.68 vs. embryonal 5.68 ± 7.20 , $p < 0.01$). An example of mixed fetal and embryonal HB and their perilipin-2 expression is shown in **Figure 3**. Furthermore, the perilipin-2 staining pattern in these two components was obviously different. In the fetal components, vesicular staining was the predominant staining pattern (55/79, 69.62%). In comparison, the most common staining pattern was granular-staining and only a small proportion (7/41, 17.07%) of the embryonal components showed vesicular-staining (**Figure 2 and Figure 3**).

Correlation of the perilipin-2 expression with the HB prognosis

Although the histological pattern of SCU and initial AFP < 100 ng/ml are two risk factors for the prognosis of HB (31, 43), the number of these cases in our study population was very small (SCU, 2 cases; AFP < 100 ng/ml, 0 cases). We excluded these cases in a survival analysis for the sake of convenience and a total of 85 cases were finally included. They were then divided into the perilipin-2 strong ($n=33$) and weak ($n=52$) expression groups based on the cut-off value (12.50%) that offered the best predictive value for the prognosis (EFS) in the receiver operating characteristic (ROC) curve. The clinical characteristics were compared between these two groups and we found that a histopathological pattern of fetal predominant HB was more common in the perilipin-2 strong expression group than in the weak expression group

(strong expression vs. weak expression: 25/33, 75.76% vs. 29/52, 55.77%; $p=0.062$) (**Table 2**). Although the incidence of metastasis was increased in the strong expression group, the subsequent multivariate logistic regression analysis denied their correlation (**Table 3**). Furthermore, there was no significant difference in the perilipin-2 expression of HB cases with and without metastasis (Table 1). Univariate Kaplan-Meier analysis for event free survival (EFS) revealed that the perilipin-2 strong expression group seemed to have a better prognosis in comparison to the weak expression group; however, the difference was not statistically significant (**Figure 4A**). Further multivariate Cox regression analysis verified that the strong expression of perilipin-2 was positively correlated with better EFS (**Figure 4B**), while the presence of metastasis was the most powerful factor for predicting a poor prognosis (**Table 4**). We could not conduct an overall survival (OS) analysis because of the very small number of deaths in our study population ($n=6$ in all 87 HB cases).

Discussion

Perilipin-2, an intracytoplasmic lipid droplet coating protein, has been found to be involved in hepatocellular carcinoma (HCC). Macrovesicular LDs were reported to be associated with decreased recurrence *in vivo* (44), while the overexpression of perilipin-2 enhanced tumor cell proliferation *in vitro* (45). However, the role of perilipin-2 in HB is still not well-defined. Although *perilipin-2* gene expression has been found to be decreased in HB (extract data from GEO database, shown in **supplementary materials**), the relationship between perilipin-2 expression and diagnosis or prognosis is still unclear. In the present study, we demonstrated that

perilipin-2 was differentially expressed in fetal and embryonal HB. In detail, the expression of perilipin-2 was elevated in the fetal component in comparison to the embryonal component. Furthermore, the predominant perilipin-2 staining pattern was vesicular in fetal HB, while it was mainly granular in embryonal HB. The differential expression of perilipin-2 in these two most common HB components makes it a useful marker for the histopathological diagnosis of the epithelial subtypes of HB. With respect to the prognosis, we found that the strong expression of perilipin-2 was associated with a better prognosis in a multivariate analysis. No correlation was found between the perilipin-2 expression and clinical features including metastasis, advanced tumor stage (PRETEXT IV), and tumor size. In summary, strong and vesicular-staining pattern of perilipin-2 can differentiate fetal from embryonal HB. Patients with this characteristic are more often diagnosed as fetal HB and associated with a better prognosis. In the absence of other risk factors, physicians could consider de-escalating the treatment and prolonging follow-up interval in these patients.

Two hypotheses may support the findings of our study. The first is that the expression of perilipin-2 is associated with tumor cell differentiation. In lung adenocarcinoma, perilipin-2 positive staining was associated with the poorly differentiated cancer type (19). In contrast, the high expression of perilipin-2 was linked to better tumor differentiation in clear cell renal cell carcinoma (23). Another study found that increased plasma perilipin-2 indicated better tumor differentiation in colorectal cancer (46). In our study, the expression of perilipin-2 was enhanced in better-differentiated HB (fetal HB) in comparison to less-differentiated HB (embryonal HB). Furthermore,

the expression of perilipin-2 in these two HB components exhibited different patterns. Our findings strengthened the hypothesis that perilipin-2 was associated with tumor differentiation and the better differentiated histological type in the perilipin-2 high expression group may have been responsible for the improved prognosis. The other hypothesis that may explain our findings is that perilipin-2 plays a role in the regulation of autophagy (47). It has been demonstrated that perilipin-2 regulates autophagy in the liver. The overexpression of perilipin-2 protects against autophagy, whereas perilipin-2 deficiency enhances autophagy and inhibits severe endoplasmic reticulum stress-induced hepatocyte apoptosis (48). In our study, the high expression of perilipin-2 may inhibit HB tumor cell autophagy. This dysregulation of autophagy may cause the enhancement of tumor apoptosis and finally result in a better prognosis. As a reliable protein marker for LDs, the staining of perilipin-2 visualizes LD and reflects lipid accumulation. Increased intracellular LDs have been observed in many cancers (49). However, the role of increased LDs in tumor cells is unclear. Whether it is a lipid reservoir to better sustain cancer cell survival in the tumor microenvironment, where there is a relative shortage of oxygen and nutrients, or a marker of autophagy dysfunction is still debatable. In breast cancer, lung adenocarcinoma, and pancreatic ductal adenocarcinoma, the accumulation of LDs may support tumor progression by supplementing unsaturated fatty acids instead of potentially toxic saturated lipids. In clear cell renal cell carcinoma and HB, the accumulation of LDs may reflect autophagy dysfunction. Tumor cells with increased LDs may be more susceptible to apoptosis under the stressful environment caused by

high proliferation or chemotherapy. Furthermore, the pattern of LD accumulation may also define its role in cancer. Granular LDs may represent a lipid reservoir that promotes tumor development. In contrast, vesicular LDs—especially macrovesicular LDs—may reflect autophagy dysfunction. One previous study revealed that macrovesicular steatosis was associated with a better prognosis in HCC (44). In combination with our study in HB, it may be reasonable to hypothesize that both the amount and pattern of LDs can determine the role of LD accumulation in cancer. The findings of the present study may explain the diverse role of PRDX4 (an antioxidative enzyme) in HB, which we reported in a previous study (42). In embryonal HB, the oxidative stress-induced overexpression of PRDX4 may lead to the accumulation of granular LDs, which promote tumor migration via the supplementation of unsaturated fatty acids. In contrast, the overexpression of PRDX4 in fetal HB may accompany the accumulation of macrovesicular LDs in well-differentiated fetal HB and represent autophagy dysfunction. The positive correlation between the PRDX4 and perilipin-2 expression may support this hypothesis (correlation coefficient=0.46, $p<0.01$, unpublished data).

In conclusion, perilipin-2 is differentially expressed in HB and the strong expression of perilipin-2 predicts a better prognosis.

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Tables

Table 1. Clinical characteristics of patients with hepatoblastoma (n=87)

Clinical characteristics	Study population n (%)	Perilipin-2	p
Sex			0.36
Female	29 (33.33%)	12.34±13.19	
Male	58 (66.67%)	16.50±22.37	
Age (years)			0.17
0-3	66 (75.86%)	12.85±17.15	
3-7	15 (17.24%)	22.73±22.92	
≥8	6 (6.90%)	15.11±19.80	
Tumor diameter (cm)			0.42
<10	34 (40.00%)	13.21±17.01	
≥10	51 (60.00%)	16.76±21.77	
Serum AFP (ng/ml)			0.59
≤1000	2 (2.38%)	1.00±0.00	
1001-1000000	62 (73.81%)	15.90±22.00	
>1000000	20 (23.81%)	15.65±13.35	
PRETEXT stage			0.75
I-III	60 (74.07%)	15.40±22.43	
IV	21 (25.93%)	14.19±11.42	
Metastasis			0.23
Absent	64 (73.56%)	13.55±19.41	
Present	23 (26.44%)	19.48±20.67	
VPEFR			0.68
Absent	32 (42.11%)	17.38±23.93	
Present	44 (57.89%)	15.41±17.95	

Histological category			0.01**
Predominant fetal	54 (62.07%)	19.43±22.56	
Others	33 (37.93%)	8.06±11.32	
Recurrence within 5 years			0.80
Absent	66 (77.65%)	15.05±17.42	
Present	19 (22.35%)	16.37±27.64	
Death within 5 years			0.88
Absent	79 (92.94%)	15.43±20.30	
Present	6 (7.06%)	14.17±16.30	

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 multi-Focal liver tumor or tumor Rupture at diagnosis; HB: hepatoblastoma. **p≤0.01.

Table 2. The clinical characteristics of the perilipin-2 strong and weak expression groups (n=85)

Perilipin-2 weak expression (n=52)	Perilipin-2 strong expression (n=33)	p value
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EFS (5-year)	37/50 (74.00%)	27/33 (81.82%)	0.407
OS (5-year)	47/50 (94.00%)	31/33 (93.94%)	0.991
Metastasis	9/52 (17.31%)	13/33(39.39%)	0.023*
PRETEXT Stage IV	10/48 (20.83%)	10/31 (32.26%)	0.254
Age (years)			0.451
0-3	41/52 (78.85%)	23/33 (69.70%)	
3-7	7/52 (13.46%)	8/33 (24.24%)	
≥8	4/52 (7.69%)	2/33 (6.06%)	
Male sex	34/52 (65.38%)	23/33 (69.70%)	0.680
Serum AFP (ng/ml)	6.48*10 ⁵ ±7.74*10 ⁵	6.30*10 ⁵ ±7.24*10 ⁵	0.724
Tumor size (cm)	10.18±3.30	11.39±3.18	0.104
Histological category			0.062
Predominant fetal	29/52 (55.77%)	25/33 (75.76%)	
Others	23/52 (44.23%)	8/33 (24.24%)	

EFS: event-free survival; OS: overall survival; PRETEXT: pretreatment extent of disease; AFP: alpha-fetoprotein. * p<0.05.

Table 3. Logistic regression analysis of factors associated with metastasis in HB patients

Risk factor	Hazard Ratio	95%CI	p
Age (years)	1.12	0.92-1.36	0.265
Male sex	1.67	0.38-7.28	0.494
Tumor diameter (cm)	0.99	0.82-1.20	0.959
PRETEXT stage IV	14.99	3.88-57.95	<0.001***

Perilipin2 strong expression	2.80	0.76-10.33	0.121
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HB: hepatoblastoma; CI: confidence interval; PRETEXT: pretreatment extent of disease; *** p<0.001.

Table 4. Cox regression analysis of factors associated with event-free survival in HB patients

Risk factor	Hazard ratio	95%CI	p
Strong perilipin-2 expression	0.30	0.10-0.90	0.031*
Metastasis	10.06	2.95-34.28	<0.001***
PRETEXT IV	0.85	0.26-2.80	0.793
Predominant fetal	1.79	0.69-4.62	0.233

HB: hepatoblastoma; CI: confidence interval; PRETEXT: pretreatment extent of

disease; * $p<0.05$, *** $p<0.001$.

Figure Legends

Figure 1. Representative microscopic views of hematoxylin and eosin (H&E) staining and perilipin-2 immunohistochemical staining in different hepatoblastoma (HB) components. Formalin-fixed paraffin-embedded sections (thickness: 3-5 μm) were used for staining, Bar=100 μm . SCU, small-cell undifferentiated.

Figure 2. The differential expression of perilipin-2 in fetal and embryonal hepatoblastoma (HB). Formalin-fixed paraffin-embedded sections (thickness: 3-5 μm) were used for staining, Bar=50 μm . Data are shown as the mean \pm SD, Welch's t -test

was used for the analysis. H&E, hematoxylin and eosin staining; *** $p < 0.001$.

Figure 3. Comparison of the perilipin-2 expression levels in mixed fetal and embryonal hepatoblastoma (HB). Formalin-fixed paraffin-embedded sections (thickness: 3-5 μm) were used for staining, Bar=250 μm . Data are shown as the mean \pm SD, a paired-samples *t*-test was used for the analysis. H&E, hematoxylin and eosin staining; ** $p < 0.01$.

Figure 4. Correlation of the perilipin-2 expression with event-free survival (EFS). A: Kaplan–Meier survival curve of hepatoblastoma (HB) patients with the strong and weak expression of perilipin-2 (cut-off value=12.5%). B: The Cox regression curve of HB patients with the strong and weak expression of perilipin-2 (cut-off value=12.5%). The analyses were conducted using the SPSS 16.0 software package; *p*-values were calculated using the log-rank test.

Supplementary materials. Differential expression of genes between hepatoblastoma tissues and normal liver tissues (Data were extracted from GEO database, NCBI).