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ORIGINAL PAPER

Relationship of *DDX1* and *NAG* gene amplification/ overexpression to the prognosis of patients with *MYCN*-amplified neuroblastoma

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Abstract
Purpose Amplification of the *MYCN* gene strongly correlates with advanced stage, rapid tumor progression and poor prognosis in neuroblastoma (NB). Several genes in the *MYCN* amplicon, including the DEAD box polypeptide 1 (*DDX1*) gene, and neuroblastoma-amplified gene (*NAG* gene), have been found to be frequently co-amplified with *MYCN* in NB. The aim of this study was to clarify the prognostic significance of the co-amplification or overexpression of *DDX1* and *NAG* with *MYCN*.
Procedure The gene copy numbers and mRNA expression levels of *MYCN*, *DDX1*, and *NAG* in 113 primary NBs were determined by the real-time quantitative polymerase chain reaction or quantitative reverse transcriptase/polymerase chain reaction assay. The relationships between gene co-amplification/overexpression status and stage, age at diagnosis, and overall survival were analyzed.
Results For evaluating the frequency of *DDX1* and *NAG* co-amplification, it proved appropriate to discriminate NBs with <40 copies of *MYCN* amplification from those with ≥40 copies of *MYCN* (*DDX1*, $p = 0.00058$; *NAG*, $p = 0.0242$, χ^2 for independence test). In patients with *MYCN*-amplified NB aged ≥18 months, those with tumor with enhanced *DDX1* expression and low-*NAG* expression showed a significantly better outcome than those with low-*DDX1* expression or enhanced *NAG* expression ($p = 0.0245$, log-rank test). None of the gene expression statuses had a significant relation to disease stage or survival for patients <18 months old. No relationship between any gene co-amplification status and disease stage, age at diagnosis, or overall survival was found.
Conclusions Our findings suggest that there may be a subset of NB in which enhanced *DDX1* and low-*NAG* expression consequent to *DDX1* co-amplification without *NAG* amplification contributes to susceptibility to intensive therapy. A larger study using an age cut-off of 18 months will be required.

Keywords Neuroblastoma · *MYCN* · *DDX1* · *NAG*

Abbreviations

NB	Neuroblastoma
DDX1	DEAD box polypeptide 1 gene
NAG	Neuroblastoma-amplified gene
hnRNP K	Heterogeneous nuclear ribonucleoprotein K
q-PCR	Quantitative polymerase chain reaction
q-RT-PCR	Quantitative reverse transcriptase/ polymerase chain reaction
BCM	B-cell maturation factor gene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

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Introduction

Neuroblastoma (NB) is one of the most common malignant solid tumors in childhood. It presents with

wide aggression from spontaneous regression or tumor maturation to rapid progression and fatality in most metastatic tumors diagnosed in children more than 1 year old. *MYCN* amplification occurs in about 25% of NB and is one of the most important markers in determining the aggressiveness of NB. Amplification of *MYCN* strongly correlates with advanced disease stage, rapid tumor progression and poor prognosis (Brodeur et al. 1984; Seeger et al. 1985; Brodeur and Seeger 1986). The size of the *MYCN* amplicon can span from 100 to 1,500 kb (Amler and Schwab 1989). Consequently, it is possible to suggest that additional genes being present in the amplicon and co-amplified with *MYCN* may contribute to the tumor phenotype. So far, several genes including the *DDX1* (DEAD box polypeptide 1) gene and *NAG* (NB-amplified gene) gene have been found to be frequently co-amplified with *MYCN* in NB (Beheshti et al. 2003; Scott et al. 2003a).

The *DDX1* is one of a family of genes that encode DEAD (asp-glu-ala-asp) box proteins. This gene maps to chromosome band 2p24 and 340 kb 5′ of *MYCN* (Godbout and Squire 1993; Kuroda et al. 1996). Proteins with the DEAD box motif are putative ATP-dependent RNA helicases and more than 30 proteins have been identified from bacteria to humans (De Valoir et al. 1991; Kitajima et al. 1994). By altering the RNA secondary structure, they are implicated in diverse cellular processes such as RNA splicing, ribosome assembly, and translation initiation (Tanner and Linder 2001). Some members of the family are differentially expressed during embryogenesis, cellular growth, and division (Schmid and Linder 1992; Iost and Dreyfus 1994; Godbout et al. 2002). The biological function of DDX1 remains unknown. In recent studies, DDX1 was found to associate with factors involved in 3′-end cleavage and polyadenylation of pre-mRNAs (Bleoo et al. 2001). DDX1 was also shown to have protein–protein interaction with heterogeneous nuclear ribonucleoprotein K (hnRNP K), and to have poly(A) RNA binding activity (Chen et al. 2002).

The *DDX1* gene has been known to be co-amplified with *MYCN* in 40–70% of NBs. There are reports showing a trend toward a worse clinical outcome with *MYCN* and *DDX1* co-amplification (Squire et al. 1995; George et al. 1997). Others have reported no significant difference in the clinical outcome or survival between patients with or without *DDX1* co-amplification (Manohar et al. 1995; De Preter et al. 2002). In a recent study of 98 *MYCN*-amplified NBs, a significant correlation of *DDX1* co-amplification with a better prognosis and improved patient survival was shown by using semiquantitative multiplex PCR (Weber et al. 2004). In contrast to the observations by Weber et al., De Preter et al. have concluded that *DDX1* co-amplification had no significant prognostic value in *MYCN*-amplified tumors by re-evaluating their data (De Preter et al. 2005). The prognostic significance of *MYCN* and *DDX1* co-amplification has not been determined.

Recently, Scott et al. reported that the 5′end of *NAG* is located 30 kb telomeric to *DDX1*, with the two genes lying in opposite orientations (Scott et al. 2003b). They found a significant association between low-disease stage in *MYCN*-amplified tumors and *NAG* co-amplification. The function of NAG is as yet unclear.

To date, there have been no reports of measuring and analyzing accurate copy numbers and precise mRNA expression levels of *MYCN*, *DDX1*, and *NAG* genes in NB. In order to clarify the prognostic significance of the co-amplification or gene expression of *DDX1* and *NAG* with *MYCN*, we determined gene copy numbers and mRNA expression levels of *MYCN*, *DDX1*, and *NAG* genes in 113 primary NBs using the real-time quantitative polymerase chain reaction (q-PCR) or quantitative reverse transcriptase/polymerase chain reaction (q-RT-PCR) assay. The results were analyzed in relation to stage, age at diagnosis and overall survival.

Materials and methods

Tumor samples

One hundred and thirteen primary NBs were obtained from the Department of Pediatric Surgery, University of Tsukuba, and the Division of Biochemistry, Chiba Cancer Center Research Institute, Japan. Tumors detected by mass screening were excluded. Patients were aged between 0 months and 14 years at diagnosis (median 18 months). All 52 nonsurvivors died of progressive disease, while 59 of 61 survivors are free of the disease.

Tumors with the haploid *MYCN* gene copy number of more than five and less than two by the q-PCR assay were considered as *MYCN* amplified and unamplified, respectively.

DNA or RNA extraction

Tumor DNA was isolated by proteinase K/SDS digestion followed by phenol–chloroform extraction according to the standard protocol. DNA from human

placenta and a NB cell line CHP 134 were used as templates for the reference B-cell maturation factor (*BCM*) gene and test genes, respectively. The CHP 134 cell was found to have multiple copies of *MYCN*, *DDX1*, and *NAG* genes by preliminary q-PCR.

Total RNA was prepared from frozen tumor tissue according to the Acid Guanidinium–Phenol–Chloroform method (Chomczynski and Sacchi 1987). One microgram of each RNA was incubated with random primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to yield cDNA.

Real-time q-PCR

Real-time q-PCR was carried out using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described by De Preter et al. with modification (De Preter et al. 2002). For quantification of the gene copy number, TaqMan probe assay was performed. The nucleotide sequences of the primers used are *MYCN*-f 5′-CGCAAAAGCC ACCTCTCATTA-3′ and *MYCN*-r 5′-TCCAGCAG ATGCCACATAAGG-3′, *DDX1*-f 5′-TAGGAGGAG GTGATGTACTTATGGTAA-3′ and *DDX1*-r 5′-AG CCTATGCAATTCTTAGAGTGTGT-3′, *NAG*-f 5′G ACCAAGAACTTCTTTCCCTGC-3′ and *NAG*-r 5′-GGTCAACAATACGTGGATAGAAGG-3′, and *BCM*-f 5′-CGACTCTGACCATTGCTTTCC-3′ and *BCM*-r 5′-AAGCAGCTGGCAGGCTCTT-3′.

The sequences of the TaqMan probes are *MYCN* 5′-FAM-TTCTGTAAATACCATTGACACATCCG CCTT-TAMRA-3′, *DDX1* 5′-FAM-CCCAGCTACC AATCACCTCACCAAATT-TAMRA-3′, *NAG* 5′-F AM-CAAGCTGCTGGTGAAGTGTGTCTCCA-T AMRA-3′ and *BCM* 5′-FAM-CAACCATTCTTGTC ACCACGAAAACGAA-TAMRA-3′. Twenty microliter of PCR reaction mixture for copy number determination consisted of template DNA, 1× q-PCR Mastermix (EUROGENTEC, Liege, Belgium), 300 nM of each primer and 200 nM of TaqMan probe. *MYCN*, *DDX1* or *NAG* gene assay was performed containing no-template control, standard CHP 134 DNA of five serial tenfold dilutions ranging from 100 ng to 10 pg, 10 ng of human placental DNA as a calibrator and ~10 ng of tumor DNA. The reference *BCM* gene assay included no-template control, standard human placental DNA of four serial tenfold dilutions ranging from 200 to 0.2 ng, 10 ng of human placental DNA as a calibrator and ~10 ng of tumor DNA. *BCM* gene is mapped to 16p13.1 and is located in a chromosomal region that rarely shows genetic abnormality (Vandesompele et al. 2001). Experiments were carried out in triplicate. The thermal cycling conditions for q-PCR and q-RT-PCR were: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 s and 60°C for 1 min.

Real-time q-RT-PCR

Expression levels of *MYCN*, *DDX1*, and *NAG* genes were measured in cDNA by the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control gene. cDNA from one of the 113 samples examined was used as the standard template because of its appropriate expression levels of *MYCN*, *DDX1*, *NAG*, and *GAPDH* mRNA by preliminary q-RT-PCR. The specific primers used are *MYCN*-f 5′-CACAAGGCCCTCAGTA CCTC-3′ and *MYCN*-r 5′- CAGTGACCACGTCGA TTTCTT-3′, *DDX1*-f 5′-TGGAAGAGATGGATT GGCTC-3′ and *DDX1*-r 5′-CCTGTTTCTGCAGCC ATAAGTAC-3′, *NAG*-f 5′-CAAATCACGGCAGT CACTACG-3′ and *NAG*-r 5′-ACACACTTCACCA GCAGCTTG-3′, and *GAPDH*-f 5′-GAAGGTGAA GGTCGGAGTC-3′ and *GAPDH*-r 5′-GAAGATGG TGATGGGATTTC-3′. The sequences of the TaqMan probes are *MYCN* 5′-FAM-AGAGGACACCCTGA GCGATTCAGATG-TAMRA-3′, *DDX1* 5′-FAM-C CAACTGATATCCAGGCTGAATCTATCCCA-T AMRA-3′, *NAG* 5′-FAM-TGTGACCAAGAACTTC TTTCCCTGCTCCT-TAMRA-3′ and *GAPDH* 5′-F AM-CAAGCTTCCCGTTCTCAGCC-TAMRA-3′. The primers and probes were designed to be located on exons 2–3 for *MYCN* mRNA, exons 2–4 for *DDX1* mRNA, exons 51–52 for *NAG* mRNA, and exons 4–6 for *GAPDH* mRNA.

Twenty microliter of the PCR reaction mixture for quantification contained template cDNA, 1× qPCR Mastermix (EUROGENTEC), 300 nM of each primer and 200 nM of TaqMan probe. Each assay consisted of no-template control, standard cDNA of five serial tenfold dilutions ranging from 1 μg to 0.1 ng, and ~5 ng of tumor cDNA.

Statistical analysis

The relation of *DDX1* or *NAG* gene amplification to *MYCN* gene copy number was tested using χ^2 for an independence test. Correlations between the gene amplification/expression status and disease stage or age at diagnosis were compared by χ^2 for an independence test or Fisher's exact probability test. Mann–Whitney's *U*-test was used to evaluate the relationship between the gene expression level in tumors with or without gene amplification. Survival analysis was performed according to the Kaplan–Meier method and the log-rank test.

Results

The haploid *MYCN*, *DDX1*, and *NAG* gene copy number

Seventy-two of 113 tumors examined had *MYCN* amplification. Forty-one tumors were *MYCN*-unamplified; 17 in stages 1, 2 or 4S, nine in stage 3, and 15 in stage 4. Twenty-five and 16 patients were aged <18 and ≥18 months at diagnosis, respectively. Seven of 41 patients died of disease, while all 34 survivors are free of disease. Patients with *MYCN*-amplified NB included four with stage 1, two with stage 2, two with stage 4s, 12 with stage 3, and 52 patients with stage 4 disease. Of 72 patients with *MYCN*-amplified tumor, 45 patients died of disease, while 25 of 27 survivors are free of disease. The follow-up period for *MYCN*-amplified survivors ranged from 17 to 93 months.

In 72 *MYCN*-amplified NBs, *DDX1*, and *NAG* genes were found to be co-amplified in 49 (68.1%) and 19 (26.4%) tumors, respectively (Fig. 1a, b). All 19 tumors with *NAG* amplification had also *DDX1* amplification. Forty-one tumors without *MYCN* amplification were unamplified for *DDX1* and *NAG*. By plotting precise gene copy numbers of *MYCN* and *DDX1*, and *MYCN* and *NAG* of each tumor on the same graphs, we found for the first time that NB with lower copies of *MYCN* amplification tended to a more frequent *DDX1* and *NAG* co-amplification than those with higher copies of *MYCN*. For evaluating the frequency of *DDX1* and *NAG* co-amplification, it proved appropriate to discriminate NBs with <40 copies of *MYCN* amplification from those with ≥40 copies of *MYCN* (*DDX1*, $p = 0.00058$; *NAG*, $p = 0.0242$, χ^2 for independence test) (Fig. 1a, b).

Overall survival of patients with *MYCN*-amplified NB with or without *DDX1* and *NAG* co-amplification

For the 72 patients with *MYCN*-amplified NB, no statistically significant difference in survival probability was found among three gene co-amplification statuses, patients with tumor with *MYCN* amplification alone (*MYCN* alone), those with *DDX1* co-amplification alone (*MYCN* + *DDX1*), and those with both *DDX1* and *NAG* co-amplification (*MYCN* + *DDX1* + *NAG*) (*MYCN* alone versus *MYCN* + *DDX1*, $p = 0.465$; *MYCN* alone versus *MYCN* + *DDX1* + *NAG*, $p = 0.719$; *MYCN* + *DDX1* versus *MYCN* + *DDX1* + *NAG*, $p = 0.148$, log-rank test) (Fig. 2). We found no significant difference in overall survival between patients with tumor with *MYCN* amplification alone and those with *DDX1*-co-amplified NB regardless of *NAG* co-amplification ($p = 0.763$, log-rank test).

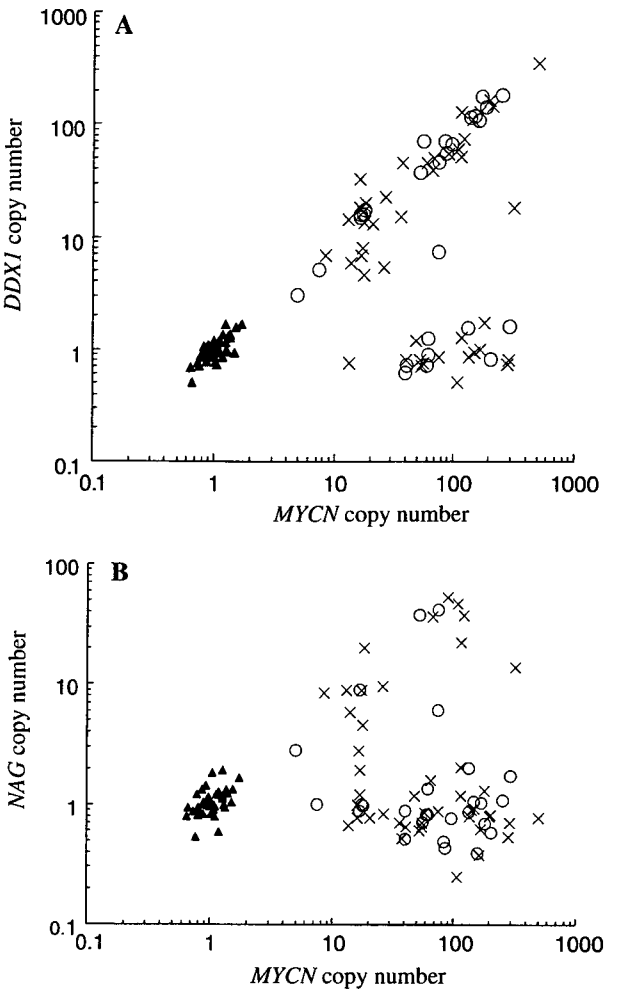

Fig. 1 The haploid *MYCN* and *DDX1* gene copy numbers (**a**), and *MYCN* and *NAG* gene copy numbers (**b**) in 113 NBs were determined by real-time q-PCR. *Open circle*, survivors with *MYCN* amplification ($n = 27$); *cross*, nonsurvivors with *MYCN* amplification ($n = 45$); *filled triangle*, patients without *MYCN* amplification ($n = 41$)

Relation of gene co-amplification status to disease stage or age at diagnosis

Table 1 shows the gene co-amplification status, disease stage and age at diagnosis of 72 patients with *MYCN*-amplified NB. Recently, an age cut-off higher than 12 months has been proposed as a prognostic predictor for comparison of survival rate in NB, suggesting an appropriate age cut-off of 18 months (London et al. 2005).

None of the gene co-amplification statuses had a significant correlation with disease stage (stages 1, 2, 3, and 4s versus stage 4) or age at diagnosis (<18 months versus ≥18 months) (data not shown), with the exception of *NAG*, which tended toward a more frequent co-amplification with *MYCN* in stage 4 (17/52, 32.7%)

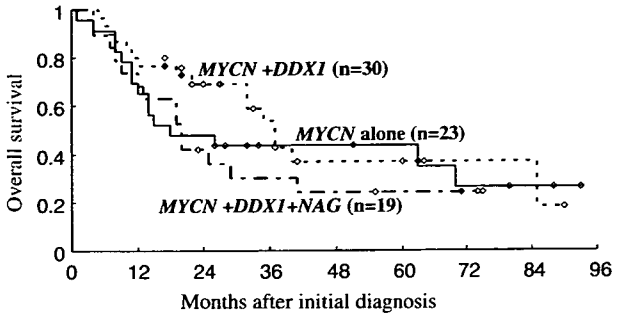

Fig. 2 Overall survival for patients with *MYCN*-amplified NB with or without *DDX1* and *NAG* co-amplification. No statistically significant difference in survival probability was found among three gene co-amplification statuses (*MYCN* alone versus *MYCN* + *DDX1*, $p = 0.465$; *MYCN* alone versus *MYCN* + *DDX1* + *NAG*, $p = 0.719$; *MYCN* + *DDX1* versus *MYCN* + *DDX1* + *NAG*, $p = 0.148$, log-rank test)

Table 1 Gene co-amplification status, disease stage and age at diagnosis of 72 patients with *MYCN*-amplified NB. There was no significant correlation between any gene co-amplification status and disease stage (stages 1, 2, 3, and 4s versus stage 4) or age at diagnosis among 72 patients with *MYCN*-amplified NB, with the exception of *NAG*, which tended toward a more frequent co-amplification with *MYCN* in stage 4 compared with stages 1, 2, 3, and 4s ($p = 0.0504$, χ^2 for independence test)

	Stage			Age (months)	
	1, 2 and 4s	3	4	<18	≥18
MYCN alone	4	4	15	10	13
MYCN+DDX1	4	6	20	11	19
MYCN+ DDX1+NAG	0	2	17	10	9
Total	8	12	52	31	41

compared with stages 1, 2, 3, and 4s (2/20, 10.0%) ($p = 0.0504$, χ^2 for independence test).

The expression level of *MYCN*, *DDX1*, and *NAG*

We determined the precise expression levels of *MYCN*, *DDX1*, and *NAG* in 108 of 113 NBs. Sixty-seven of 108 tumors had *MYCN* amplification. The *MYCN*-amplified tumors had a significantly higher expression level of *MYCN* compared with *MYCN*-unamplified tumors ($p = 8.22 \times 10^{-15}$, Mann–Whitney's *U*-test). None of the *MYCN*-unamplified tumors showed an overexpression of *MYCN*, *DDX1*, and *NAG* (data not shown). We classified *DDX1* or *NAG* gene expression levels higher than the highest in *MYCN*-unamplified tumors as enhanced.

In general, *DDX1* expression increased according to the *DDX1* copy number (Fig. 3a). The expression level of *DDX1* in tumors with *MYCN* amplification alone was as low as that without *MYCN* amplification. Enhanced DDX1 expression had no significant correlation with prognosis ($p = 0.925$, log-rank test).

The expression level of *NAG* in *NAG* co-amplified tumors was significantly higher than that in tumors without *NAG* co-amplification ($p = 5.77 \times 10^{-6}$, Mann–Whitney's *U*-test); however, *NAG* amplification was not necessarily accompanied with enhanced *NAG* expression (Fig. 3b). Enhanced *NAG* expression had no significant relation to disease stage (stage 1, 2, 3, and 4s versus stage 4) ($p = 0.462$, Fisher's exact probability test) or clinical outcome ($p = 0.0915$, log-rank test).

Relation of *DDX1* and *NAG* gene expression statuses to survival probability for patients with *MYCN*-amplified NB

None of the gene expression statuses had a significant correlation with disease stage and with survival for patients aged <18 months (data not shown).

In 41 patients with *MYCN*-amplified NB aged ≥18 months, those with tumor with enhanced *DDX1* expression and low-*NAG* expression had a significantly better outcome than those with low-*DDX1* expression or enhanced *NAG* expression ($p = 0.0245$, log-rank test) (Fig. 4a, b). The 16 tumors with enhanced *DDX1* and low-*NAG* expression included one with stage 2, four with stage 3, and 11 tumors with stage 4. All 16 tumors had *MYCN* and *DDX1* co-amplification without *NAG* amplification.

Discussion

Amplification of the *MYCN* gene is strongly associated with the rapid progression of NB and advanced disease stage (Brodeur et al. 1984; Seeger et al. 1985). The prognosis of patients with stage 4 tumors with *MYCN* amplification had been extremely poor. In 1999, Kaneko et al. reported treatment results with improved survival rate of patients with advanced NB aged 1 year or older treated with an intensive induction and consolidation chemotherapy regimens (Kaneko et al. 1999). With the use of a more intensive induction regimen followed by hematopoietic stem cell transplantation for *MYCN*-amplified stage 4 patients, survival curves for those with or without *MYCN* amplification appeared similar. In other words, the prognosis of patients with stage 4 NB without *MYCN* amplification remained poor (Kaneko et al. 2002). Their assessment of *MYCN* amplification status was based on the Southern blot technique. Measuring an accurate *MYCN* copy number in tumors is essential in

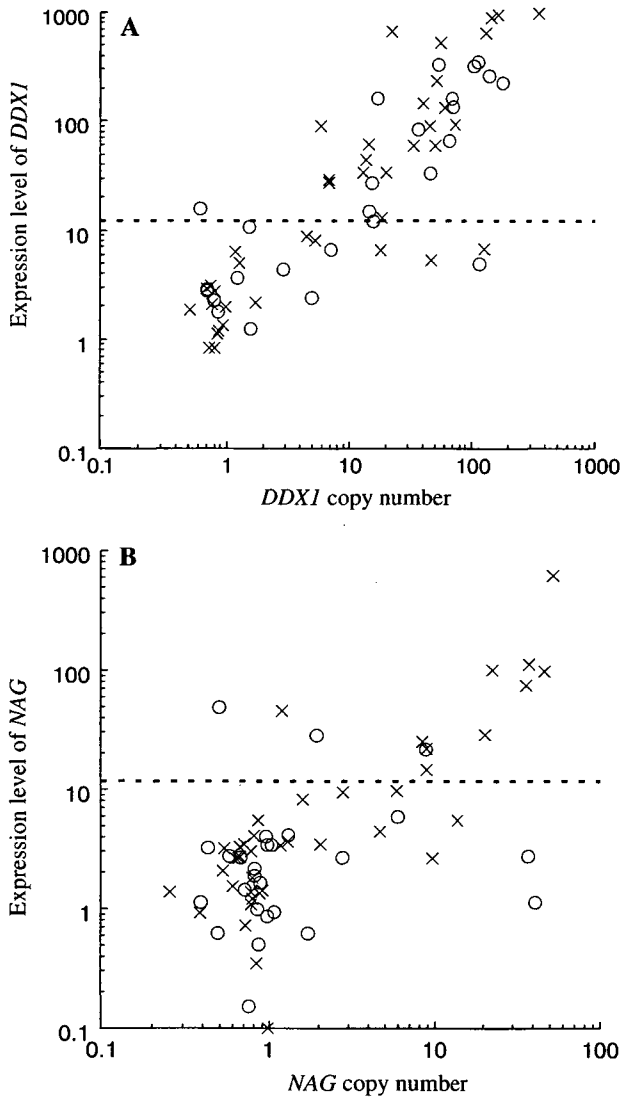

Fig. 3 Gene copy number and gene expression level of *DDX1* (**a**) and *NAG* (**b**) in 67 *MYCN*-amplified NBs. *Open circle*, survivors with *MYCN* amplification ($n = 26$); *cross*, nonsurvivors with *MYCN* amplification ($n = 41$); *dotted line*, the highest expression level of *DDX1* (**a**) or *NAG* (**b**) in *MYCN*-unamplified NBs

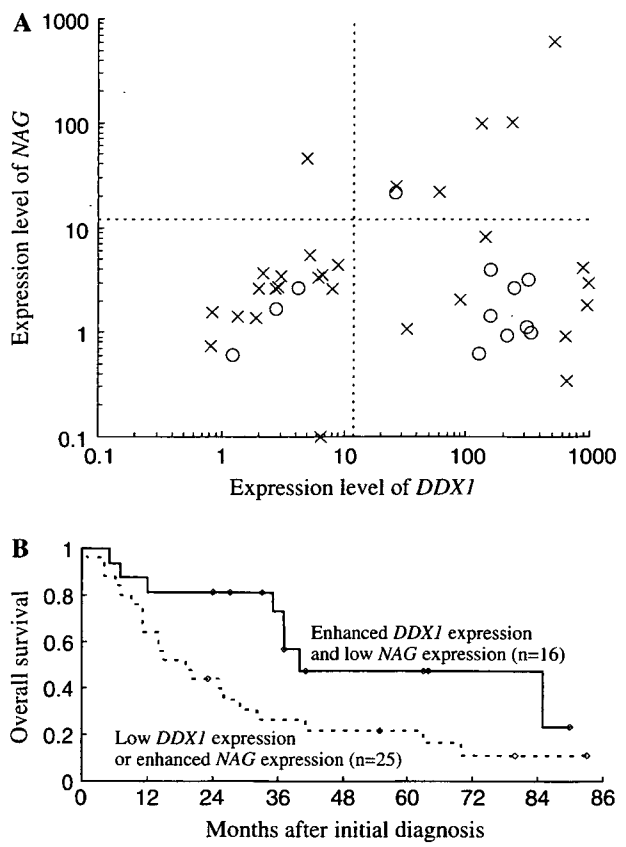

Fig. 4 **a** Expression level of *DDX1* and *NAG* mRNA in 41 *MYCN*-amplified NBs from patients aged ≥18 months. *Open circle*, survivors with *MYCN* amplification ($n = 12$); *cross*, nonsurvivors with *MYCN* amplification ($n = 29$); *dotted lines*, the highest expression levels of *DDX1* and *NAG* in *MYCN*-unamplified NBs. **b** In the 41 patients with *MYCN*-amplified NB aged ≥18 months, those with tumor with enhanced *DDX1* expression and low-*NAG* expression showed a significantly better outcome than those with low-*DDX1* expression or enhanced *NAG* expression ($p = 0.0245$, log-rank test)

order to select the optimal treatment and improve survival for patients with advanced NB. Assays for the rapid and accurate quantification of *MYCN* copy number and *MYCN* expression level in NB have been developed by real-time q-PCR or q-RT-PCR method with TaqMan probe (Raggi et al. 1999; Tajiri et al. 2001; De Preter et al. 2002). Tanaka et al. reported that in their highly sensitive analysis by a q-PCR method combined with FISH, cases with more than two *MYCN* gene dosages (*MYCN/p53* ≥2.0) were significantly associated with unfavorable prognostic factors (Tanaka et al. 2004). In our study, we did not investigate NBs with a haploid *MYCN* gene copy number of between two and five.

Recently, Scott et al. found that the 7.3 kb transcript of the *NAG* gene with 52 exons, which is predominantly expressed in NB, covers 420 kb of genomic DNA. They proposed that probes for Southern blot or FISH studies, or primers used for PCR-based methods, should include the 3′ end of the *NAG* gene over 400 kb away from *DDX1* (Scott et al. 2003b). The primers and TaqMan probe we designed were located on the 3′ end of the *NAG* gene, on exon 52, and the frequency of *NAG* co-amplification with *MYCN* was in accordance with that reported by Scott et al.

It is thought that a low copy number of *MYCN* in *MYCN*-amplified human NB cells is correlated with a low degree of recombination and large amplicon size (Amler and Schwab 1989). Consistent with the findings by Amler and Schwab, we found for the first time that NB with lower copies of *MYCN* amplification tended

to a more frequent *DDX1* and *NAG* co-amplification than those with higher copies of *MYCN*. For evaluating the frequency of *DDX1* and *NAG* co-amplification, it proved appropriate to discriminate NBs with <40 copies of *MYCN* amplification from those with ≥40 copies of *MYCN*. The information obtained from these observations may be different from a recent suggestion by De Preter et al. that the process of co-amplification within the *MYCN* amplicon occurs coincidentally, and is not subject to selection (De Preter et al. 2005).

The prognostic significance of DDX1 co-amplification with MYCN has remained unclear. Squire et al. analyzed 13 *MYCN*-amplified patients and showed a trend toward a worse clinical outcome with *DDX1* co-amplification (Squire et al. 1995). George et al. reported that with the exclusion of patients with 4S disease, those with *DDX1* co-amplification had a significantly shorter mean disease-free interval compared with *MYCN* amplification alone (George et al. 1997). However, they described that there was no significant difference in the proportion of survivors in these two groups. In contrast, Weber et al. reported that *DDX1* co-amplification correlated with an improved patient survival in 98 *MYCN*-amplified NB (Weber et al. 2004). In our study of 72 patients with *MYCN*-amplified NB, there was no significant difference in survival probability between patients with *DDX1*-co-amplified NB and those with tumor with *MYCN* amplification alone. The result was similar to those reported by Manohar et al. and De Preter et al. (Manohar et al. 1995; De Preter et al. 2002, 2005).

The *NAG* tended toward a more frequent co-amplification with *MYCN* in stage 4 compared with other stages, in contrast to the result of a significant association of *NAG* co-amplification with low-stage disease by Scott et al. (2003b). The difference was probably caused by the lower frequency of *NAG* co-amplification with *MYCN*. Amplification of *NAG* was not necessarily accompanied with enhanced *NAG* expression, and *NAG* expression level in *MYCN*-amplified tumors showed no significant relation to disease stage. The relationship between RNA expression levels of *DDX1* or *NAG* and clinical outcome for patients with *MYCN*-amplified NB has hardly been studied. Weber et al. reported that a high expression level of *DDX1* was associated with a trend toward a better survival probability while *NAG* expression was not correlated with prognosis (Weber et al. 2004). They drew the result using RNAs from 19 *MYCN*-amplified NB samples. We analyzed *DDX1* and *NAG* gene expression in 67 *MYCN*-amplified NB. Enhanced *DDX1* and *NAG* expression had no significant correlation with prognosis, respectively.

For the discrimination of prognosis for patients with NB, an age cut-off of 12 months was adopted worldwide. However, the International Neuroblastoma Pathology Classification, established for the prognostic evaluation of patients with neuroblastic tumors, has incorporated age factor of 18 months in the system (Shimada et al. 2001; Sano et al. 2006). The Children's Cancer Group in the USA has already chosen the 18 months as an age cut-off (Schmidt et al. 2005). Recent evidence supports the age cut-off ranging 15–18 months based on the results from the German analysis and two Children's Oncology Group analyses (London et al. 2005). In our study, the *DDX1* and *NAG* gene expression status in *MYCN*-amplified NBs revealed an age cut-off of 18 months to be an appropriate prognostic predictor of survival. We found that older patients with enhanced *DDX1* expression and low-*NAG* expression had a significantly better prognosis than those with low-*DDX1* expression or enhanced *NAG* expression. These findings indicate that, for *MYCN*-amplified NB from patients aged ≥18 months, both enhanced *DDX1* expression and low-*NAG* expression may be associated with a better response to intensive therapy. It is also possible to suggest a subset of NB in which enhanced *DDX1* and low-*NAG* expression consequent to *MYCN* and *DDX1* co-amplification without *NAG* amplification contributes to patient survival.

A larger cohort of patients and longer follow-up period using an age cut-off of 18 months will be required to clarify the clinical and prognostic significance of the expression status of both *DDX1* and *NAG* genes with *MYCN*.

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Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas ☆

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Abstract

The CpG island methylator phenotype (CIMP) was closely associated with poor overall survival (OS) in Japanese neuroblastoma (NBL) cases in our previous study. Here, in German NBL cases, CIMP(+) cases ($n = 95$) showed markedly poorer OS (hazard ratio (HR) = 9.5; $P < 0.0001$) and disease-free survival (DFS) (HR = 5.4; $P < 0.0001$) than CIMP(−) cases ($n = 50$). All the 23 cases with N-*myc* amplification had CIMP. Among the remaining cases without N-*myc* amplification, CIMP(+) cases ($n = 27$) had a poorer OS (HR = 4.5; $P = 0.02$) and DFS (HR = 5.2; $P < 0.0001$) than CIMP(−) cases ($n = 95$). In multivariate analysis, CIMP and N-*myc* amplification had an influence on OS and DFS independent of age and disease stage. CIMP had a stronger influence on DFS than N-*myc* amplification while N-*myc* had a stronger influence on OS.

Keywords: Neuroblastoma; Methylation; CIMP; MS-RDA; N-*myc*

1. Introduction

Neuroblastoma (NBL) is one of the most common pediatric solid tumors, and is characterized by two extreme disease courses, spontaneous regression and life-threatening progression. To implement adequate and necessary therapeutics, NBL cases are stratified into low-, intermediate- and high-risk groups based upon clinical and genetic information, such as disease stage, age at diagnosis, Shimada histology, N-*myc* amplification status, DNA ploidy, and *TrkA* expression level [1–6]. Especially, N-*myc* amplification, present in approximately 20–30% of NBL cases, is a powerful molecular marker for the stratification [1–4]. Nevertheless, more precise risk estimation is necessary for cases currently stratified

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into the intermediate-risk group, and development of a novel prognostic marker is awaited [1,2].

Recently, using a genome-wide screening method for differences in DNA methylation, methylation-sensitive representational difference analysis [7–9], we found that multiple CGIs were methylated in NBL cases with poor prognosis [10]. By analysis of 140 Japanese NBL cases, methylation of the multiple CGIs was shown to be dependent upon each other, and conformed to the concept of the CGI methylator phenotype (CIMP), originally established in colorectal cancers [11]. Cases could be classified as either CIMP(+) or CIMP(−), and a very limited number of cases had an intermediate phenotype. CIMP(+) cases had a markedly poorer overall survival (OS) than CIMP(−) cases with a hazard ratio (HR) of 22.1 [95% confidence interval (95%CI) = 5.3–93.4; $P < 0.0001$]. Its influence was independent of *TrkA* expression status, DNA ploidy, and age at diagnosis. Notably, almost all cases with N-*myc* amplification exhibited CIMP (37 of 38 cases), and, even among the cases without N-*myc* amplification, CIMP(+) cases had a poorer OS than CIMP(−) cases (HR = 12.4; 95%CI = 2.6–58.9; $P = 0.002$). CIMP status was well associated with the methylation level of the *Protocadherin β* (*PCDHB*) gene family, followed by methylation levels of *hepatocyte growth factor-like protein* (*HLP*) gene and *Cytochrome p450 CYP26C1* (*CYP26C1*).

Considering that there could be potential ethnic differences and that genome-wide screenings tend to produce "too good" results [12], here we took advantage of archived materials of German NBL cases. If the strong influence of CIMP on OS is also observed in German cases, we can establish CIMP as a prognostic marker that can be universally used. Also, the German NBL cases have information on disease-free survival (DFS), which was not available for Japanese NBL cases, and the influence of CIMP on DFS can be clarified.

2. Materials and methods

2.1. Tissue samples

A total of 152 cases were collected between 1998 and 2004, and all patients were enrolled in the German NBL Trial. The mean age at initial diagnosis was 1082 days (range 0–9607 days). Thirty-seven, 29, 17, 51 and 17 cases belonged to stages 1, 2, 3, 4, and 4S (International Neuroblastoma Staging System), respectively, although information was not available for one case. The composition of the cohort in terms of stage, N-*myc* status and age at diagnosis was in agreement with the composition of unselected cohort of 1741 patients diagnosed betwe 1990 and 2003 in Germany [13]. DNA was extracted the standard phenol/chloroform procedure, and used f this study under approval of Institutional Review Boar

2.2. Sodium bisulfite modification and quantitative methylation-specific PCR (MSP)

One microgram of DNA restricted with *Bam*I underwent sodium bisulfite modification [14], and w suspended in 20 μl of TE buffer. For quantitative MS 1 μl of the solution was used for PCR using SYE Green PCR Core Reagents (PE Biosystems) and an iC cler Thermal Cycler (Bio-Rad Laboratories). PCR w performed separately for methylated (M) DNA mo cules and for unmethylated (U) DNA molecules wi primers specific to each sequence, and the numbers M and U molecules in a test sample were determin by comparing their amplification with those of standa samples containing 10–10^6 molecules. Primer sequenc and standard DNA were previously described [10]. T "methylation level" was calculated as the fraction of molecules in the total DNA molecules (# of M mo cules + # of U molecules). All the molecular analys were performed blind to clinical information, and met ylation level for a case was obtained as an average two independent measurements.

2.3. Statistical analysis

Reproducibility of methylation levels between tv measurements was assessed using the Pearson correlati coefficient. Survival time was measured from the date initial diagnosis to the date of death or last contact. Ka lan–Meier analysis and log-rank tests were performed compare overall survival (OS) and disease-free surviv (DFS) between groups. HRs were estimated by the C proportional hazards model. These statistical analys were performed using SPSS, version 13.0 (SPSS Inc., Cl cago, IL).

3. Results

3.1. Determination of CIMP statuses in German NBL cas

Methylation levels were measured in 152 Germ NBLs for three CGI (group)s – (i) the 17 *PCDHB* fami genes, (ii) *HLP*, and (iii) *CYP26C1*. They were high reproducible with a correlation coefficient ⩾0.99, a the average levels were used hereafter. The methylati level of the *PCDHB* gene family showed a clear bimod distribution (Fig. 1A). To avoid artificial bias, CIMP st tuses were diagnosed before having access to clinic information of the cases. First, since cut-off valu between 40% and 60% gave high HRs in our previo

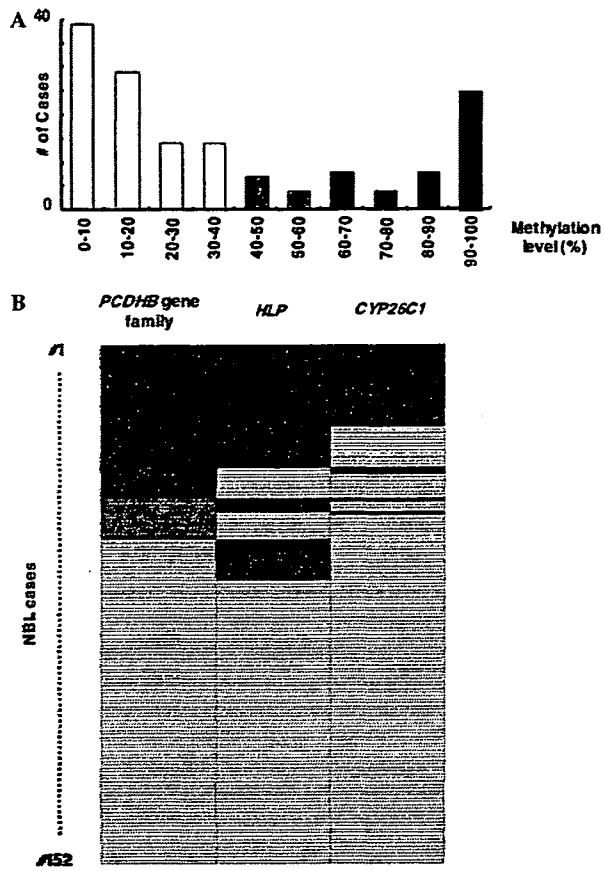

Fig. 1. Bimodal distribution of methylation levels of the *PCDHB* gene family, and diagnosis of CIMP status. (A) Histogram of number of cases according to *PCDHB* methylation levels. The methylation level of the *PCDHB* gene family was measured exactly as in our previous study [10], and its bimodal distribution in German NBLs was confirmed. (B) Methylation statuses of the three CGIs (groups) among the 152 NBLs. Cut-off values for the *PCDHB* gene family, *HLP* and *CYP26C1* were set based on the previous study, which were 40–60%, 10%, and 70%, respectively. Closed and open boxes show high and low methylation levels, and methylation levels of the *PCDHB* gene family between 40% and 60% are shown by grey boxes. Methylation levels of these three CGIs were closely associated with each other.

study [10], cases with methylation levels lower than 40% and higher than 60% were diagnosed as CIMP(−) ($n = 95$) and CIMP(+) ($n = 45$), respectively. Only 12 cases had methylation levels between 40% and 60%.

Then, for these 12 cases, methylation levels of *HLP* and *CYP26C1*, whose predictive powers followed that of the *PCDHB* gene family in our previous study [10], were taken into account. Five of the 12 cases had high levels of methylation of *HLP* and/or *CYP26C1*, and were considered to have CIMP, and seven other cases were left as unknown for CIMP status (Fig. 1B). Cut-off values for *HLP* and *CYP26C1* were set at the same levels as in our previous study, which were 10% and 70%, respectively. As a result, 50, 95, and 7 cases of the 152 cases were diagnosed as CIMP(+), CIMP(−), and unknown, respectively. Methylation statuses of the three CGI (groups) showed close correlation with methylation statuses of the other CGIs.

3.2. Univariate analysis with OS and DFS

In univariate analysis, the 50 CIMP(+) cases exhibited markedly and significantly poorer OS (HR = 9.5; 95%CI = 3.2–28.1; $P < 0.0001$) and DFS (HR = 5.4; 95%CI = 2.9–10.3; $P < 0.0001$) than the 95 CIMP(−) cases. Cases with N-*myc* amplification ($n = 23$) also exhibited markedly and significantly poorer OS (HR = 11.8; 95%CI = 4.9–28.7; $P < 0.0001$) and DFS (HR = 3.1; 95%CI = 1.6–6.0; $P = 0.0007$) than 122 cases without N-*myc* amplification. All of the 23 German cases with N-*myc* amplification had CIMP, as observed in a Japanese population.

Therefore, the German NBL cases were classified into three groups: (a) CIMP(−) cases ($n = 95$), all of which were without N-*myc* amplification, (b) CIMP(+) cases without N-*myc* amplification ($n = 27$), and (c) CIMP(+) cases with N-*myc* amplification ($n = 23$). As for OS (Fig. 2A), the three groups exhibited a step-wise increase of risk, showing the influence of N-*myc* amplification in addition to CIMP. Among the cases without N-*myc* amplification (groups (a) and (b)), CIMP had a significant influence on OS (HR = 4.5; 95%CI = 1.3–16.1; $P = 0.02$). As for DFS (Fig. 2B), CIMP had a significant influence (HR = 5.2; 95%CI = 2.6–10.6; $P < 0.0001$) by comparison of groups (a) and (b). However, additional influence by N-*myc* amplification was unclear by comparison of groups (b) and (c). These suggested that N-*myc* amplification had a strong influence on OS while CIMP had a strong influence on DFS.

3.3. Multivariate analysis

Since CIMP and N-*myc* amplification were dependent upon each other, multivariate analysis was first performed using age at diagnosis, disease stage, and either CIMP or N-*myc* amplification (Table 1A and B). It was confirmed that either CIMP or N-*myc* amplification had a significant influence on OS and DFS independent of age at diagnosis and disease stage.

Then, multivariate analysis was performed using age at diagnosis, disease stage, and both CIMP and N-*myc* amplification to compare the influences of them (Table 1C). As for OS, N-*myc* amplification retained its power while CIMP lost its power. In contrast, as for DFS, CIMP retained its power while N-*myc* amplification lost its power. This result was in accordance with the finding that CIMP had a strong influence on DFS while N-*myc* amplification had a strong influence on OS.

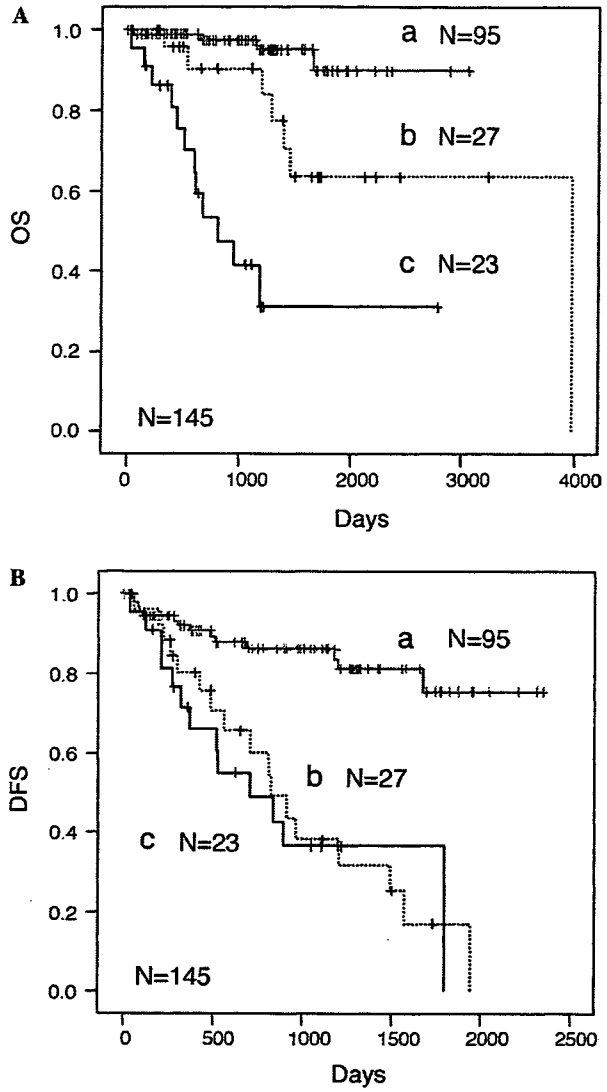

Fig. 2. Kaplan–Meier analysis of (a) CIMP(−) cases without N-*myc* amplification ($n = 95$), (b) CIMP(+) cases without N-*myc* amplification ($n = 27$), and (c) CIMP(+) cases with N-*myc* amplification ($n = 23$). (A) Kaplan–Meier analysis using OS. Using group (a) as a reference, group (b) had a HR of 4.5 (95%CI = 1.3–16.1; and $P = 0.02$), and group (c) had a HR of 21.7 (6.8–69.3; <0.0001). Using group (b) as a reference, group (c) had a HR of 4.8 (1.7–13.6; 0.003). (B) Kaplan–Meier analysis using DFS. Using group (a) as a reference, group (b) had a HR of 5.2 (2.6–10.6; <0.0001), and group (c) had a HR of 5.7 (2.6–12.2; <0.0001). There was no significant difference between groups (b) and (c) ($P = 0.82$).

4. Discussion

Methylation levels of the *PCDHB* gene family showed a bimodal distribution in German NBL cases, as in our initial analysis of Japanese NBL cases [10], and the presence of two groups of NBLs from the viewpoint of CIMP was confirmed. T CIMP statuses of individual German NBL ca were determined using criteria established in Ja nese NBL cases to avoid falsely "too good" resu which tend to happen in genome-wide analyses [1 Nevertheless, the strong influence of CIMP on in all the NBL cases (HR = 9.5) and also in th without N-*myc* amplification (HR = 4.5) was c firmed. After finishing all the analysis we search for a *PCDHB* methylation level that would g the highest HR for the 152 German NBL cas and it was 30% with a HR of 9.8 (95%CI = 2. 33.0; $P < 0.0001$), followed by 40% with a HR 9.4 (95%CI = 3.2–27.6; $P < 0.0001$). Based on t precise reproduction of the initial findings in Ja nese NBL cases in German NBL cases, CIMP highly likely to be a novel prognostic marker t can be universally used in cases without N-*m* amplification. A prospective study is warranted.

A strong influence of CIMP on DFS w revealed for the first time in this study because da on DFS were available only for German NBL cas In univariate analysis, CIMP had a strong influen on DFS in all the NBL cases (HR = 5.4) and in t cases without N-*myc* amplification (HR = 5. (groups (a) and (b) in Fig. 2B). In multivariate an ysis involving age at diagnosis, disease stage, a both N-*myc* amplification and CIMP, CIM retained its power on DFS while N-*myc* amplific tion retained its power on OS. This suggested th the recurrence of NBL cases was strongly associat with CIMP, but that NBL cases without N-*m* amplification had higher chances to be induced in the second remission.

The almost complete inclusion of cases with N *myc* amplification within the CIMP(+) cases our two independent studies indicates that the two abnormalities are very closely associated wi each other. If we assume a single abnormality th underlies a poor prognosis of NBL cases, it is like that CIMP is caused by it, and some of CIMP(- NBLs develop N-*myc* amplification. If we assur multiple abnormalities, it is likely that CIMP consistently associated with the devastating stat of NBLs, which can be induced by N-*myc* ampli cation and other causes. Clarification of wh molecular abnormality causes CIMP and hc CIMP and N-*myc* amplification are related important.

The presence of CIMP was considered to lead tc poor prognosis by induction of methylation promoter CGIs of various tumor-related genes. V

Table 1
Multivariate analysis of prognostic factors for overall and disease-free survival

Variable	OS			DFS		
	HR	95% CI for HR	*P*	HR	95% CI for HR	*P*
(A)						
Age at diagnosis	6.2	0.8–48.7	0.082	1.8	0.8–4.1	0.171
Disease stage	1.8	0.6–5.8	0.319	1.8	0.8–4.0	0.152
CIMP	4.9	1.5–15.8	0.008	3.3	1.5–7.0	0.002
(B)						
Age at diagnosis	13.6	1.8–104.3	0.012	2.5	1.1–5.6	0.025
Disease stage	1.5	0.5–5.0	0.501	2.6	1.2–5.4	0.013
N-*myc* amplification	11.5	3.9–33.8	<0.001	2.1	1.0–4.2	0.043
(C)						
Age at diagnosis	12.1	1.6–94.4	0.017	1.9	0.8–4.5	0.137
Disease stage	1.2	0.3–4.1	0.796	1.7	0.8–3.9	0.179
N-*myc* amplification	8.0	2.5–25.8	<0.001	1.3	0.6–2.7	0.563
CIMP	2.3	0.6–8.9	0.226	3.0	1.3–6.9	0.009

HR, hazard ratio; CI, confidence interval; OS, overall survival; DFS, disease-free survival.

previously observed association between CIMP and promoter methylation of tumor-suppressor *RASSF1A* and *BLU* genes [10]. It is reported that an anti-apoptotic gene, *TMS1*, a homeobox gene, *HOXA9*, a cell cycle gene, *CCND2*, and candidate tumor-suppressor genes, *EMP3* and *NR1I2*, are more frequently methylated in NBL cases with a poor prognosis [15–17]. However, the risk given by methylation of one of these individual genes is much smaller than that given by CIMP. This is in accordance with our hypothesis that CIMP leads to consistent methylation of marker CGIs, such as exonic CGIs of the *PCDHB* gene family, and occasional methylation of promoter CGIs of tumor-related genes. Silencing of an individual gene accounts for a poor prognosis of only a fraction of NBL cases with CIMP. It is known that exonic CGIs are more susceptible to methylation than promoter CGIs [9], and it is expected that they are more useful as a prognostic marker.

In summary, the faithful reproduction in German NBL cases of the highly significant findings obtained in Japanese cases demonstrated that CIMP is a strong and universal prognostic marker for NBL cases, especially for those without N-*myc* amplification. The close association between CIMP and DFS was revealed for the first time in this study.

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Reciprocal expression of CCAAT/enhancer binding proteins α and ß 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 ß-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 α (C/EBPα) and C/EBPß, 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/EBPα mRNA was significantly up-regulated in tumors 223% (p=0.013) as compared with that in adjacent normal livers, while expression of C/EBPß was down-regulated to 27% (p=0.002). Of interest, the immunohistochemical analysis showed that expression of C/EBPα was higher and that of C/EBPß lower in the poorly differentiated tumor cells than in the well-differentiated cells within the same tumor. Furthermore, high expression of C/EBPα (p=0.047) as well as low expression of C/EBPß (p=0.025) was significantly associated with poor prognosis of the patients. Cox hazard model suggested that expression of C/EBPα and that of C/EBPß 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.

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Key words: hepatoblast, differentiation, Cox proportional hazard ratio

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 α-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)α and C/EBPß are liver-enriched transcription factors, regulating the expression of liver-specific genes. Expression of C/EBPα is observed on day 9.5 of gestation, and C/EBPß on day17.5 in the fetal liver of rodents, suggesting that they may be involved in hepatocyte differentiation (4). The hepatocytes in C/EBPα-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/EBPα may play a role in the growth regulation of HBL cells. C/EBPα is down-regulated while C/EBPß is up-regulated in the remnant liver after partial hepatectomy (6).

We studied expression of C/EBPα and C/EBPß 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

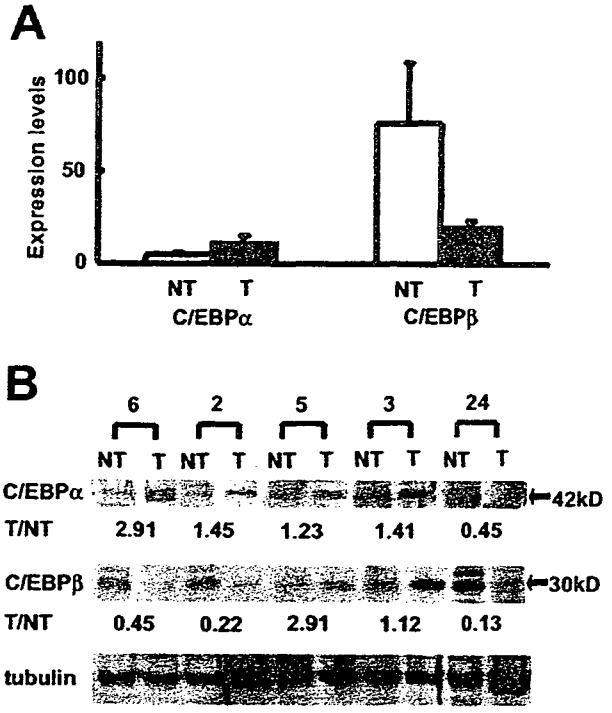

Figure 1. Real-time quantitiative PCR and Western blot analysis of hepatoblastoma with C/EBPα and C/EBPß. (A) Expression levels of C/EBPα and C/EBPß were analyzed with real-time quantitative PCR (x1000) (mean ± standard error). NT, non-tumorous tissue; T, tumorous tissue; n=24. (B) Western blot analysis was performed with representative patients (patient numbers; 6, 2, 5, 3 and 24). The intensities of C/EBPα (42 kD) and C/EBPß (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.

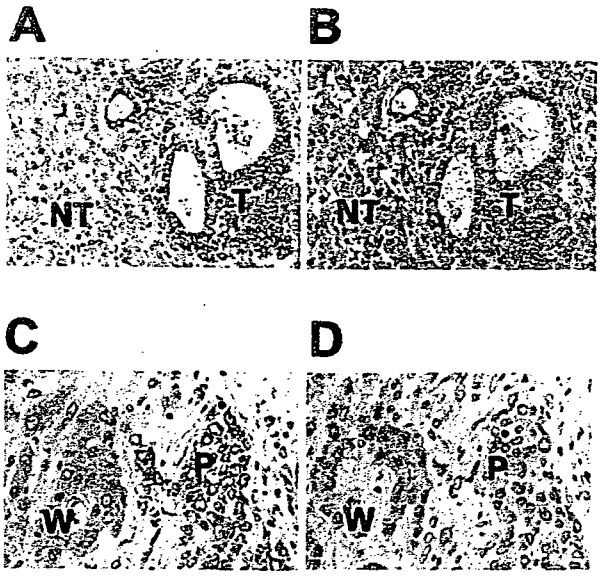

Figure 2. Immunohistochemistry of C/EBPα and C/EBPß in hepatoblastomas. Surgical specimens were immunohistochemically stained with anti-C/EBPα or -C/EBPß antibody. (A) C/EBPα was weakly positive in the cytoplasm and nuclei of normal hepatocytes in non-tumorous tissues (NT). C/EBPα was strongly positive in the cytoplasm and the nuclei of tumor cells (T). (B) C/EBPß was strongly positive in the cytoplasm and nuclei of hepatocytes (NT). C/EBPß 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/EBPß 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).

(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 TGCGTCTAAG-3' for 5', 5'-GAGGCAGGAAACCTCC AAAT-3' for 3', and 5'-GAGGCAGGAAACCTCCAAAT-3' for the detection probe; the primers for C/EBPß were 5'-AG CGCGGCGACGAGTACAAGATC-3' for 5', 5'-ACCTTGT GCTGCGTCTCCA-3' for 3', and 5'-CGCGAGCGCAACA ACATCGC-3' for the detection probe. Taq-Man ß-actin control reagents (Perkin Elmer Inc., Wellesley, MA) were used for the amplification of ß-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/EBPß (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/EBPß, 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/EBPß mRNA were measured in primary HBLs and their adjacent normal liver tissues by using quantitative real-time RT-PCR (Fig. 1A).

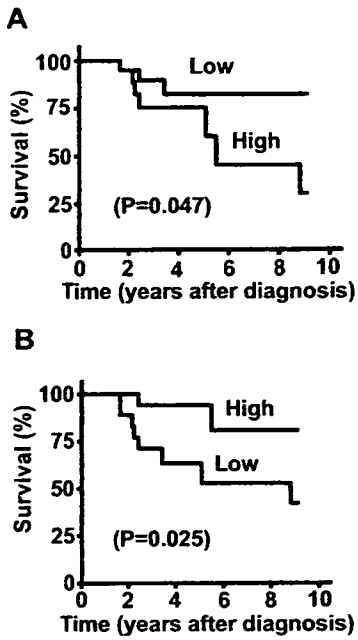

Figure 3. Kaplan-Meier survival curves for patients with hepatoblastoma. Survival of patients with hepatoblastoma after surgery was analyzed (Kaplan-Meier). Patients were divided into two groups for each factor. Patients with higher expression levels of C/EBPα than the median (A) (P=0.047), and lower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

Table I. Proportional Cox regression models using C/EBPα and C/EBPß 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/EBPß	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/EBPß	0.18 (0.04-0.86)	0.031
F	C/EBPα	5.66 (1.32-24.2)	0.019
	C/EBPß	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/EBPß	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/EBPß, high vs. low expression levels of C/EBPα and C/EBPß in tumor with real-time quantitative PCR; n=46.

The C/EBPα expression was significantly high in the tumors (mean ± SEM: 11.6±3.2, n=24) as compared with that in the normal livers (5.1±0.9, n=24; p=0.013). On the other hand, the expression of C/EBPß was significantly lower in HBL tissues (20.4±2.7, n=24) than that in the normal liver tissues (75.8±33, n=24; p=0.002). To confirm these results, we next measured expression levels of C/EBPα and C/EBPß 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/EBPß 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/EBPß 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/EBPß 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/EBPß mRNA were significantly correlated with favorable prognosis (92.6% 5-year survival rate, n=27, vs. 57.9%, n=19; p=0.025). Univariate Cox regression analysis of 46 patients with HBL showed that expression of C/EBPß (high vs. low expression; p=0.044), age (<1-year vs. ≥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/EBPß were significantly related to survival in a model jointly with each factor (P<0.05, model E). C/EBPα and C/EBPß 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/EBPß 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 ß-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 ß-*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 indentified Plk1 as a highly expressed gene in HBLs (11). However, expression of most of the genes was not predictive for prognosis.

The C/EBPα 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/EBPα may function as a tumor suppressor (13,14). In addition, our previous study suggested that both C/EBPα and C/EBPß were down-regulated in hepatocellular carcinomas as compared with the adjacent non-tumorous liver tissues (15). These suggest that C/EBPα may play a different role in HBLs from other cancers.

C/EBPß 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/EBPß in HepG2 cells, a human HCC cell line, suppressed proliferation of the cells (17). Therefore, C/EBPß might function as a tumor suppressor in HBL cells. In addition, C/EBPß 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 lever-specific genes.

Thus, both C/EBPα and C/EBPß 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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Oxidative stress induces p53-dependent apoptosis in hepatoblastoma cell through its nuclear translocation

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Hepatoblastoma (HBL) is the most common malignant liver tumor in children. Since tumor suppressor p53 is rarely mutated in HBL, it remains unknown whether p53 could contribute to the hepatocarcinogenesis. In the present study, we have found for the first time that, like neuroblastoma (NBL), wild-type p53 was abnormally accumulated in the cytoplasm of the human HBL-derived Huh6 cells. In accordance with this notion, immunohistochemical analysis demonstrated that p53 is largely expressed in cytoplasm of human primary HBLs. In response to the oxidative stress, Huh6 cells underwent apoptotic cell death in association with the nuclear translocation of p53 and the transactivation of its target gene implicated in apoptotic cell death. siRNA-mediated knockdown of the endogenous p53 conferred the resistance of Huh6 cells to oxidative stress. Intriguingly, histone deacetylase inhibitor (nicotinamide) treatment strongly inhibited the oxidative stress-induced nuclear translocation of p53 as well as the p53-dependent apoptosis in Huh6 cells. In contrast to the previous observations, the cytoplasmic anchor protein for p53 termed Parc had undetectable effect on the cytoplasmic retention of p53. Collectively, our present results suggest that the abnormal cytoplasmic localization of p53 might contribute at least in part to the development of HBL.

Introduction

Hepatoblastoma (HBL) is one of the most frequent malignant liver tumors of childhood. Indeed, its incidence is higher than that of hepatocellular carcinoma (HCC) in children. HBL arises from the hepatic precursor cells and displays a morphological similarity to the immature hepatocytes of the developing liver. In a sharp contrast to HCC, which is associated with hepatitis virus infection (Llovet *et al.* 2003), it has been shown that the incidence of HBL is highly elevated in patients with familial adenomatous polyposis (FAP), which carry germ-line mutations in the APC (adenomatous polyposis coli) tumor suppressor gene (Hughes & Michels 1992; Nagase & Nakamura 1993). APC protein forms a cytoplasmic multiprotein complex involved in the Wnt signaling pathway, which regulates the stability of β-catenin (Henderson & Fagotto 2002). Although APC is rarely mutated in sporadic HBL, accumulating evidence demonstrated that the frequent mutations or deletions of β-catenin at hot-spot regions within the exon 3 encoding its degradation targeting box are detectable in HBL, suggesting that the abnormal nuclear accumulation of the stabilized β-catenin which collaborates with Tcf/Lef complex plays a central role in the genesis of HBL (Koch *et al.* 1999). Consistent with this notion, Takayasu *et al.* (2001) revealed that β-catenin mutation is significantly correlated with the up-regulation of its target genes, including cyclin D1 and fibronectin. However, Harada *et al.* (2002, 2004) described that β-catenin mutation alone is not sufficient for the hepatocarcinogenesis, indicating that the additional mutations or epigenetic changes might be required for the genesis of HBL. The detailed molecular mechanism(s) behind the pathogenesis and development of HBL remains unknown.

The p53 tumor suppressor is a nuclear transcription factor, which has an ability to transactivate various p53-target genes implicated in the regulation of G1 cell cycle arrest and/or apoptosis such as $p21^{WAF1}$, MDM2, Bax and NOXA (Prives & Hall 1999; Sionov & Haupt 1999; Vousden & Lu 2002). The importance of p53 in the tumorigenesis has been emphasized by the observations

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