

# Reciprocal expression of CCAAT/enhancer binding proteins $\alpha$ and $\beta$ in hepatoblastomas and its prognostic significance

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**Abstract.** Hepatoblastoma is one of the common pediatric solid tumors with frequent mutation of the  $\beta$ -catenin gene which might be an early event of its carcinogenesis. However, the detailed molecular mechanism is still unknown. We studied the expression levels of CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) and C/EBP $\beta$ , which regulate differentiation and growth of embryonic hepatocytes, to establish whether or not they were involved in affecting the clinical behavior of hepatoblastoma. The quantitative real-time reverse transcriptase-PCR revealed that expression of C/EBP $\alpha$  mRNA was significantly up-regulated in tumors 223% ( $p=0.013$ ) as compared with that in adjacent normal livers, while expression of C/EBP $\beta$  was down-regulated to 27% ( $p=0.002$ ). Of interest, the immunohistochemical analysis showed that expression of C/EBP $\alpha$  was higher and that of C/EBP $\beta$  lower in the poorly differentiated tumor cells than in the well-differentiated cells within the same tumor. Furthermore, high expression of C/EBP $\alpha$  ( $p=0.047$ ) as well as low expression of C/EBP $\beta$  ( $p=0.025$ ) was significantly associated with poor prognosis of the patients. Cox hazard model suggested that expression of C/EBP $\alpha$  and that of C/EBP $\beta$  were independent indicators to predict the prognosis from age but not from histology. Thus, expression of C/EBP proteins may play an important role in the genesis and clinical behavior of hepatoblastoma probably by inducing different stages of arrest of differentiation.

## Introduction

Hepatoblastoma (HBL) is an embryonal tumor and derives from the progenitor cells of the infantile or even the fetal liver which may include hepatoblasts or immature hepatocytes (1). Microscopically, HBL is usually composed of a mixture of well-differentiated tumor cells (fetal type) resembling immature hepatocytes and poorly differentiated cells (embryonal type) similar to embryonic cell components with different proportion (1). Moreover, HBL cells are positive for CK-18 and CK-19, bile duct epithelial markers, as well as  $\alpha$ -feto-protein (AFP), a hepatocyte marker, suggesting that HBL also has the components with a potential to differentiate into both directions (2,3).

Hepatocyte differentiation is controlled by coordinated transcription factors. Both CCAAT/enhancer binding protein (C/EBP) $\alpha$  and C/EBP $\beta$  are liver-enriched transcription factors, regulating the expression of liver-specific genes. Expression of C/EBP $\alpha$  is observed on day 9.5 of gestation, and C/EBP $\beta$  on day 17.5 in the fetal liver of rodents, suggesting that they may be involved in hepatocyte differentiation (4). The hepatocytes in C/EBP $\alpha$ -deficient mice resemble the embryonal type of HBL cells which may have bipotential ability to differentiate into hepatocytes and bile duct epithelial cells (5). This indicates that C/EBP $\alpha$  may play a role in the growth regulation of HBL cells. C/EBP $\alpha$  is down-regulated while C/EBP $\beta$  is up-regulated in the remnant liver after partial hepatectomy (6).

We studied expression of C/EBP $\alpha$  and C/EBP $\beta$  in primary HBLs and found that they were expressed in an opposite manner in HBL and significantly associated with the patient prognosis.

## Materials and methods

**Tissue samples and RNA isolation.** The patients underwent surgical treatment at various hospitals or institutions under the framework of the Japanese Study Group for Pediatric Liver Tumor (JPLT) between 1991 and 2005. The extent of the disease (stage) was classified according to that of SIOPEL

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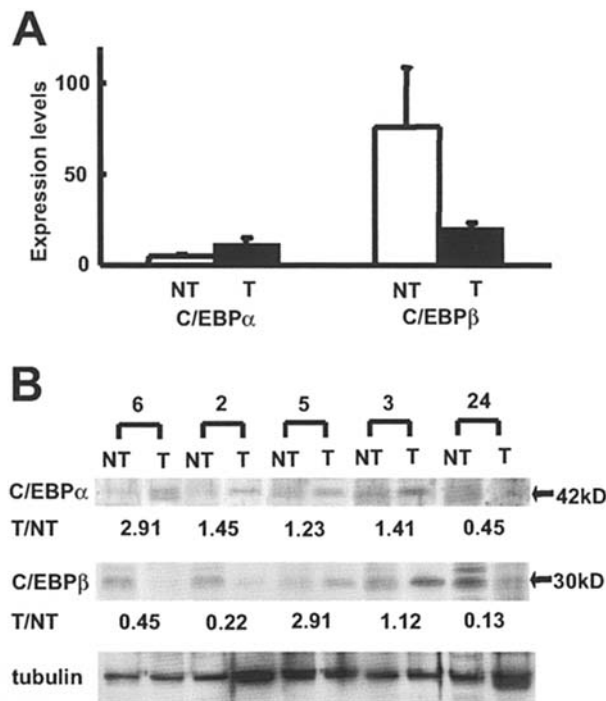


Figure 1. Real-time quantitative PCR and Western blot analysis of hepatoblastoma with C/EBPα and C/EBPβ. (A) Expression levels of C/EBPα and C/EBPβ were analyzed with real-time quantitative PCR (x1000) (mean ± standard error). NT, non-tumorous tissue; T, tumorous tissue; n=24. (B) Western blot analysis was performed with representative patients (patient numbers; 6, 2, 5, 3 and 24). The intensities of C/EBPα (42 kD) and C/EBPβ (30 kD) proteins expression were normalized against α-tubulin, and the ratio of T to NT was calculated. T/NT, a ratio of the C/EBP expression level in tumorous tissue divided by that in non-tumorous tissue.

(7). Histopathology of HBL was according to the classification by the Japanese Society of Pathology which includes well differentiated (fetal) and poorly differentiated (embryonal) types. With informed consent, tumor tissues and their corresponding normal liver tissues were obtained at surgery, immediately frozen, and stored at -80°C until use. Frozen tumor tissues were obtained from 46 patients with HBL, and corresponding normal liver tissues were available from the rejected tissues of 24 patients. All specimens used in this study were provided by the Tissue Bank of JPLT. The JPLT Review Board as well as the Chiba Cancer Center institutional committee approved the analysis with the specimens. Total RNA was prepared by the conventional guanidine thiocyanate-phenol-chloroform procedure.

**Real-time quantitative PCR.** First-strand cDNA was prepared with 5 μg of total RNA from the surgical specimens, with 200 units of Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA), and 160 pmol of random primers (Takara, Ohtsu, Japan). Synthesized cDNA was subjected to a quantitative real-time PCR (PE Biosystems, Foster City, CA) (8). The primers for C/EBPα were 5'-CGGACTTGG TCGTCTAAG-3' for 5', 5'-GAGGCAGGAAACCTCC AAAT-3' for 3', and 5'-GAGGCAGGAAACCTCCAAAT-3' for the detection probe; the primers for C/EBPβ were 5'-AG CGCGGCGACGAGTACAAGATC-3' for 5', 5'-ACCTTGT GCTGCGTCTCCA-3' for 3', and 5'-CGCGAGCGCAACA ACATCGC-3' for the detection probe. Taq-Man β-actin

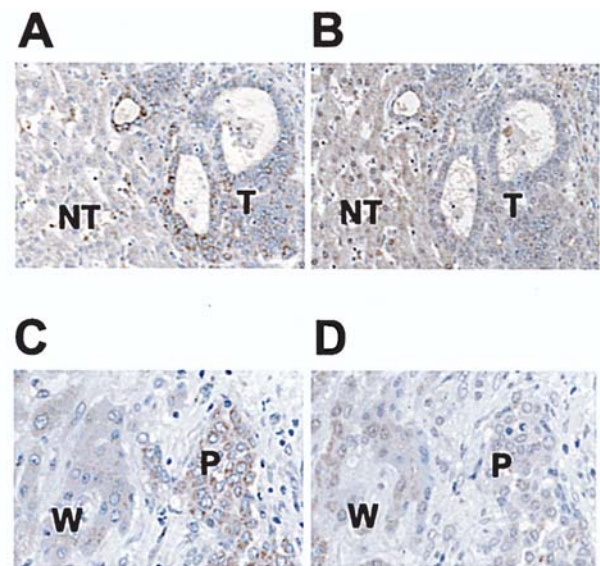


Figure 2. Immunohistochemistry of C/EBPα and C/EBPβ in hepatoblastomas. Surgical specimens were immunohistochemically stained with anti-C/EBPα or -C/EBPβ antibody. (A) C/EBPα was weakly positive in the cytoplasm and nuclei of normal hepatocytes in non-tumorous tissues (NT). C/EBPα was strongly positive in the cytoplasm and the nuclei of tumor cells (T). (B) C/EBPβ was strongly positive in the cytoplasm and nuclei of hepatocytes (NT). C/EBPβ was weakly positive in the cytoplasm of tumor cells. (T) (C) C/EBPα was more strongly positive in the cytoplasm of poorly differentiated tumor cells (P) than in that of well differentiated tumor cells (W). (D) C/EBPβ was more weakly positive in the cytoplasm of poorly differentiated tumor cells (P) than in that of well differentiated tumor cells (W). Original magnification: x100 (A and B), x400 (C and D).

control reagents (Perkin Elmer Inc., Wellesley, MA) were used for the amplification of β-actin as recommended by the manufacturer.

**Immunohistochemistry and Western blot analysis.** Nine HBL tissues were used for immunohistochemistry, and 5 paired (HBL tissue and its adjacent normal tissue) samples were used for Western blot analysis. Primary antibodies were polyclonal rabbit anti-rat C/EBPα (1:100 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA), polyclonal rabbit anti-rat C/EBPβ (1:100, dilution, Santa Cruz Biotechnology Inc.), and mouse monoclonal anti-α-tubulin antibody (Lab Vision, Fremont, CA). The exposed films from Western blot analysis were scanned, and the images were analyzed with the software program ImageJ 1.34s (NIH, Bethesda, MD).

**Statistical analysis.** Kaplan-Meier survival curves were calculated, and survival distributions were compared using the log-rank test. Proportional Cox regression models were used to explore associations among C/EBPα, C/EBPβ, age, stage, alpha-feto protein, pathology, and survival. Statistical significance was declared at P-value <0.05. Statistical analysis was performed using Stata 7.0 (Stata Corp., College Station, TX).

## Results

The expression levels of C/EBPα and C/EBPβ mRNA were measured in primary HBLs and their adjacent normal liver tissues by using quantitative real-time RT-PCR (Fig. 1A).

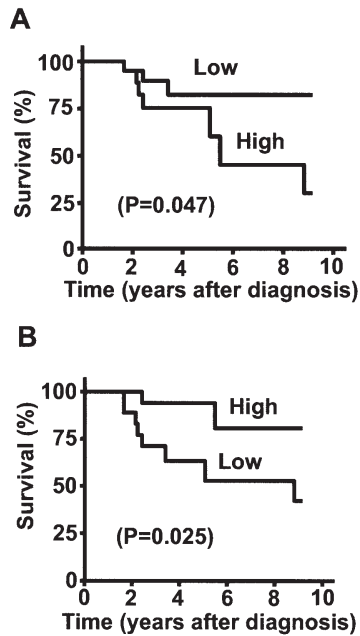


Figure 3. Kaplan-Meier survival curves for patients with hepatoblastoma. Survival of patients with hepatoblastoma after surgery was analyzed (Kaplan-Meier). Patients were divided into two groups for each factor. Patients with higher expression levels of C/EBPα than the median (A) ( $P=0.047$ ), and lower expression levels of C/EBPβ than the median (B) ( $P=0.025$ ) were associated with shorter survival. P-value for log-rank test is shown in parentheses;  $n=46$ .

The C/EBPα expression was significantly high in the tumors (mean  $\pm$  SEM:  $11.6 \pm 3.2$ ,  $n=24$ ) as compared with that in the normal livers ( $5.1 \pm 0.9$ ,  $n=24$ ;  $p=0.013$ ). On the other hand, the expression of C/EBPβ was significantly lower in HBL tissues ( $20.4 \pm 2.7$ ,  $n=24$ ) than that in the normal liver tissues ( $75.8 \pm 33$ ,  $n=24$ ;  $p=0.002$ ). To confirm these results, we next measured expression levels of C/EBPα and C/EBPβ proteins in 5 paired samples of tumor and its corresponding normal liver by Western blot analysis. The data obtained showed a tendency of up-regulation of C/EBPα and down-regulation of C/EBPβ in the tumor tissues as compared with the paired normal livers (Fig. 1B).

Fig. 2 shows immunohistochemical stainings of primary HBLs. C/EBPα was weakly positive in the cytoplasm and nuclei of normal hepatocytes, and strongly positive mainly in the cytoplasm of tumor cells (Fig. 2A). By contrast, C/EBPβ was rather positive in the cytoplasm and nuclei of hepatocytes in the adjacent normal livers, whereas it was weakly positive in the cytoplasm of the tumor cells (Fig. 2B). In the same tumor tissues, C/EBPα was more strongly positive in the poorly differentiated tumor cells than the adjacent well-differentiated tumor cells (Fig. 2C). C/EBPβ was weakly positive in the poorly differentiated tumor cells, whereas it was almost negative in the well-differentiated tumor cells (Fig. 2D).

The Kaplan-Meier cumulative survival curves are shown in Fig. 3. The high levels of expression of C/EBPα mRNA were significantly associated with poor patient survival (68.2% 5-year survival rate,  $n=22$ , vs. 87.5%,  $n=24$ ;  $p=0.047$ ), whereas high levels of expression of C/EBPβ mRNA were significantly correlated with favorable prognosis (92.6% 5-year survival rate,  $n=27$ , vs. 57.9%,  $n=19$ ;  $p=0.025$ ).

Table I. Proportional Cox regression models using C/EBPα and C/EBPβ and dichotomous factors of age and pathology.

Model	Factor	HR (95% C.I.)	P-value
A	C/EBPα	3.57 (0.91-14.0)	0.068
B	C/EBPβ	0.20 (0.04-0.96)	0.044
C	Age	0.27 (0.07-1.02)	0.054
D	Pathology	13.5 (1.70-107)	0.014
E	C/EBPα	4.14 (1.04-16.5)	0.044
	C/EBPβ	0.18 (0.04-0.86)	0.031
F	C/EBPα	5.66 (1.32-24.2)	0.019
	C/EBPβ	0.17 (0.04-0.81)	0.027
	Age	0.15 (0.03-0.76)	0.022
G	C/EBPα	2.30 (0.56-9.41)	0.25
	C/EBPβ	0.40 (0.08-2.07)	0.28
	Pathology	7.95 (0.87-72.6)	0.066

HR, hazard ratio showing the relative risk of death of the first category relative to the second; parenthesis, 95% confidence interval (C.I.); C/EBPα and C/EBPβ, high vs. low expression levels of C/EBPα and C/EBPβ in tumor with real-time quantitative PCR;  $n=46$ .

Univariate Cox regression analysis of 46 patients with HBL showed that expression of C/EBPβ (high vs. low expression;  $p=0.044$ ), age (<1-year vs.  $\geq 1$ -year;  $p=0.054$ ) and histopathology (well vs. poorly differentiated;  $p=0.014$ ) were significant indicators of the prognosis, while expression of C/EBPα ( $p=0.068$ ) was marginally significant as a prognostic factor of HBLs (Table I). Multivariate analysis using Cox model showed that C/EBPα and C/EBPβ were significantly related to survival in a model jointly with each factor ( $P<0.05$ , model E). C/EBPα and C/EBPβ were significantly related to survival ( $P<0.047$ ) even after controlling age ( $P=0.022$ , model F). Finally, since 9 out of 10 deceased patients had poorly differentiated histopathology, C/EBPα and C/EBPβ would lose significance in a model including pathology (model G).

## Discussion

The basic studies of hepatoblastoma have recently provided important information to understanding of genesis and progression of HBL. The β-catenin gene was discovered to be mutated and translocated into the cellular nucleus (9). However, high frequency of aberration of the Wnt signaling appeared to be an early event and the β-catenin mutation itself did not have prognostic significance (8). The studies using comparative genomic hybridization (CGH) presented an interesting pattern of the chromosomal aberrations in HBLs (10). The comprehensive cDNA project of HBLs has also given some insights into the understanding of the molecular aspect of HBLs by identifying a large number of



differentially expressed genes between the HBL tumors and their corresponding normal livers, that identified Plk1 as a highly expressed gene in HBLs (11). However, expression of most of the genes was not predictive for prognosis.

The C/EBP $\alpha$  gene is mapped to chromosome 19q, which is often gained in HBLs and has been found to be up-regulated in HBLs (12). However, the gene is mutated in acute myeloid leukemia and is often down-regulated in some other cancers, suggesting that C/EBP $\alpha$  may function as a tumor suppressor (13,14). In addition, our previous study suggested that both C/EBP $\alpha$  and C/EBP $\beta$  were down-regulated in hepatocellular carcinomas as compared with the adjacent non-tumorous liver tissues (15). These suggest that C/EBP $\alpha$  may play a different role in HBLs from other cancers.

C/EBP $\beta$  was down-regulated in HBLs as compared with the corresponding normal livers and correlated with poor prognosis in the HBL patients, that was similar to HCC and other cancers (15,16). Buck *et al* reported that overexpression of C/EBP $\beta$  in HepG2 cells, a human HCC cell line, suppressed proliferation of the cells (17). Therefore, C/EBP $\beta$  might function as a tumor suppressor in HBL cells. In addition, C/EBP $\beta$  may also play a role in regulating differentiation of HBL cells because the gene is expressed in mature hepatocytes and is reported to be indispensable for induction of the liver-specific genes.

Thus, both C/EBP $\alpha$  and C/EBP $\beta$  may play important roles in regulating growth and differentiation of HBLs. Moreover, a small amount of tumor biopsy samples could be used for measuring the mRNA expression levels of both genes for predicting aggressiveness of the HBL tumors.

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