Table 2 Comparison of the known genes frequently appeared in hepatoblastomas with or without secretion of AFP and a non-tumorous infant's liver

Gene symbol	*Acc. no.*	*Gene name*	*No. of appearance of the genes*		
			HBL with positive AFP	*Normal infant's liver*	*HBL with negative AFP*
Total number of genes			2289	2768	2537
Protein synthesis, metabolism, transport					
ALB	NM_000477	Albumin	558	482	8
AFP	NM_001134	Alpha-feto protein	67	0	0
AGT	NM_000029	Angiotensinogen	43	16	0
EEF1A1	X03558	Eukaryotic translation elongation factor 1 alpha 1	35	20	87
RPL27A	NM_000990	60S ribosomal protein L27a	31	4	52
FTL	M11147	Ferritin	24	11	3
FGA	NM_021871	Fibrinogen, A alpha polypeptide	20	38	2
HP	K01763	Haptoglobin	19	6	1
ORM1	X02544	Orosomucoid-1	12	8	0
RPS27	NM_001030	Ribosomal protein S27	11	4	31
F2	J00307	Coagulation factor 2	11	26	0
TF	NM_001063	Transferrin	8	6	0
PAH	U49897	Phenylalanine hydroxylase	6	6	0
PLG	NM_000301	Plasminogen	5	8	0
SERPINA1	X01683	Serine proteinase inhibitor, clade A, member 1	5	6	0
GC	NM_000583	Group-specific component	4	21	1
RPS29	NM_001032	Ribosomal protein S29	3	1	0
CTSB	NM_147783	Cathepsin B	2	5	3
SERPING1	BC011171	Serine proteinase inhibitor, clade G, menber 1	2	33	0
CRP	X56692	C-reactive protein	1	8	0
ITIH2	NM_002216	Inter-alpha (globulin) inhibitor, H2 polypeptide	0	25	0
Growth factor					
MST1	M74178	Macrophage stimulating 1	8	16	0
Cell signaling					
WIF1	NM_007191	Wnt inhibitory factor 1	0	0	11
DKK1	NM_012242	Dickkopf	0	0	7
Cell structure, adhesion					
VTN	NM_000638	Vitronectin	7	30	0
ACTB	BC013380	Actin	6	17	6
LRG	AF403428	Leucine-rich alpha-2-glycoprotein	6	11	0
VIM	NM_003380	Vimentin	0	3	38
Cell cycle					
RBM4	NM_002896	RNA binding motif protein	2	0	21
RAP1B	NM_015646	RAP1B	0	0	11
Organism defense					
BF	L15702	B-factor, properdin	5	13	0
GPX1	NM_000581	Glutathione peroxidase	4	0	0
C1R	NM_001733	Complement component 1	1	21	1
Glycometabolism					
LDHA	NM_005566	Lactate dehydrogenase	19	28	7
ADH1B	AF153821	Alcohol dehydrogenase	15	29	1
CES1	L07764	Carboxylesterase	9	22	2
ALDH1A1	NM_000689	Aldehyde dehydrogenase	2	13	2
Lipid metabolism					
EPHX1	NM_000120	Epoxide hydrolase 1	7	12	0
APOA2	NM_001643	Apolipoprotein A-II	6	2	0
ADFP	BC005127	Adipose differentiation-related protein	5	14	1
Heat shock protein, metabolic enzyme					
UGT2B4	Y00317	UDP-glucuronosyltransferase	11	32	2
HSPA8	NM_006597	Heat shock 70 kDa protein	1	6	1
Unknown, others					
ATP5A1	NM_004046	ATP synthase	18	11	23
SEPP1	NM_005410	Selenoprotein P	7	10	2

Table 2 (*continued*)

Gene symbol	*Acc. no.*	*Gene name*	*No. of appearance of the genes*		
			HBL with positive AFP	*Normal infant's liver*	*HBL with negative AFP*
CYP3A4	M18907	P450	6	81	3
AHSG	M16961	Alpha-2-HS-glycoprotein	6	5	2
TPT1	X16064	Translationally controlled tumor protein	6	0	3
CYP2C9	M61855	P4502C9	1	10	1

other hand, genes involved in protein synthesis such as elongation factors and ribosomal proteins were observed more frequently in HBLs than in normal liver. The expression profile in the library of the tumor without AFP secretion was very different from that with positive AFP (HMFT *vs* HKMT). As expected, *AFP* gene did not appear in the HKMT library. Intriguingly, *Wnt Inhibitory factor-1* and *dickkopf*, both of which are inhibitors of Wnt signaling (Hsieh *et al*., 1999; Wang *et al*., 2000), frequently appeared in the HKMT library. In addition, *vimentin*, *RNA-binding motif protein*, and *RAP1B* also frequently appeared in the HKMT library, but hardly in the HMFT library with AFP secretion. Thus, HBL with positive AFP and that with negative AFP seem to have a distinct gene expression profile, resulting in different biological characteristics.

Identification of the differentially expressed genes between HBLs and normal livers

To identify differentially expressed genes between HBLs and their corresponding normal livers, 1188 independent genes which included all of the 847 genes with unknown function and 341 known genes that were related to cellular functions including cell growth and differentiation among the 10431 cDNAs were selected and subjected to semiquantitative RT–PCR analysis (Figure 1a). The complementary DNAs reverse-transcribed from total RNA obtained from eight tumors and their corresponding normal livers were used as PCR templates after normalization with *GAPDH* expression. As a result, we found that 75 genes were expressed at higher levels in normal livers than in HBLs, whereas only 11 genes were expressed at higher levels in the tumors than in normal livers. Figure 1a shows the representatives of the results of differential screening using semi-quantitative RT–PCR and Table 3 lists 46 differentially expressed genes with known functions. We classified those differentially expressed genes into 12 categories according to their known functions. The genes preferentially expressed in normal liver showed the profiles which reflected normal liver function. Consistent with the previous reports about HBL and hepatocellular carcinoma (von Horn *et al*., 2001; Xu *et al*., 2001; Kinoshita and Miyata, 2002), *Insulin-like growth factor binding protein-3* (*IGFBP-3*), *aldolase B*, *ceruloplasmin*, and *c-reactive protein* were downregulated in HBLs as compared with the normal livers. The expression of *IGF2*, whose product has mitogenic

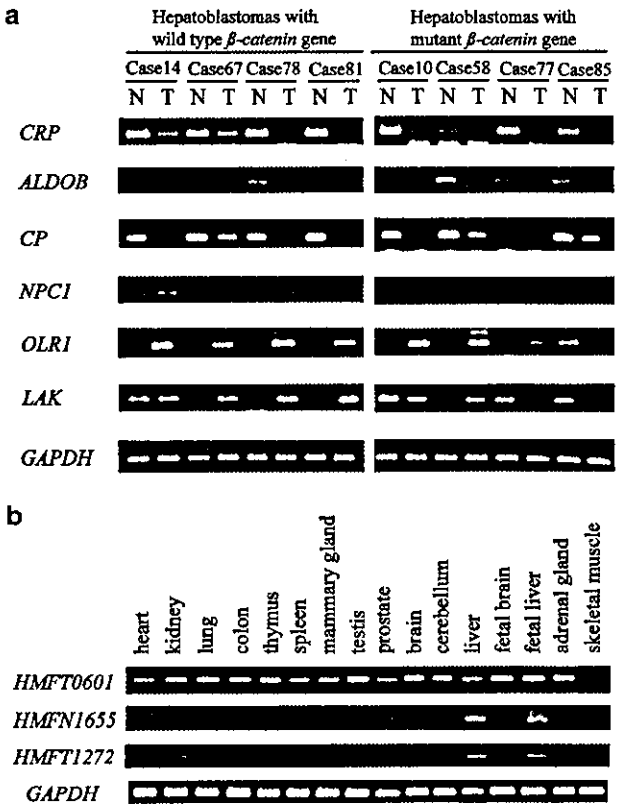

Figure 1 Expression of the representative genes by semi-quantitative RT–PCR. (**a**) Differentially expressed genes between HBLs with or without *β-catenin* mutation and the corresponding normal livers. cDNA was synthesized from RNAs prepared from eight pairs of tumors and their corresponding normal livers, and was used as a PCR template. Amount of cDNAs was normalized to that of *GAPDH*. Four tumors (cases 14, 67, 78, and 81) were with wild-type *β-catenin* gene, while the other four tumors (cases 10, 58, 77, and 85) were with mutant *β-catenin* gene. Gene symbols were shown on the left; *CRP*: C-reactive protein, *ALDOB*: aldolase, *CP*: ceruloplasmin, *NPC1*: Niemann–Pick disease, type C1, *OLR1*: oxidized low-density lipoprotein receptor 1, *LAK*: lymphocyte alpha-kinase. N: normal, T: tumor. (**b**) Semiquantitative RT–PCR of multiple human tissues. *HMFT0601* exhibited ubiquitous expression in all tissues examined, whereas *HMFN1655* and *HMFT1272* showed specific expression in liver and fetal liver

activity, is upregulated in HBLs, suggesting that the IGF axis may be involved in development of the tumor (Gray *et al*., 2000).

Four known genes which were expressed at high levels in HBLs (tumor > normal liver) include GTP-binding

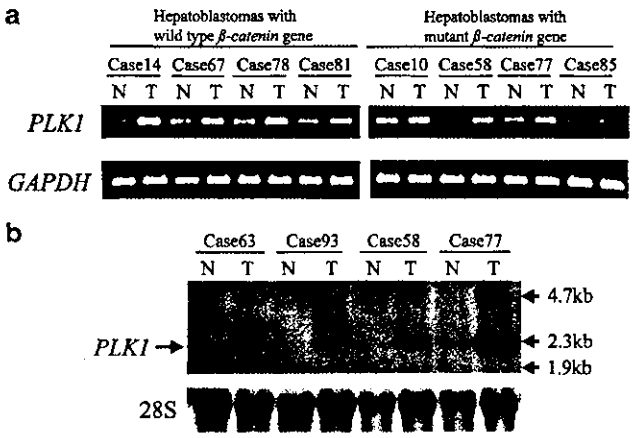

Figure 2 Increased expression of *PLK1* in HBLs. (**a**) Semiquantitative RT–PCR of *PLK1* gene in eight HBL cases. Preferential expression of the *PLK1* was seen in all sample pairs with and without *β-catenin* mutation. (**b**) Northern blot analysis of *PLK1* in primary HBLs, The 28S ribosomal band is shown as a control of each RNA amount

nuclear protein gene *RAN*, *PLK1* onocgene, and two cholesterol metabolism-associated protein genes, *low-density lipoprotein (LDL) receptor 1* and *Niemann-Pick disease type C1 (NPC1)*. The RAN protein is involved in the control of nucleo-cytoplasmic traffic of many nuclear proteins through formation of the transport nuclear pore complex (Ribbeck *et al.*, 1998). Nagata *et al.* (2003) also reported that *RAN* is upregulated in HBLs by oligonucleotide DNA array experiment. The LDL receptor 1 binds LDL, a major plasma cholesterol-carrying lipoprotein, and plays an important role in cholesterol homeostasis (Sudhof *et al.*, 1987; Goldstein and Brown, 1990; Hamanaka *et al.*, 1992). *NPC1* is a causal gene of Niemann–Pick type C disease which is an autosomal recessive lipid storage disorder that affects the viscera and central nervous system (Brady *et al.*, 1989). It encodes a protein with sequence similarity to the morphogen receptor 'patched', and to the cholesterol-sensing regions of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Loftus *et al.*, 1997) and is involved in the intracellular trafficking of cholesterol. Concerning the differentially expressed genes which contained unknown sequences, those cDNA sequences have been submitted to the public database (Genbank/DDBJ Accession numbers: AB07 3346-AB073347, AB073382-AB073387, AB073599-AB0 73614, and AB075869-AB075881). Interestingly, only one known gene, *lymphocyte alpha-kinase (LAK)*, showed distinct expression pattern between HBLs with mutant *β-catenin* and those with wild type *β-catenin* (Figure 1a).

We next examined expression pattern of the novel genes in human multiple tissues by semi-quantitative RT–PCR and found that at least five genes were specifically expressed in the liver (a part of the data is shown in Figure 1b). Since the oncogene *PLK1 (polo-like kinase-1)* was expressed in HBLs at significantly high levels as compared with the corresponding normal livers, we further examined the role of its expression in HBL.

PLK1 oncogene is overexpressed in HBLs

Recent studies have demonstrated that the preferential expression of *PLK1* mRNA is associated with some cancers including non-small-cell lung cancer (Wolf *et al.*, 1997), squamous cell carcinoma of the head and neck (Knecht *et al.*, 1999), and esophageal carcinoma (Tokumitsu *et al.*, 1999). However, the role of *PLK1* in HBL has never been reported. As indicated by semi-quantitative RT–PCR described above, we found that *PLK1* mRNA expression in HBLs is higher than in normal livers (Figure 2a). Northern blot analysis also confirmed its higher expression in HBLs (Figure 2b). We also performed Southern blot analysis by using the genomic DNAs obtained from primary HBLs and human placenta as a control, and probed with the *PLK1*-specific DNA fragment. However, we failed to find any clue of rearrangements or amplification of the *PLK1* gene locus (data not shown).

To examine the clinical significance of the expression level of *PLK1*, we performed quantitative real-time RT–PCR analysis using 74 primary hepatoblastomas and 29 corresponding normal liver samples (Figure 3a). The average arbitrary values of *PLK1* expression in HBLs and normal livers were 28.9 ± 6.7 and 4.1 ± 0.76, respectively (mean $\pm$ s.e.m., $P < 0.01$). The average values in alive and dead cases were 21.7 ± 5.2 ($n = 61$) and 62.4 ± 28.2 ($n = 13$), respectively ($p = 0.021$). When we compared the expression levels of *PLK1* between 24-paired HBLs and their corresponding normal livers, the former in HBL samples was significantly higher in comparison with the latter ($P < 0.01$) (Figure 3b). We also examined the relationship between the expression levels of *PLK1* and clinicopathological data of HBLs. Statistically significant correlation was observed only between histology and *PLK1* expression ($p = 0.041$). The expression level of *PLK1* in the tumors with poorly differentiated histology was higher than those with the well-differentiated one. The other clinicopathological factors such as age, clinical stage, and *β-catenin* mutation did not show a statistical significance with *PLK1* expression.

To further examine whether the *PLK1* expression was associated with the outcome of the patients with HBL, we performed a Kaplan–Meier analysis (Figure 4). The distinction between high and low levels of *PLK1* expression was based on the median value (low, *PLK1* < 13 d.u.; high, *PLK1* $\geq$ 13 d.u.). Since the overall survivals of 15 out of 74 cases were unknown, 59 cases were applied to the analysis. The 5-year survival rates of the groups with high and low *PLK1* expression were 55.9 and 87.0%, respectively ($P = 0.042$). The univariate analysis showed that both *PLK1* expression ($P = 0.015$) and histology ($P = 0.025$) have a significant prognostic importance (Table 4). The multivariate analysis demonstrated that *PLK1* expression was significantly related to survival, after controlling *β-catenin* mutation, age, stage,

Table 3 The known genes differentially expressed between hepatoblastomas and normal livers

	Gene symbol	*Acc. no*	*Gene name*
Protein synthesis, metabolism, transport			
T>N	RAN	NM_006325	GTP-binding nuclear protein RAN
N>T	LBP	AF105067	Lipopolysaccharide-binding protein
N>T	TDO2	BC005355	Tryptophan 2,3-dioxygenase
N>T	CRP	X56692	C-reactive protein
N>T	GC	NM_000583	Group-specific component
N>T	HP	K01763	Haptoglobin
N>T	HPX	NM_000613	Hemopexin
N>T	SQSTM1	NM_003900	Sequestosome 1
N>T	PHDGH	AF171237	A2-53-73 3-phosphoglycerate dehydrogenase
N>T	PPP1R3C	XM_005398	Protein phosphatase 1, regulatory (inhibitor) subunit 3C
N>T	ITIH4	D38595	Inter-alpha-trypsin inhibitor family heavy chain-related protein
N>T	G1P2	M13755	Interferon-induced 17-kDa/15-kDa protein
Cytokine, growth factor, hormones			
N>T	HABP2	D49742	Hyaluronan binding protein 2
N>T	IGFBP3	NM_000598	Insulin-like growth factor binding protein 3
N>T	GOT1	AF052153	Glutamic-oxaloacetic transaminase 1
Cell signaling			
N>T	CSNK2B	M30448	Casein kinase II, beta polypeptide
N>T	TPD52	NM_005079	Tumor protein D52
cell cycle			
T>N	PLK1	X73458	PLK1
Cell structure, adhesion			
N>T	LRG	AF403428	Leucine-rich alpha-2-glycoprotein
N>T	PGRP-L	AF384856	Peptidoglycan recognition protein L precursor
N>T	CLDN4	NM_001305	Claudin4
N>T	VTN	NM_000638	Vitronection
Organism defense			
N>T	RODH-4	NM_003708	Retinol dehydrogenase 4
N>T	MASP1	AF284421	Mannan-binding lectin serine protease 1
N>T	C4BPA	M31452	Complement component 4 binding protein, alpha
Glycometabolism			
N>T	ADH1B	AF153821	Alcohol dehydrogenase 1B, beta polypeptide
N>T	ALDOB	M15657	Aldolase B
Lipid metabolism			
T>N	NPC1	NM_000271	Niemann–Pick disease, type C1
T>N	OLR1	NM_002543	Oxidized low density lipoprotein (lectin-like) receptor 1
N>T	DGAT2	AF384161	Diacylglycerol acyltransferase
N>T	SCP2	NM_002979	Sterol carrier protein 2
N>T	APOA5	AF202890	Apolipoprotein A-V
N>T	AADAC	L32179	Arylacetamide deacetylase
N>T	SAA4	M81349	Amyloid A protein
Transcription			
N>T	BZW1	NM_014670	Basic leucine zipper and W2 domains 1
N>T	CREB-H	NM_032607	CREB/ATF family transcription factor
RNA biogenesis, metabolism			
N>T	HNRPDL	AB017018	Heterogeneous nuclear ribonucleoprotein D-like
Homeostasis, heat shock protein, metabolic enzymes			
N>T	UGT1A	AF297093	UGT1 gene locus
N>T	ALPL	X14174	Liver-type alkaline phosphatase
N>T	SLC10A1	L21893	Solute carrier family 10
N>T	CES1	AF177775	Carboxylestelase
N>T	AKR1D1	Z28339	Aldo-keto reductase family 1, member D1
N>T	AKR1C2	U05598	Aldo-keto reductase family 1, member C2
N>T	CP	D45045	Ceruloplasmin
Others			
N>T	DGCR6L	NM_033257	DiGeorge syndrome critical region gene 6 like
N>T	A1BG	AF414429	Alpha-1-B glycoprotein

T>N: highly expressed in the tumors as compared to normal livers. N>T: highly expressed in normal livers as compared to the tumors

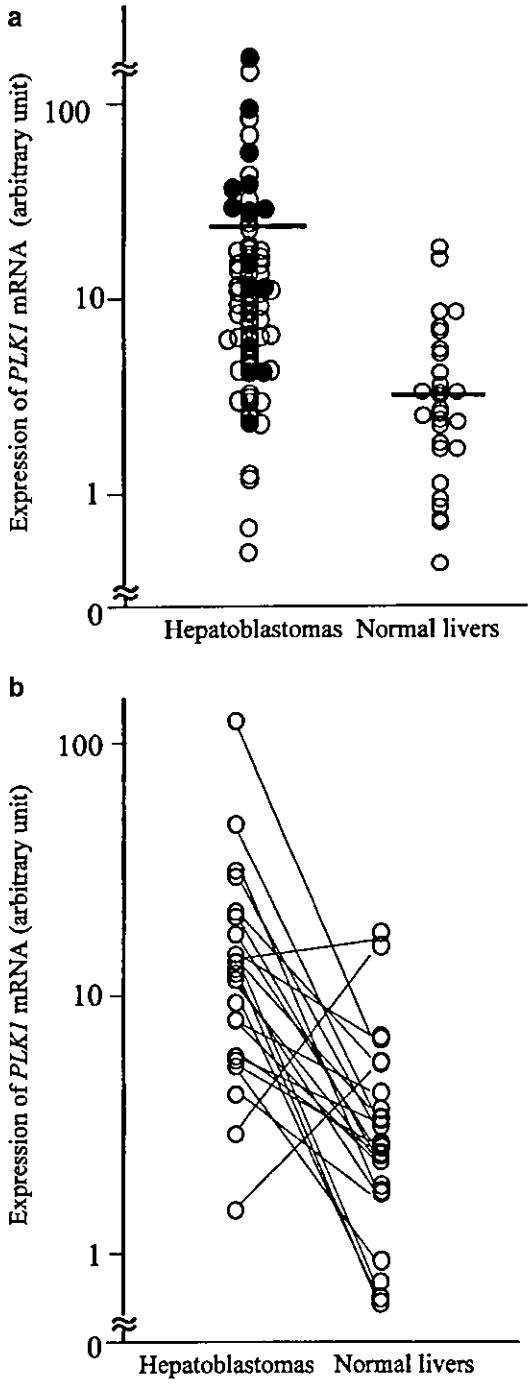

Figure 3 mRNA expression of *PLK1* in HBLs and the corresponding normal livers measured by quantitative real-time RT–PCR. (**a**) The levels of *PLK1* mRNA expression in HBLs and normal livers. The expression levels of *PLK1* were determined by quantitative real-time RT–PCR analysis using 74 HBL tissues and 29 normal livers (see Materials and methods). The *PLK1* expression values were normalized by *GAPDH*. Open and closed circles represent alive and dead, respectively. Since the values of the *PLK1* expression were skewed, a log transformation was used for the expression values. The bars show mean values. (**b**) Correlation of *PLK1* expression between HBL and its corresponding normal liver in 24 paired samples

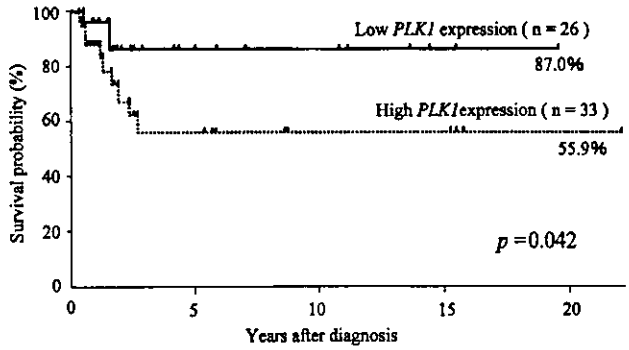

Figure 4 Kaplan–Meier survival curves ($n = 59$) in relation to the expression levels of *PLK1* (median cutoff). The arbitrary median cutoff value was set as 13. The patients with high expression of *PLK1* represented significantly poor prognosis than those with its low expression

Table 4 Univariate Cox regression analysis using *PLK1*(log) and dichotomous factors of *β-catenin* mutation, age, stage, and histology ($n = 59$)

Factor	n	P-*value*	*HR (95% CI)*
PLK1(log)	59	0.015	1.62 (1.10, 2.40)
β-catenin (mutant vs wild type)	58	0.27	1.85 (0.62, .5.56)
Age (>1 vs ≤1 year)	55	0.76	1.22 (0.33, 4.52)
Stage (3, 4 vs 1, 2)	56	0.083	3.81 (0.84, 17.2)
Histology (poorly vs well)	53	0.025	4.48 (1.21, 16.6)

All variables with two categories, except *PLK1*(log); HR = hazard ratio shows the relative of death of first category relative to second; CI = confidence interval

or histology, but marginally related to survival after controlling both histology and stage (Table 5).

Discussion

HBL is one of the embryonal tumors in close relation to the normal as well as abnormal tissue development. To understand the molecular basis of the genesis of HBL, here we randomly cloned a large number of genes expressed in HBLs with or without AFP production and in a non-tumorous infant's liver. Extensive screening for the differentially expressed genes between the tumors and their corresponding normal livers has successfully identified at least 86 genes including 40 with unknown function, which may potentially contribute to develop new therapeutic strategies against HBLs with poor prognosis.

HBL cDNA libraries

We have identified the genes with unknown function in approximately 8% of the total 10431 clones obtained from our oligo-capping cDNA libraries. The comparison of the frequently appeared genes in each libraries shows that expression profile is relatively similar between AFP-positive HBL and the normal part of the infant's liver, whereas it is very different between AFP-positive and AFP-negative tumors, in which many genes

Table 5 Multivariable Cox regression analysis using *PLK1*(log) and dichotomous factors of *β-catenin* mutation, age, stage, and histology ($n = 50$)

Variable	P-*value*	*Variable*	P-*value*	*Variable*	P-*value*
PLK1(log)	0.009	*β-catenin* (mutant vs. wild type)	0.51		
PLK1(log)	0.005	Age (>1 vs ≤1 year)	0.92		
PLK1(log)	0.019	Stage (3, 4 vs 1, 2)	0.46		
PLK1(log)	0.027	Histology (poorly vs well)	0.12		
PLK1(log)	0.052	Histology (poorly vs well)	0.12	Stage (3, 4 vs 1, 2)	0.47

All variables with two categories, except *PLK1*(log)

are downregulated (Table 2). In the library of the latter tumor, *vimentin*, *RNA-binding motif protein*, *Wnt inhibitory factor-1*, *dickkopf*, and *RAP1B* are frequently appeared, whereas they are hardly appeared in the other libraries. Wissmann *et al.* (2003) have recently reported that *WIF1* is downregulated in various cancers (prostate cancer, breast cancer, non-small-cell lung cancer, and bladder cancer), and suggested that loss of WIF1 expression may be an early event in tumorigenesis in those tissues. It is notable that, in contrast to AFP-positive HBLs, the patient's outcome of the tumor with negative AFP is very poor, though the incidence of the latter tumor is low (von Schweinitz *et al.*, 1995). This suggests that AFP-positive and AFP-negative HBLs have a different genetic as well as biological background. In addition, recent reports have demonstrated that frequent mutation of the *β-catenin* gene and nuclear accumulation of its protein product are one of the main causes of the tumorigenesis of HBL. The *APC* and *Axin* genes are also mutated in some HBLs (Oda *et al.*, 1996; Miao *et al.*, 2003; Thomas *et al.*, 2003), indicating that Wnt signaling pathway plays an important role in causing the tumors, most of which are AFP-positive. Therefore, the poor-prognostic HBL without producing AFP might be caused by the particular mechanism additional to or other than the abnormality of Wnt signaling pathway. Although the appearance frequency of the genes in each library does not always reflect the actual expression levels of each gene, it may at least in part show the differences among the tumor subsets with different genetic abnormalities. As our libraries contain many genes involved in liver development, normal liver functions, and carcinogenesis, they must be useful for making a liver-proper cDNA microarray to analyse expression profiles of HBL, viral infection-induced hepatitis, liver cirrhosis, and HCC.

Differentially expressed genes between HBLs and the corresponding normal livers

cDNA microarray, which is often applied to a comprehensive gene expression analysis, is able to detect many genes that are differentially expressed between tumors and normal tissues (Okabe *et al.*, 2001; Nagata *et al.*, 2003). However, it is expensive and needs further confirmation of the selected genes by a semi-quantitative RT–PCR or a real-time RT–PCR method. Therefore, using semi-quantitative RT–PCR and the specific primers of 1188 cDNAs, we have identified 86 genes differentially expressed between HBLs and their corresponding normal livers. Surprisingly, 75 out of 86 genes are preferentially expressed in the latter tissues, and only 11 including *RAN*, *PLK1*, *NPC1*, and *OLR1* known genes are expressed at high levels in HBLs. One of the reasons of this result may be that many gene products, which are necessary for full function in the matured liver metabolism, are dispensable for the malignant growth of the tumor except for the very limited genes. The results of some differentially expressed genes are consistent with those in the previous reports. von Horn *et al.* (2001) have shown that the mRNA levels of *insulin-like growth factor-binding proteins* including *IGFBP-3* are decreased in HBLs than in normal livers. Kinoshita and Miyata (2002) have also reported that *aldolase B* mRNA is downregulated in over 50% of 20 HCCs examined. They proposed that the measurement of aldolase activity in serum is useful to determine the number of collapsed hepatic cells in cirrhosis. Recently, evidences suggest that not only mutant β-catenin but also wild-type β-catenin localize in the cellular nuclei of HBL as well as some other cancers (Rimm *et al.*, 1999; Takayasu *et al.*, 2001). The increased expression of the *Ran* gene in HBLs might be correlated with the shuttling of β-catenin and/or other related proteins between cytoplasm and nucleus in the tumor cells.

Owing to constitutive activation of Wnt signaling in most of the HBLs, the 86 genes differentially expressed between the tumor and its corresponding normal liver were expected to contain downstream target genes of Wnt signaling pathway that might regulate early stage of the hepatic development. In this study, however, only the *lymphocyte alpha-kinase* (*LAK*) gene was found to be differentially expressed at high levels in HBLs with wild-type *β-catenin* and at low levels in those with *β-catenin* mutation. LAK is a new class of protein kinases with a novel catalytic domain, but its precise function is currently unknown (Ryazanov *et al.*, 1999). Thus, our result may suggest that the target genes of the Wnt signaling pathway are commonly affected in HBLs, regardless of the presence or absence of *β-catenin* mutation.

PLK1 as a prognostic indicator of HBL

PLK1 (*polo-like kinase 1*), the human counterpart of *polo* in *Drosophila melanogaster* and of *CDC5* in *Saccharomyces cerevisiae*, encodes a serine/threonine kinase with polo-box domains (Clay *et al.*, 1993). PLK1 is crucial for various events of mitotic progression including centrosome maturation (Lane and Nigg,

1996), spindle function (Glover *et al.*, 1996), activation of cyclin B/Cdc2 (Qian *et al.*, 1998; Toyoshima-Morimoto *et al.*, 2001), and regulation of anaphase-promoting complex (Kotani *et al.*, 1998; Nigg, 1998). Elevated expression of *PLK1* is also found in different types of adult cancers including non-small-cell lung cancer, head and neck tumors, esophageal carcinomas, melanomas, and colorectal cancers (Wolf *et al.*, 1997; Knecht *et al.*, 1999; Tokumitsu *et al.*, 1999; Dietzmann *et al.*, 2001; Takai *et al.*, 2001), implying its critical role in tumorigenesis. In the present study, we have found that *PLK1* is upregulated in primary HBLs, and that its mRNA expression levels are significantly correlated with poor outcome of the patients. Multivariate Cox regression analysis indicated that *PLK1* expression could be an independent prognostic factor from *β-catenin* mutation, age, stage, or histology. However, clinical stage did not show a significant correlation with *PLK1* expression, though it is one of the critical prognostic markers. One of the possible reasons may be that the 59 tumors we used for statistical analysis include two unusual patients, one had stage 4 tumor with good prognosis and another case had stage 1 tumor with poor prognosis. These might have reduced the significance of the tumor stage in patients' survival in our sample set.

It is notable that, among the 1188 genes we have screened for differential expression, *PLK1* is the only one known oncogene overexpressed in the HBL tissues. Smith *et al.* (1997) have reported that constitutive expression of *PLK1* in NIH3T3 cells causes oncogenic focus formation and forms tumors in nude mice. Furthermore, Liu and Erikson (2003) have recently shown that the application of small interfering RNA which specifically depletes *PLK1* expression in cancer cells inhibits cell proliferation, arrests cell cycle, and induces apoptosis. Thus, PLK1 may play a crucial role in causing HBL and other cancers. It may be interesting to examine whether *PLK1* is a target of β-catenin transported from the cytosol into the nucleus. The disruption of PLK1 function could be a future therapeutic tool for the aggressive type of hepatoblastomas.

In conclusion, our HBL cDNA project has provided a large number of genes related to liver development, metabolism, and carcinogenesis. We are currently applying these genes to the cDNA microarray system. Our cDNA resource should be an important tool to understand the molecular mechanism of the genesis of HBL as well as to develop new diagnostic and therapeutic strategies against the aggressive tumors in the future.

Materials and methods

Clinical materials

Tumor tissues and their corresponding normal liver tissues were frozen at the time of surgery and stored at −80°C until use. All specimens were provided from the Tissue Bank of the Japanese Study Group for Pediatric Liver Tumor (JPLT) (Uotani *et al.*, 1998). A total of 74 HBL samples (seven were classified as being stage 1, 17 as stage 2, 26 as stage 3, 15 as stage 4, and nine were unknown stages) were used in this study. The tumors were staged according to the Japanese histopathological classification of HBL (Hata, 1990). From 1991 to 1999, HBLs had been treated by combination chemotherapy using cisplatin and THP-adriamycin according to the JPLT-1 protocol (Sasaki *et al.*, 2002). After 2000, a more intensive chemotherapeutic regimen, ITEC (ifosfamide, THP-adriamycin, etoposide, and carboplatin), has been utilized for tumors that prove resistant to the combination chemotherapy in the JPLT-2 study. Among the 74 tumor samples we examined, 36 and 35 tumor tissues were obtained prior to and after chemotherapy, respectively, and the remaining three were unknown. In the same sample set, 59 tumors were accompanied by outcome information and used for making survival curves, among which 31 and 28 tissues were obtained prior to and after chemotherapy, respectively. Tumor histology was also classified according to Hata (1990). 'Poor histology' indicates 'poorly differentiated (embryonal type)', and 'well histology' indicates 'well-differentiated (fetal type)'. The informed consents were obtained in each institution or hospital. High molecular weight DNA and total RNA of these samples were prepared as described previously (Ichimiya *et al.*, 1999).

Construction of oligo-capping cDNA libraries

Four oligo-capping cDNA libraries, two (HMFT, HYST) derived from HBLs with secretion of AFP, one (HKMT) from HBL without AFP secretion, and one (HMFN) from the corresponding normal liver, were constructed according to the method previously described (Suzuki *et al.*, 1997). These were approved by the institutional review board. The oligo-capping method enables full-length cDNA cloning with high efficiency. The 12 000 cDNA clones in total were randomly picked up and single-run sequencing was performed. Nucleotide sequence of both ends for each cDNA clone was homology-searched against the public nucleotide database using the BLAST program at the National Center for Biotechnology Information (NCBI) (Genbank release 122, January 2001).

Differential screening of the genes by semi-quantitative RT–PCR

The eight samples were selected as PCR templates to screen for the differentially expressed genes. Cases 58 and 81 were defined as stage 2 HBL, cases 10, 67, 78, and 85 were in stage 3, case 14 was in stage 4. Among those eight tumors, four (cases 14, 67, 78, and 81) had the mutant *β-catenin*, and the others (cases 10, 58, 77, and 85) not. Mutation analysis for *β-catenin* was performed as described previously (Takayasu *et al.*, 2001). The differential expression of the genes between the HBL and normal livers was confirmed at least twice using semi-quantitative RT–PCR. The individual gene-specific PCR primer sequences were determined by using Primer3 program (provided at Washington University). For cDNA templates, 5 μg of total RNA was converted to cDNA using random primers (Takara, Otsu, Japan) with SuperScript II RNaseH⁻ reverse transcriptase (Gibco BRL, Rockville, MD, USA). Those cDNAs were at first amplified with *GAPDH* primers for 27 cycles and the amounts of the PCR products were measured by ALF Express™ sequencer and normalized. The amplification was performed 35 or 40 cycles of 95°C for 30 s, 57 or 59 or 61°C for 15 s and 72°C for 60 s, and the final extension was at 72°C for 5 min, using a Perkin-Elmer Thermalcycler 9700 (Perkin-Elmer, Foster City, CA, USA). The PCR products

were run on 2% agarose gels and stained with ethidium bromide. We defined the gene as differentially expressed when it exhibits differential expression between the tumor and its corresponding normal liver in more than four out of the eight samples.

Northern blot analysis

In all, 25 μg of total RNA from the primary HBLs, HCC, and their corresponding normal livers were subjected to Northern analysis. Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Total RNA was fractionated by electrophoresis on 1% agarose gel containing formaldehyde, transferred onto a nylon membrane filter, and immobilized by UV crosslinking. The hybridization cDNA probe was a 976-base pair human *PLK1* cDNA fragment and labeled with [α-^{32}P]-dCTP using the BcaBEST random priming kit (Takara Biomedicals). The filter was hybridized at 65°C in a solution containing 1 M NaCl, 1% SDS, 7.5% dextran sulfate, 100 μg/ml of heat-denatured salmon sperm DNA, and radiolabeled probe DNA.

Quantitative real-time RT–PCR of PLK1

The primer set for amplification of the *PLK1* and probe sequence are as follows: forward primer, 5′-GCTGCACAAG AGGAGGAAA-3′; reverse primer, 5′-AGCTTGAGGTCTC-GATGAATAAC-3′; probe, 5′-CCTGACTGAGCCTGAGG CCCGATAC-TA-3′. Taqman *GAPDH* control reagents (Perkin-Elmer/Applied Biosystems) were used for the amplification of *GAPDH* as recommended by the manufacturer. PCR was performed using ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems). In all, 2 μl of cDNA was amplified in a final volume of 25 μl containing 1 × Taqman PCR reaction buffer, 200 μM each dNTP, 0.9 μM each primer, and 200 nM Taqman probe. The optional thermal cycling condition was as follows: 40 cycles of a two-step PCR (95°C for 15 s, 60°C for 60 s) after the initial denaturation (95°C for 10 min). Experiments were carried out in triplicate for each data point.

Statistical analysis

Statistical analyses were performed using Mann–Whitney's *U*-test and Cox regression. A *P*-value of less than 0.05 was considered significant.

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Identification of novel human neuronal leucine-rich repeat (h*NLRR*) family genes and inverse association of expression of *Nbla10449*/h*NLRR-1* and *Nbla10677*/h*NLRR-3* with the prognosis of primary neuroblastomas

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Abstract. To search for novel prognostic indicators, we previously cloned >2,000 novel genes from primary neuroblastoma (NBL) cDNA libraries and screened for differential expression between the subsets with favorable (stage 1 or 2 with a single copy of *MYCN*) and unfavorable (stage 3 or 4 with amplification of *MYCN*) prognosis. From them, we have identified 3 genes of human neuronal leucine-rich repeat protein (NLRR) family: *Nbla10449*/h*NLRR-1*, *Nbla00061*/h*NLRR-2*/*GAC1* and *Nbla10677*/h*NLRR-3*. An additional family member, h*NLRR-5*, was also found by homology search against public database. NLRR family proteins have been proposed to function as a neuronal adhesion molecule or soluble ligand binding receptor like *Drosophila toll* and *slit* with multiple domains including 11 sets of extracellular leucine-rich repeat (LRR)-motifs. However, the functional role of the NLRR protein family has been elusive. Our present study shows that h*NLRR* mRNAs are preferentially expressed in nervous system and/or adrenal gland. In cancer cell lines, h*NLRR-1*, h*NLRR-3* and h*NLRR-5* are expressed at high levels in the neural crest-derived cells. Most remarkably, in primary NBLs, h*NLRR-1* is significantly expressed at high levels in unfavorable subsets as compared to favorable ones, whereas the expression pattern of h*NLRR-3* and h*NLRR-5* is the opposite. In order to understand the function of these receptors, we have used newborn mouse superior cervical ganglion (SCG) cells which are dependent on nerve growth factor (NGF) for their survival. Expression of the mouse counterparts of h*NLRR-2* and h*NLRR-3* is up-regulated after NGF-induced differentiation and down-regulated after NGF depletion-induced apoptosis. On the other hand, expression of h*NLRR-1* and h*NLRR-5* is inversely regulated in the same system. These results have suggested that the regulation of the h*NLRR* family genes may be associated with NGF signaling pathway in both SCG cells and neuroblastoma. Our quantitative real-time RT-PCR analysis using 99 primary NBLs has revealed that high levels of h*NLRR-1* expression are significantly associated with older age (>1 year, p=0.0001), advanced stages (p=0.0007), low expression of *TrkA* (p=0.011), and *MYCN* amplification (p=0.0001), while those of h*NLRR-3* expression are significantly correlated with the favorable prognostic indicators. Furthermore, multivariate analysis reveals that expression of h*NLRR-1* is an independent prognostic indicator in human neuroblastoma. Thus, our results demonstrate that, despite being members of the same family, h*NLRR-1* and h*NLRR-3* may share different biological function among the NBL subsets, and that their expression level becomes novel prognostic indicators of NBL.

Introduction

Neuroblastoma (NBL) is one of the most common pediatric tumors originating from sympathoadrenal lineage of the neural crest. NBL shows variable biological behavior which characterizes different clinical subsets (1). The tumors found in young children, <1 year of age usually regress spontaneously, while those in the older children are often aggressive leading to poor outcome. Recent advances in molecular biology have identified the important molecules involved in the regulation of growth, differentiation and programmed cell death during development of the sympathoadrenal cells (2), some of which link to the modulation of NBL biology. These include Trk family tyrosine kinase receptors and *MYCN* proto-oncogene. TrkA, a high-affinity receptor for nerve growth factor (NGF), is expressed in favorable subsets of NBL and regulates differentiation and/or regression of the tumor cells (3). On the other hand, TrkB, a receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4), is expressed in NBLs with unfavorable prognosis. An autocrine loop of BDNF/NT-4 and TrkB may promote tumor cell survival and increase their invasiveness (4). Amplification of *MYCN* is significantly associated with allelic loss of the

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distal region of chromosome 1, and both are indicators of poor prognosis. A recent report suggests that *MYCN* oncoprotein induces expression of *Id-2* with a helix-loop-helix domain and in turn negatively regulates Rb tumor suppressor in NBL (5). However, many important genes may still be missing for better understanding of NBL biology as well as predicting the prognosis. In order to identify novel NBL-related genes and promote better understanding of the molecular mechanism of NBL genesis and its biology, differential screening method has been applied (6).

We have previously constructed full-length-enriched oligocapping cDNA libraries from different subsets of primary NBL (6,7). One derived from the mixture of favorable NBLs in stage 1 with single-copy of *MYCN*, and the other from unfavorable NBLs in stage 3 and 4 with *MYCN* amplification. We have finished end-sequencing of 2,500 clones obtained from each library, and found that the expression profile is markedly different between the subsets. So far, 1,800 independent genes from these libraries have been subjected to semi-quantitative RT-PCR using 16 favorable and 16 unfavorable NBLs to find the genes differentially expressed between favorable (F) and unfavorable (UF) subsets (8,9).

In this study, we have identified novel human *NLRR* family genes that are differentially expressed among the NBL subsets. NLRRs are proteins with leucine-rich repeat (LRR) domains which may be involved in protein-protein interactions (10). They may also function as cell-adhesion molecules or signaling receptors implicated in regulation of the neural development. Expression of the h*NLRR-1/Nbla10449* gene is significantly associated with short survival as well as conventional poor-prognostic factors, whereas that of the h*NLRR-3/Nbla10677* gene is increased in favorable subset of NBL. Our results suggest that the differential expression of h*NLRR* genes among the NBL subsets is involved in the regulation of growth, differentiation and cell death of human NBL.

Materials and methods

Patients. We studied tumors from 99 children with NBL diagnosed between 1995-1999. Fifty-four Japanese patients were identified by a mass screening program started in 1985 (9,10). The selection of tumors for this study was solely based on the availability of a sufficient amount of tumor tissue, from which DNA and mRNA could be prepared for the analyses described below.

The diagnosis of NBL was confirmed by histologic assessment of the tumor specimen obtained at surgery according to the classification of Shimada *et al* (11). There were 57 tumors with favorable histology, and 42 with unfavorable histology. The tumors were staged according to the International Neuroblastoma Staging System (INSS) (12). Thirty-eight tumors (36 identified by mass screening) were stage 1, 14 (11 identified by mass screening) stage 2, 5 (3 identified by mass screening) stage 4s, 10 (3 identified by mass screening) stage 3, and 32 (1 identified by mass screening) stage 4. The patients were treated according to the protocols previously described (13).

Tumor samples and cell lines. Fresh, frozen tumorous tissues were sent to the Division of Biochemistry, Chiba Cancer Center Research Institute, from various hospitals in Japan with informed consent from the patient's parents. All samples were obtained by surgery (or biopsy) and stored at -80°C. Studies were approved by the Institutional Review Board of the Chiba Cancer Center. Human cell lines which we used included NBL (CHP134, CHP901, GANB, GOTO, IMR32, SMS-KAN, SMS-KCN, KP-N-NS, LAN-5, NB-1, NB-9, NBKM-1, NB (Tu)-1, NLF, NMB, RTBM1, SMS-SAN, SK-N-BE, SK-N-DZ, TNB, TGW, LHN, NGP, NB69, NBL-S, OAN, SK-N-AS, SK-N-SH, SH-SY5Y, and CNB-RT), osteosarcoma (OST, Saos2, and NOS1), rhabdomyosarcoma (RMS-MK and ASPS-KY), colorectal adenocarcinoma (COLO320, SW480, and LOVO), a hepatocellular cancer (HepG2), breast cancer (MOA-MB-453 and MB231), melanoma (G361, G32TG, A875), a thyroid cancer (TTC11), a gastric cancer (KATO3), esophageal cancer (ECGI10), a pancreatic cancer (ASPC1) and a lung cancer cell lines (A549). The cells were cultured in the RPMI-1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) with 10% fetal bovine serum and 50 μg/ml penicillin/streptomycin at humidified 5% CO_2/95% air at 37°C.

Primary culture of newborn mouse superior cervical ganglion cells. The SCG neurons were isolated from newborn mice, and treated with 50 ng/ml of NGF for 5 days, as previously reported (14). RNAs were isolated 12, 24, and 48 h after depleting NGF and adding anti-NGF antibody (1% v/v).

Northern blot analysis. Multiple Tissue Northern blot purchased from Clontech (Palo Alto, CA, USA) was used for Northern analysis with cDNA fragments labeled with α-[^{32}P]dCTP as probes. Hybridization was performed in the ExpressHyb hybridization buffer (Clontech) at 68°C for 1 h. Membrane was washed twice in 2X SSC/0.05% SDS at room temperature for 30 min, twice in 0.1X SSC/0.1% SDS at 50°C for 40 min. After washing, the filter was autoradiographed with X-ray film. The membrane was boiled in 0.1% SDS for 10 min for reprobing, and rehybridized with ß-actin as a control.

Semi-quantitative RT-PCR. cDNA was synthesized from 5 μg of total RNA in a 20 μl reaction mixture containing 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) and pd$(N)_6$ random hexamer (Takara Shuzo Co., Ltd., Ohtsu, Japan). The resulting cDNA fragments were diluted to be a 1:10 solution for PCR templates. The following pairs of forward and reverse primer sets were prepared for amplification: *NLRR-1*, 5'-GTCGATGTCCATGAATACAAC CT-3' and 5'-CAAGGCTAATGACGGCAAAC-3'; *NLRR-2*, 5'-TGACCTATTCCTGACGG-3' and 5'-AAATCACAGTCT CGGGC-3'; *NLRR-3*, 5'-ACTCTTGCCTAATACCCTGAC-3' and 5'-AGATGGTATTCGAGCACTTTG-3'; *GAPDH*, 5'-CTGCACCAACAATATCCC-3' and 5'-GTAGAGACA GGGTTTCAC-3'. All PCR amplifications were performed with a Perkin-Elmer Corp. GeneAmp PCR 9700, using rTaq polymerase (Takara Shuzo Co., Ltd.) with 35 cycles of sequential denaturation (95°C for 15 sec) and annealing-extension (58°C for 15 sec and 72°C for 1 min). *GAPDH* was used as a control and amplified under the same condition except for reduced amplification cycles to 28. PCR templates

were standardized by its *GAPDH* expression before performing semi-quantitative PCR. The products were electrophoresed on 2.0% agarose gels and stained with ethdium bromide for visualization.

Quantitative real-time RT-PCR. cDNA was prepared by the same method as in the semi-quantitative RT-PCR and 2 µl of the 40-fold dilution was used for each PCR reaction. Primers and TaqMan probes for *Nbla10449* and *Nbla10677* were designed using the primer design software Primer Express™ (Perkin-Elmer Applied Biosystems). TaqMan GAPDH control reagent kit (Perkin-Elmer Applied Biosystems) was used for *GAPDH* expression as a control. Reaction mixture (25 µl), containing 2 µl of cDNA, 1X TaqMan mixture, 0.3 µM forward and reverse primers, and 0.2 µM TaqMan probe were used for PCR. The condition of PCR was as follows: 2 min at 50°C (stage 1), 10 min at 95°C (stage 2), and then 50 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (stage 3).

Statistical analysis. The student's t-tests were used to explore possible associations between *Nbla10449/hNLRR-1* expression and other factors, such as age. Since the values of the *Nbla10449/hNLRR-1* and *Nbla10677/hNLRR-3* expression were skewed, a log transformation was used to achieve the normality when using t-test and Cox regression. The distinction between high and low levels of *Nbla10449* was based on the median value (low, *Nbla10449* <0.31 d.u.; high, Nbla10449 >0.31 d.u.), regardless of tumor stage, *MYCN* copy number, or survival. The distinction between high and low levels of *Nbla10677* was based on the median value (low, *Nbla10677* <1.04 d.u.; high, *Nbla10677*>1.04 d.u.), regardless of tumor stage, *MYCN* copy number, or survival. Kaplan-Meier survival curves were calculated, and survival distributions were compared using the log-rank test. Cox regression models were used to explore associations between *Nbla10449/Nbla10677*, age, *MYCN* copy number, mass screening, tumor origin and survival. Statistical significance was declared if the p value was <0.05. Statistical analysis was performed using Stata 6.0. (Stata Statistical Software: Release 6.0 College Station, Stata Corporation, TX, 1999).

Results

Identification of novel human homologues of NLRR family genes, Nbla10449/hNLRR-1 and Nbla10677/hNLRR-3, and their differential expression between favorable and unfavorable subsets of neuroblastoma. To identify the genes differentially expressed between favorable and unfavorable NBLs, semi-quantitative RT-PCR analyses were performed. Sixteen favorable (F) and 16 unfavorable (UF) NBLs were used as PCR templates after normalization by GAPDH expression. So far, ~1,800 independent genes from the NBL cDNA libraries have been surveyed, resulting in the approximately 300 genes with differential expression between the subsets (8,9). Among them, we found *Nbla10449* and *Nbla10677* genes that are highly homologous to the mouse *NLRR-1* and *NLRR-3* genes, respectively. *Nbla10449/hNLRR-1* was preferentially expressed in UF NBLs, whereas *Nbla10677/hNLRR-3* was highly expressed in F NBLs (Fig. 3A).

Full-length cDNA cloning and structure of human NLRR-1, NLRR-2, NLRR-3 and NLRR-5 genes. We performed sequencing of whole inserts of *Nbla10449* and *Nbla10677* and defined their full-length cDNA sequences. In addition, during the process, we also identified human *NLRR-5* by homology search on the database. Furthermore, the other clone, *Nbla00061*, was found to be the same gene as *GAC1* which we renamed as h*NLRR-2*. *NLRR-4* has recently been reported by another group (15).

Nbla10449/hNLRR-1. A full-length *Nbla10449* genes comprised 3,060 bp, with an open reading frame (ORF) of 2,151 bp. The deduced protein was 716 a.a. in length, and included 2 hydrophobic stretches corresponding to a signal peptide at the extreme N-terminal region and a deduced transmembrane domain close to the C-terminal region (Fig. 1A). Analysis of the extracellular domain revealed the presence of 11 leucine-rich repeats encompassed by flanking cystein cluster, a leucine-rich repeat N-terminal domain (LRRNT) and a leucine-rich repeat C-terminal domain (LRRCT), a single immunogloblin C2 type domain, and a fibronectin type III domain (Fig. 1). Homology search against public database showed that *Nbla10449* was identical to the human *EST KIAA1497* (GenBank/DDBJ accession number AB040930) which lacked the N-terminal region and was similar to 2 leucine-rich repeat proteins, m*NLRR-1* (acc. no. D45913) and *Xenopus* x*NLRR-1* (acc. no. AB014462). The identities of deduced Nbla10449 protein to mNLRR-1 and xNLRR-1 were 92 and 75%, respectively. We also analyzed genomic structure of *Nbla10449*, and found that this gene comprised of single exon without any intron and mapped to chromosome 3p region.

Nbla10677/hNLRR-3. Nbla10677 comprised 2,471 bp with an ORF of 2,127bp (acc. no. AB060967) without intron, and mapped to chromosome 7q31. The deduced protein contained 708 a.a. and had a similar structure to Nbla10449/hNLRR-1 (Fig. 1A). In addition, the RGD sequence, an integrin-binding domain, was found in the leucine-rich repeats. Homology search showed that *Nbla10677* was identical to human cDNA FLJ11129 (acc. no. AK001991) and highly similar to the leucine-rich repeat proteins of mouse (mNLRR-3; acc. no. D49802) and rat (rNLRR-3; acc. no. AF291437). Therefore, Nbla10677 seemed to be a human NLRR-3. The Nbla10677/hNLRR-3 showed 85 and 83% similarity to mNLRR-3 and rNLRR-3 proteins, respectively.

Nbla00061/GAC1/hNLRR-2. The *Nbla00061* cDNA clone comprised 3,206 bp including a partial ORF of 2,142 bp. Sequence analysis revealed that it is identical to a *glioma amplified on chromosome 1* gene, *GAC1* (acc. no. AF030435), mapped to chromosome 1q32.1. The GAC1 protein, which was previously reported to be a member of an NLRR protein family (15), had 713 a.a. with a similar structure to NLRR-1 and 3 (Fig. 1). GAC1 showed 98% identity to mNLRR-2, although the latter was reported as only a partial sequence (16). It showed only 54 and 50% identities to mNLRR-1 and mNLRR-3, respectively, indicating that Nbla00061/GAC1 is a human counterpart of mNLRR-2.

m*NLRR-4* was cloned by another group from hemangioblast-like cell line derived from E11.5 mouse AGM and its predicted protein has 4 LRRs, fibronectin 3 and EGF-like motives in the extracellular region (Rump A *et al*, The Molecular Biology Society of Japan Conference, Yokohama,

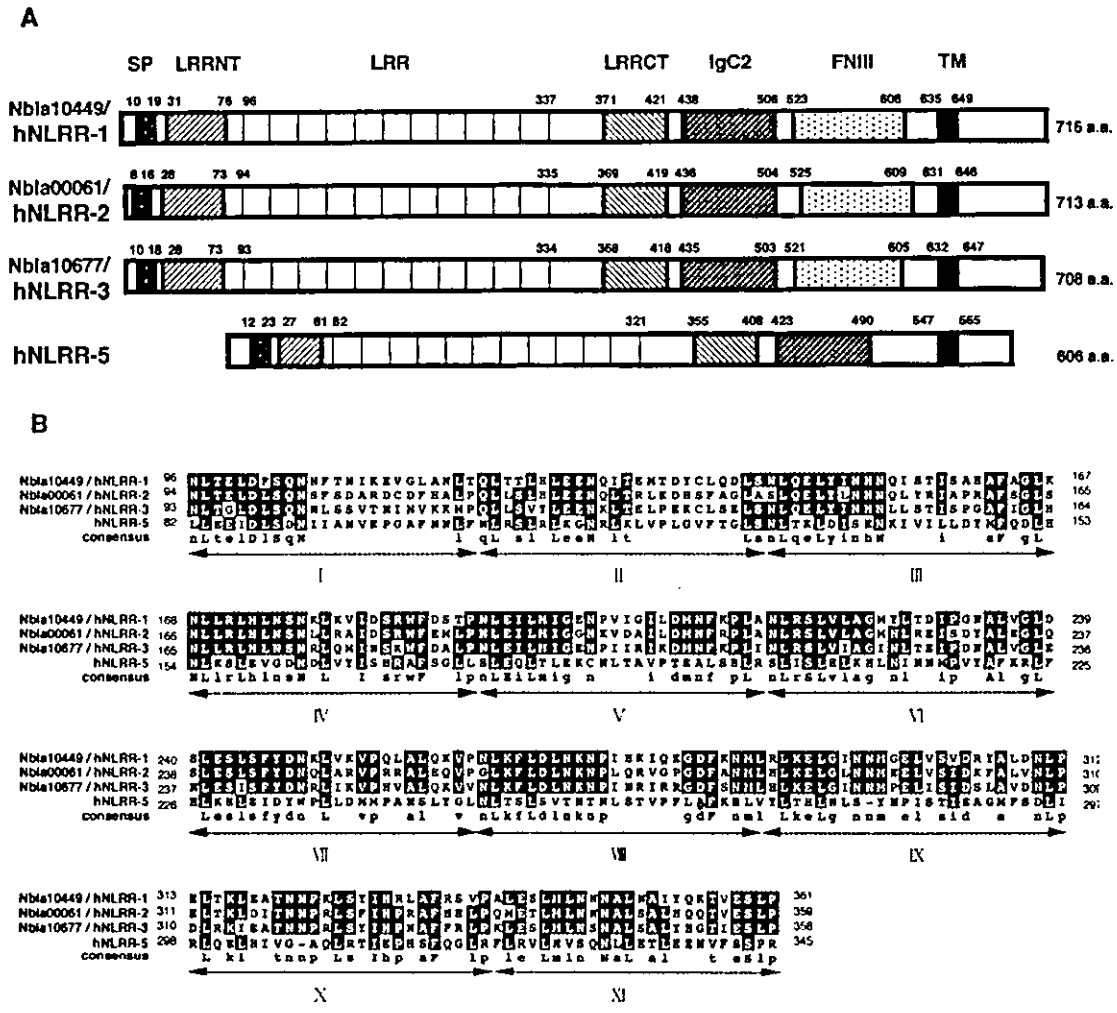

Figure 1. Structures and deduced amino acid sequences of hNLRR families. A, Schematic representation of hNLRR-1, hNLRR-2, hNLRR-3 and hNLRR-5 whose proteins consist of 716, 713, 708 and 606 a.a., respectively. SP, predicted signal peptide; TM, predicted transmembrane region; LRRNT, leucine-rich repeat N-terminal domain; LRRCT, leucine-rich repeat C-terminal domain; LRR, leucine-rich repeat; Igc2, immunoglobulin C-2 type domain; FN III, fibronectin type III domain. B, Amino acid alignment of the LRR domains of hNLRR families. Eleven repeats of LRR motif are shown by Roman numerals. Consensus sequences are highlighted and shown below.

abs. 4P-500 and 4P-501, 2001). h*NLRR-5* has no EGF-like motif and has 11 LRRs, and we failed to identify its human counterpart in the database or our NBL cDNA libraries.

h*NLRR-5*. Homology search against proteins deduced from genomic sequences on chromosome 9p revealed the presence of another family member of NLRR (acc. no. CAC22713). Its deduced protein was 606 a.a. in length and had a similar structure to the other NLRR members. However, a fibronectin domain was not included in this product. It showed 56 and 53% identities to mouse hypothetical protein (acc. no. BAB32403) and *Macaca fascicularis* hypothetical protein (acc. no. BAB03557), respectively, suggesting that they were mouse and *Macaca fascicularis* counterparts of hNLRR-5.

Expression of hNLRR family genes in human tissues. To examine whether *hNLRR* genes display neuron-specific expression, Northern analysis and semi-quantitative RT-PCR were performed. Among several human fetal tissues, h*NLRR-1*, h*NLRR-2* and h*NLRR-3* mRNAs were strongly expressed in brain at the size of 4.0-4.5 kb (Fig. 2A). By contrast, h*NLRR-5* was ubiquitously expressed in all main fetal organs. The size of h*NLRR-2* transcript in the liver was smaller than that in the other tissues. In adult human tissues, all h*NLRR-1*, h*NLRR-2*, h*NLRR-3* and h*NLRR-5* were also preferentially expressed at high levels in the nerve tissues (Fig. 2B).

Expression of hNLRR family genes in neuroblastoma and cell lines. Expression of h*NLRR* family genes was measured in primary neuroblastomas and cell lines using semi-quantitative RT-PCR. As shown in Fig 3A, *Nbla10449/hNLRR-1* was highly expressed in UF NBLs, whereas *Nbla10677/hNLRR-3* and h*NLRR-5* were preferentially expressed in the F NBLs. *Nbla00061/hNLRR-2* seemed to be equally expressed between both subsets. In NBL cell lines, expression of *NLRR-1* was observed relatively more frequently in the lines with *MYCN* amplification than in those with a single copy of the gene. On the other hand, *NLRR-3* appeared to be expressed rather frequently in the cell lines without *MYCN* amplification. Interestingly, however, there was a tendency that the cells with high expression of *NLRR-1* also had a high levels of expression of *NLRR-3* (Fig. 3B). The expression of both h*NLRR-2* and h*NLRR-5* was found in most NBL cell lines.

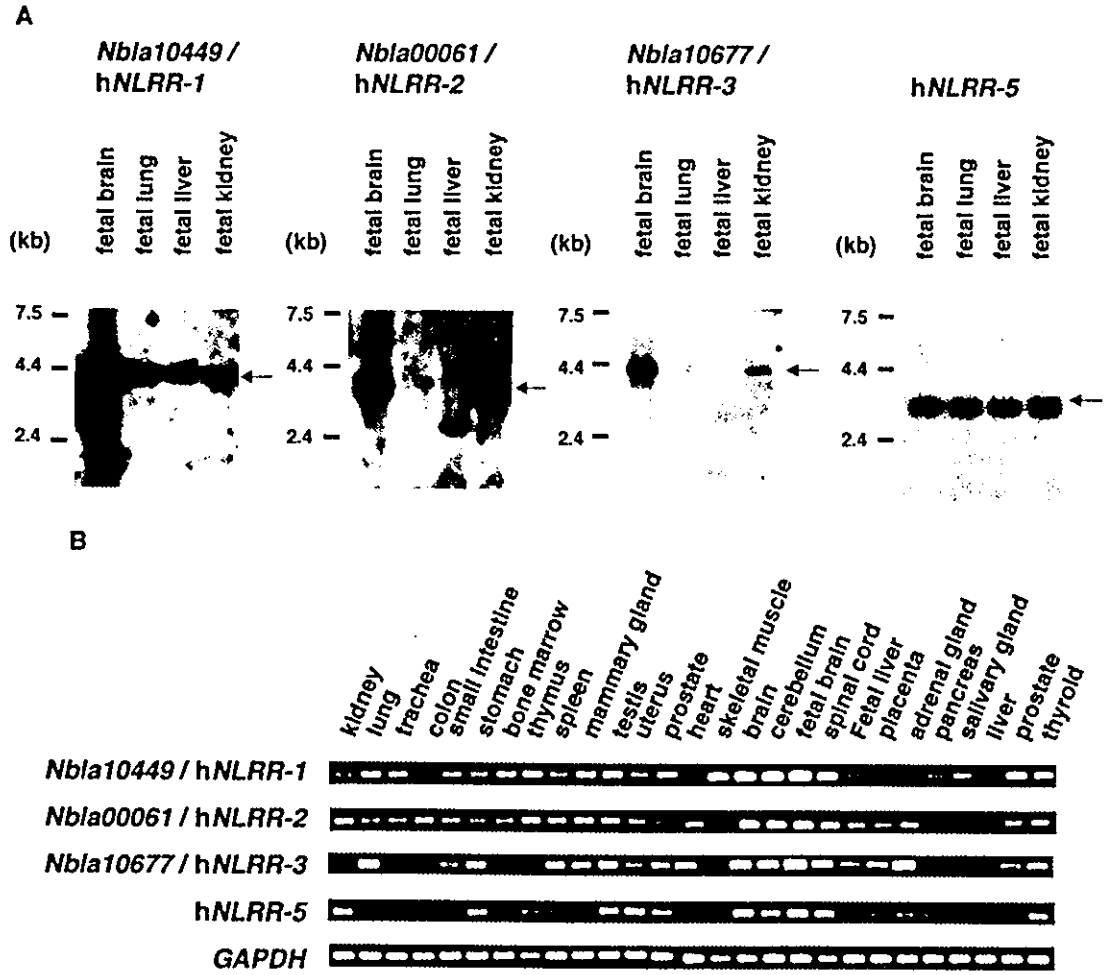

Figure 2. Expression of h*NLRR*s mRNA in human normal tissues. A, Northern blot analysis of h*NLRR*s mRNA in human fetal tissues. As a control for the amount of RNA, the same filter was rehybridized with ß-actin. B, Semi-quantitative RT-PCR of h*NLRR*s in multiple human tissues. Total RNA of 25 adult and 2 fetal tissues. As a control, same cDNA templates were amplified by *GAPDH* primers.

We then examined whether or not there was any genomic amplification of h*NLRR-1* or h*NLRR*-2 because that of *Nbla10449/*h*NLRR-1* was preferentially expressed in UF NBLs, and that of *GAC1/*h*NLRR-2* was reported to be amplified in the primary glioblastoma and anaplastic astrocytoma (15). However, our Southern blot analysis showed that neither of both genes was amplified in NBL cell lines so far examined (CHP134, IMR32, NB-9, NLF, TGW, NGP, NB69, NBL-S, SK-N-AS and SH-SY5Y) (data not shown). As regards the other cancer cell lines, expression of h*NLRR* family members was relatively restricted to the osteosarcoma and rhabdomyosarcoma cell lines (Fig. 3C). The low levels of h*NLRR*-3 and h*NLRR-5* expression were also seen in melanoma cell lines. Furthermore, expression of h*NLRR-2* was observed in the cell lines of colon, thyroid (medullary thyroid cancer), esophagus and lung. These results suggested that h*NLRR*s were preferentially expressed in the cell lines derived from neural crest cells.

Changes in expression of the NLRR family genes during NGF-induced differentiation and NGF-depletion-induced apoptosis in newborn mouse SCG neurons in primary culture. To investigate the role of NLRR family molecules in NGF/TrkA-mediated signaling, we next used newborn mouse SCG neurons, from which NBL is derived. As reported previously, NGF induced marked morphological differentiation of SCG neurons (14). NGF-induced neurite extension was observed on day 2 and was enhanced thereafter by increasing in number and length (Fig. 4A, NGF$^+$). The depletion of NGF by treating the cells with anti-NGF antibody induced neuronal programmed cell death (Fig. 4A, NGF$^-$). As shown in Fig. 4B, expression of m*NLRR-1* and m*NLRR-5* was down-regulated during NGF-induced neuronal differentiation, and was up-regulated after NGF deprivation (Fig. 4B). On the other hand, expression of m*NLRR-2* and m*NLRR-3* was slightly up-regulated when they were treated with NGF, and was significantly down-regulated after NGF deprivation (Fig. 4B), suggesting that expression of m*NLRR* genes might be related to the NGF signaling.

*Prognostic significance of expression of Nbla10449/*h*NLRR-1 and Nbla10677/*h*NLRR-3 in primary neuroblastomas.* To evaluate the clinical significance, expression of *Nbla10449/*h*NLRR-1* and *Nbla10677/*h*NLRR-3* in 99 NBLs was statistically analyzed. Table I gives the mean and standard error (SEM) of h*NLRR-1/Nbla10449* and h*NLRR-3/Nbla10677*

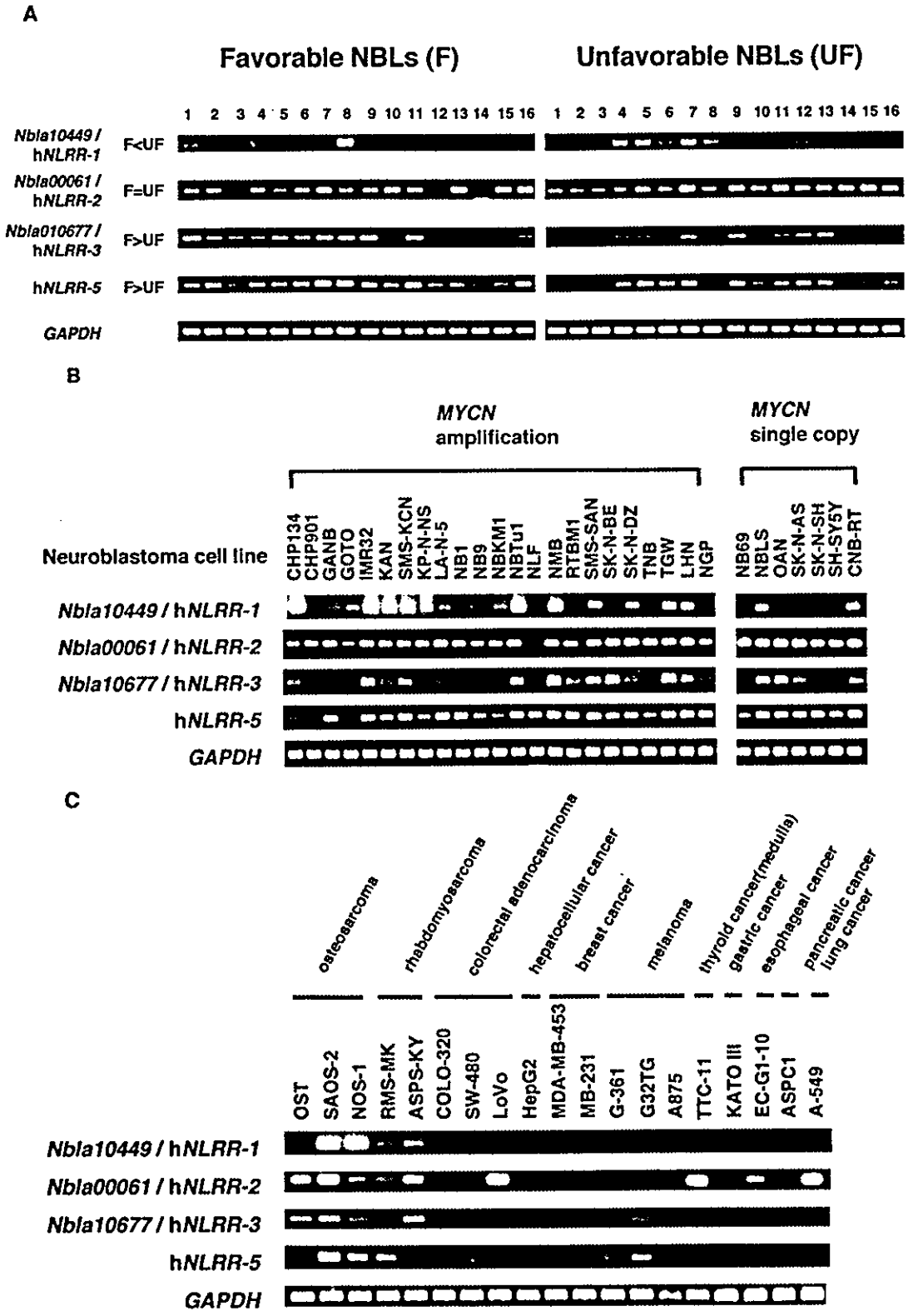

Figure 3. Expression of h*NLRR* family genes in primary NBLs, NBL cell lines and other cancer cell lines. A, Differential expression of h*NLRR* family genes in 16 favorable and 16 unfavorable NBLs. mRNA expression was detected by semi-quantitative RT-PCR procedure. The expression of *GAPDH* is shown as a control. Lanes 1-16: favorable NBLs (F, stage 1 or 2, with a single copy of *MYCN*), lanes 17-32: unfavorable NBLs (UF, stage 3 or 4, with *MYCN* amplification). B, Expression of h*NLRRs* mRNA in NBL cell lines. Twenty-three NBL cell lines with *MYCN* amplification and 7 cell lines with a single copy of *MYCN* were used for semi-quantitative RT-PCR as templates. C, Expression of h*NLRRs* mRNA in the other cancer cell lines. Semi-quantitative RT-PCR analysis was performed using cDNA and control *GAPDH* primers. Tumor origins are shown on the top.

expression by age, tumor stage, *TrkA* expression, *MYCN* copy number, origin, and mass screening. High expression of h*NLRR-1/Nbla10449* were significantly associated with >1 year of age (p=0.0001), advanced stage (p=0.0007), low expression of *TrkA* (p=0.011), *MYCN* amplification (p=0.0001) and sporadic tumors (p=0.0004), but not with the tumor origin (p=0.4). The results of log-rank test showed that a high level of h*NLRR-1/Nbla10449* expression was significantly associated

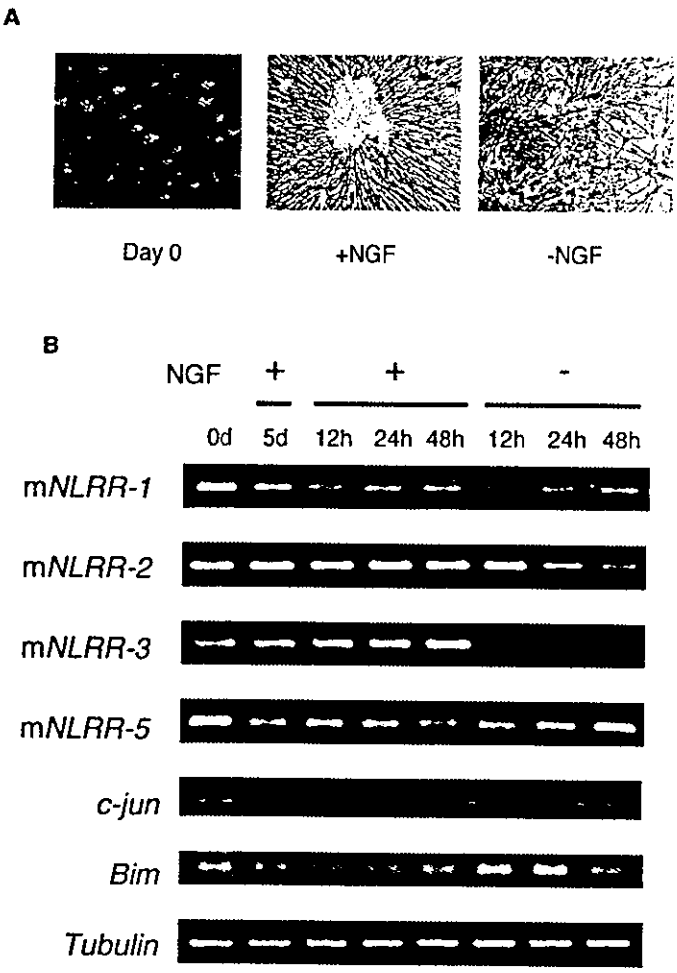

Figure 4. Changes in mRNA expression of mouse *NLRR* family genes in mouse superior cervical ganglion (SCG) cells treated with NGF in primary culture. A, Effect of NGF on newborn mouse SCG neurons in primary culture. The pictures were taken on day 0 and 5 (NGF$^+$) in the presence of 50 ng/ml NGF. NGF was then depleted from the medium by adding 1% v/v anti-NGF antibody for 36 h (NGF$^-$). B, Changes in expression of m*NLRR*s mRNA during NGF-induced differentiation and NGF depletion-induced apoptosis in newborn mouse SCG neurons in primary culture. SCG neurons were cultured for 5 days with NGF and then further cultured with or without NGF for 12, 24, 48 h. *c-jun* and *Bim*, positive control gene; Tubulin, used for standardization of the cDNA concentration.

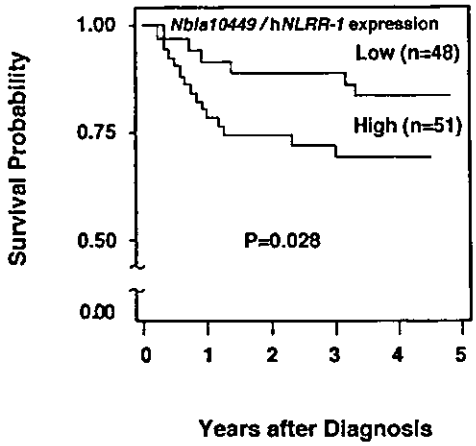

Figure 5. Kaplan-Meier survival curves for the 48 patients with low expression and the 51 patients with high expression.

with an unfavorable outcome (p=0.028). On the other hand, there was significant correlation between high levels of *Nbla10677/hNLRR-3* expression and younger age (p=0.0018), favorable stage (p=0.0007), high levels of *TrkA* expression (p=0.021), single copy of *MYCN* (p=0.0002) and the tumors found by mass screening (p=0.0049), but not with the tumor origin (p=0.33).

The univariate Cox regression was employed to examine the individual relationship of each variable to survival (Table II). These variables were: h*NLRR-1/Nbla10449* (log), h*NLRR-3/Nbla10677* (log), age (>1 year vs. <1 year), tumor stage (3+4 vs. 1+2+4s, *MYCN* copy number (1 copy vs. >1 copy), mass screening (+ vs. -), and origin (adrenal gland vs. others). Expression of h*NLRR-1/Nbla10449* (p=0.005), age (p<0.0005), *MYCN* copy number (p<0.0005), mass screening (p=0.001) were found to be statistically of prognostic importance. The results in Table II show that h*NLRR-1/Nbla10449* expression was an independent prognostic factor from age, *MYCN* copy number and mass screening in primary NBLs.

Discussion

In the present study, we identified the full-length human neuronal leucine-rich repeat protein (*NLRR*) family genes preferentially expressed in the nervous system and adrenal gland: *Nbla10449/hNLRR-1*, *Nbla00061/hNLRR-2/GAC1*, *Nbla10677/hNLRR-3* and h*NLRR-5*. In primary NBLs, the levels of h*NLRR-1* expression are significantly higher in the unfavorable subsets than those in the favorable tumors, whereas the expression pattern of h*NLRR-3* and h*NLRR-5* is the opposite. The results from the experiments using mouse SCG neurons treated with NGF in the primary culture have suggested that both m*NLRR-2* and *NLRR-3* are the molecules relating to promotion of neuronal survival or differentiation, while m*NLRR-1* and m*NLRR-5* function as those promoting cell growth or enhancing apoptosis. Furthermore, expression of h*NLRR-1* has been found as a significant indicator of poor outcome of NBLs, whereas that of h*NLRR-3* is associated with other favorable prognostic factors. Thus, hNLRR family members appear to differently regulate functions of neuronal cells as well as those of neuroblastoma.

A protein with leucine-rich repeat (LRR) domains was first identified in an α-2-glycoprotein of human serum (17). LRR-containing proteins represent a diverse group of molecules with different functions and cellular locations in a variety of organs. LRR domains provide an ideal conformation for binding to other proteins and this structure is thought to be involved in protein-protein interaction (10). Many LRR-containing proteins have been shown to function as cell-adhesion molecules or signaling receptors and are implicated in a variety of events in neural development. For example, adhesive LRR-containing proteins and small proteoglycans such as osteoinductive factor (OIF) bind various components of the extracellular matrix and growth factors. Interestingly, OIF binds the transforming growth factors, TGF-ß and TGF-ß2, and is involved in bone formation (18). The neurotrophin receptors, Trks, also possess the LRR domains in the extra-

Table I. Correlation between expression of *Nbla10449/hNLRR-1* or *Nbla10677/hNLRR-3* and other prognostic factors (Student's t-test).

Variable	No.	*Nbla10449/hNLRR-1* Mean ± SEM	p-value	*Nbla10677/hNLRR-3* Mean ± SEM	p-value
Age					
<1 year	63	0.84±0.21	0.0001	5.05±0.93	0.0018
>1 year	36	3.97±1.44		2.53±0.77	
Tumor stage					
1, 2, 4s	57	0.68±0.17	0.0007	5.36±1.00	0.0007
3, 4	42	3.74±1.25		2.48±0.68	
TrkA expression					
Low	45	3.50±1.17	0.011	3.13±0.77	0.021
High	54	0.71±0.18		4.97±1.02	
MYCN copy no.					
Amplified	29	5.19±1.75	0.0001	1.71±0.90	0.0002
Single	70	0.65±0.14		5.14±0.86	
Origin					
Adrenal gland	63	2.16±0.75	0.4	4.18±0.90	0.33
Others	36	1.67±0.79		4.06±0.93	
Mass screening					
+	54	0.67±0.18	0.0004	5.09±1.02	0.0049
-	45	3.55±1.17		2.98±0.76	

Table II. Cox regression models using *Nbla10449/hNLRR-1* expression and dichotomous factors of age, *TrkA* expression, *MYCN* amplification, and origin (n=99).

Model	Variable	p-value
A	*Nbla10449/hNLRR-1*	0.005
B	*Nbla10677/hNLRR-3*	0.15
C	Age (>1 vs. <1 year)	<0.005
D	*MYCN* (1 copy vs. amplification)	<0.005
E	Origin (adrenal gland vs. others)	0.079
F	Mass screening (+ vs. -)	0.001

cellular region. In *Drosophila*, some LRR domain-containing molecules such as toll, slit, connectin, chaoptin and tartan play an important role in regulating neural development (19-23).

The LRR motif includes highly hydrophobic amino acids and a repeat structure consisting of about 24 residues (20). NLRR family proteins contain in its extracellular region an immunoglobulin C-2 type domain and a fibronectin type III domain in addition to 11 sets of LRR motif (24). *NLRR* family genes were first isolated from a mouse brain cDNA library (16,25), and then 3 distinct isoforms (m*NLRR-1*, m*NLRR-2* and m*NLRR-3*) have been identified in zebrafish, *Xenopus*, mouse, rat and *Macaca facicularis* (16,24-26). The function of these NLRR proteins is poorly understood except that expression of m*NLRR-3* was increased after cortical brain injury (27) and that *rNLRR-3* expression is regulated through the Ras-MAPK signaling pathway in fibroblasts (28).

The deduced amino acid sequences of hNLRRs are highly conserved in the domains of LRR, LRRNT, LRRCT, Igc2 and FNIII, except that h*NLRR-5* does not have the FNIII domain. Many LRR proteins with LRRNT and LRRCT domains have been proposed to function in the regulation of neural differentiation and/or developmental processes as adhesive proteins and/or receptors (10). In addition to LRR, the Igc2 and FNIII domains in the extracellular region are often found in the molecules expressed in the central nervous system (26) and in several neuronal cell-adhesion molecules of the immunoglobulin superfamily such as N-CAM and L1 (29). Although hNLRRs and other NLRRs have no known signaling domain in the cytoplasmic region, a number of conserved stretches are found (Fig. 1). Especially, NLRR-1 and NLRR-3 have been shown to have a conserved stretch of 11 amino acids (ELYPPLINLWE) with 2 clathrin mediated endocytosis motifs, a tyrosine-based signal conforming to the YXRF motif (30,31), and a dileucine-type motif (32). Endocytosis and recycling mechanisms are relevant for cell adhesion molecules like integrins during cell migration (33,34).

Although the function of NLRR protein is poorly understood, there are some clues in recent reports. mNLRR-3 expression is increased in layers 2-3 in cerebral cortex after cortical injury, suggesting that this molecule plays a role in

the regulation of synaptic re-organization (27). zNLRR has also been proposed to have function as a neuronal-specific adhesion molecule or soluble ligand binding receptor during regeneration of the zebrafish central nervous system after injury, because retinal ganglion cells and descending spinal cord neurons strongly increased expression of z*NLRR* after axotomy in the adult (24).

The SCG/NGF system utilized in this study also provides a helpful hint to consider the neuronal function of hNLRRs. NLRR-1 may be involved in growth promotion in NBL by suppressing neuronal differentiation according to the result showing that the expression of m*NLRR-1* is down-regulated when the cells were treated with NGF. On the other hand, NLRR-3 may play a role in regulating differentiation to extend neurites and in neuronal survival of NBL cells since the expression of m*NLRR-3* was up-regulated by NGF and down-regulated after deprivation of NGF. These results are consistent with their differential expression pattern between favorable and unfavorable subsets of NBL.

In favorable NBLs as well as the cell lines with a single copy of *MYCN*, h*NLRR-1* expression was low as compared with the *MYCN*-amplified cells, suggesting that MYCN could influence the h*NLRR-1* expression. Interestingly, we have identified MYCN transcription factor-binding motifs (E-boxes) in the promoter region of the *NLRR-1* gene. Like h*NLRR-3*, h*NLRR-2* may also be involved in controlling neural cell survival as supposed from the result obtained in the NGF/SCG system. Ubiquitous h*NLRR-2* expression in NBLs suggests that h*NLRR-2* plays a role in maintaining cell survival. Of interest, h*NLRR-2* is often amplified in glioma as described below. h*NLRR-5* shows similar change in expression to h*NLRR-1* in the system of NGF-treated SCG neurons, albeit it is highly expressed in favorable NBLs. This suggests that hNLRR-5 may function as a proapoptotic molecule in NBL. Thus, each hNLRR member may have distinct biological function in NBL as well as neuronal cells. As the deduced intracellular region at the extreme C-terminus of hNLRR proteins has variable amino acid sequences, it may play a role in determining the differential function of hNLRR family receptors.

There are a few reports showing the relationship between LRR or NLRR and human cancer. *GAC1* (*hNLRR-2*), mapped to chromosome 1q32.1, is amplified and overexpressed in glioblastoma multiforme and anaplastic astrocytoma (15). Another report shows that expression of r*NLRR-3*, which was cloned by the subtractive screening using fibrosarcoma cells overexpressing c-Ha-ras, is regulated through the Ras-MAPK pathway, albeit the role in cancer cells is unknown (28). Trk family receptor tyrosine kinases have 3 LRRs in the extracellular domain, whose alteration can cause onco genic activation in some cancers (35). Interestingly, TrkA and TrkB also show an inverse expression pattern between favorable and unfavorable NBLs, that is very similar to the pattern of h*NLRR-1* and h*NLRR-3* expression. Since expression levels of TrkA and TrkB are powerful prognostic factors in NBLs, those of h*NLRR-1* and h*NLRR-3* may also be important in predicting the patient's outcome. Indeed, our present data suggest that expression of both hNLRR-1 and hNLRR-3 is inversely associated with the prognosis as well as other prognostic factors.

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【総論：ウイルムス腫瘍】

Wilms' Tumor

秦　順一

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Key words
Wilms tumor, Embryonal tumor,
Congenital anoma ly

はじめに

神経芽腫，ウイルムス腫瘍，肝芽腫，胚細胞腫瘍に代表される胎児性腫瘍は，主に乳幼児期に発生する特有な腫瘍群である。これら胎児性腫瘍は器官形成途上の母細胞から発生し，腫瘍細胞が発生母地の有する分化・成熟能を潜在的に有している。一方，腫瘍発生機構においては遺伝子異常が直接的な要因であり時に，環境の付加的要因により修飾されることが明らかになっている。このような発癌に関連する遺伝子の多くが，正常組織での細胞の増殖や分化または器官形成に強く関わっていることが，明らかにされつつある。ウイルムス腫瘍は胎児性腫瘍の代表的な腫瘍である。本腫瘍はネフロンの形成に与る後腎組織の細胞を発生母地とし，形態学的にも後腎芽細胞が尿細管，糸球体に分化する過程を模倣している。

本腫瘍の歴史は古く，1814年にイギリスの臨床医であったRanceが17ヶ月男児の急激に増大した腎腫瘍を，また1828年には同じく小児の腎腫瘍をGardnerが記載している。詳細な臨床的記載を初めて行ったのはVan der Bylで，巨大な腎腫瘍として解剖まで行っている。このような報告から本腫瘍が患児に死をもたらす腫瘍であり，治療の必要性が認識されるようになった。1877年にこの腫瘍に対して，最初の腎摘術が行われた。その時期には，本腫瘍はembryonal sarcoma, sarcoma of the kidney, addenomyosarcomaなどと呼ばれていた。病理学的所見の最初の記述は，1872年，Eberthによって行われた。彼は17ヶ月女児の両側性腫瘍について検索したが，本腫瘍が極めて豊富な横紋筋成分を含んでいることを認めている。胎児性横紋筋腫性腎芽腫(fetal rhabdomyomatous nephrobalastoma, FRN)であったと考えられる。彼は本腫瘍の由来をWolffian bodyであると述べている。その後，Cohnheimが横紋筋細胞とともに他の間葉系細胞の未熟な細胞や上皮様細胞が混じった腫瘍であることを記載した。このような初期の病理学的記載から判断すると，当時は発生途上の腎生殖隆起および筋節と密接に関連して発生する腫瘍であると考えられていたのであろう。このような報告にもあるように，本腫瘍の組織像は極めて多彩である。また，後述されているように多種の腫瘍が含まれており，どこまでを真のウイルムス腫瘍に含めるかは議論の多いところである。

1899年にドイツの医学者Max Wilmsが彼の医学書に本腫瘍が記載したことによって，以後ウイルムス腫瘍(Wilms' tumor)と呼ばれるようになった。本腫瘍は疫学的にも極めて興味深い。すなわち白人種に多く，日本人を含めて「有色人種」に少ない。因みに，米国の統計では16歳以下の10,000人に1人の割合で発生し，年間460名内外の患者が発生する。すなわち，小児がんの6%を占めている。また，白人種は有色人種の3倍の頻度で多い。わが国の正確な発生数は明らかではないが，年間100名以下の発生と推定される。このような人種による発生頻度の差異は本腫瘍の発生や生物学的特異性を明らかにする上で，重要な所見である。本腫瘍の発生母地や腫瘍発生機序に関しては，ウイルムス腫瘍の歴史を反映して多くの説が提唱されてきた。その詳細は，各論を参照して頂きたい。

一方，発生母地に関しては組織像の類似性から腎発生途上に生じる後腎芽細胞に由来するという見解

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