

Fig. 2. GnT-V activities in various NBL cell lines and retinoic acid-induced apoptosis of CHP134 cells. (A) GnT-V activity of each of the NBL cells was measured using a whole cell lysate as an enzyme source. (B) Western blot of whole cell lysate of CHP134 cells. Cell apoptosis was observed by staining of cleaved PARP after treatment of retinoic acid (RA) at indicated concentrations and times. The expression levels of ERK1/2 were confirmed as a loading control.

in vivo [16,17]. Thus, we chose this cell line for further analysis of the effects of GnT-V on apoptosis.

3.3. Knockdown of GnT-V expression in CHP134 cells

We prepared a retroviral siRNA vector containing a small hairpin construct capable of generating a duplex RNAi oligonucleotide corresponding to human GnT-V. After retroviral infection, CHP134 cells were selected based on their resistance to G418, and clones with decreased GnT-V activities were chosen. The GnT-V activities were effectively downregulated by 80%, compared with those in parent or mock cells (Fig. 3A), while GnT-III activity, as a control, showed no significant changes between those cells. A quantitative real-time PCR analysis also indicated the downregulation of RNAi-directed

GnT-V mRNA expression in these cells (Fig. 3B). It is noteworthy that the cells in GnT-V-knockdown clones showed more spreading on the culture dishes, rather than the spindle shapes of the parent and mock cells (Fig. 4), suggesting that GnT-V may affect cellular cytoskeletal formation. In fact, Guo et al. reported that the overexpression of GnT-V in human HT1080 cells resulted in a decrease in cell adhesion on fibronectin [18].

3.4. Decreased susceptibility to retinoic acid-induced apoptosis in GnT-V-knockdown cells

To evaluate the effects of GnT-V expression on susceptibility to apoptosis induction in CHP134 cells, we examined cell viabilities in the presence of retinoic acid. After treatment with different concentrations of retinoic acid, we found that GnT-V-knockdown cells (KD1 and KD2 in Fig. 5A) had a tendency to be resistant to stimulation by retinoic acid. We further assessed the apoptosis level in retinoic acid-treated cells by PARP cleavage. The GnT-V-knockdown cells showed dramatically reduced levels of PARP cleavage (Fig. 5B). Collectively, these results suggest that GnT-V may sensitize cells to apoptotic signals, which partly contribute to the favorable prognosis of NBL.

4. Discussion

Previous studies demonstrated that an increased amount of β 1,6-branched oligosaccharides, formed by the action of GnT-V, are correlated with metastatic potential [2], and this has been shown to be a marker of tumor progression in human breast and colon neoplasia [19], and a prognostic marker in human colorectal carcinoma [20,21]. However, it is not always the case, as evidenced by the fact that Dosaka-Akita et al. reported that the lower expression of GnT-V is associated with a shorter survival and a poor prognosis in non-small cell lung cancers [22]. The present study also suggested that a higher expression of GnT-V is related to a favorable prognosis in NBLs.

GnT-V and GnT-IX, two closely related glycosyltransferases, are expressed in both the adult and fetal brain [7,9]. GnT-V expression is upregulated in E9.5 embryos, and is then

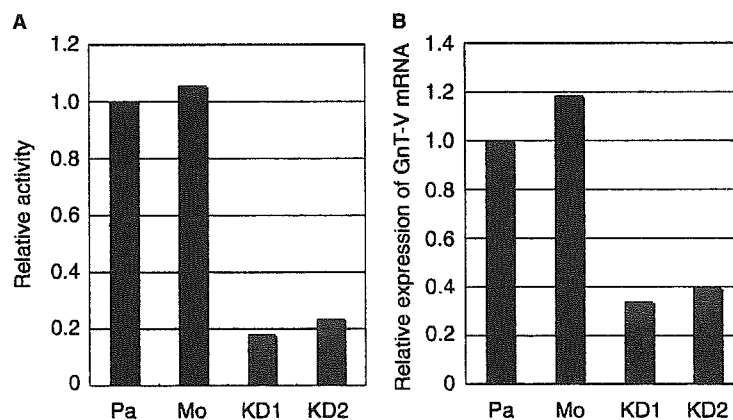


Fig. 3. Enzyme activities and mRNA expression levels in siRNA-mediated GnT-V-knockdown cells. (A) GnT-V activities of GnT-V-knockdown CHP134 cells. The microsomal fraction was used as an enzyme source in the assay. (B) mRNA expression of GnT-V in knockdown cells. Quantitative analysis was performed by real-time PCR. Pa, parent cells; Mo, mock cells; KD1 and KD2, GnT-V-knockdown cells.

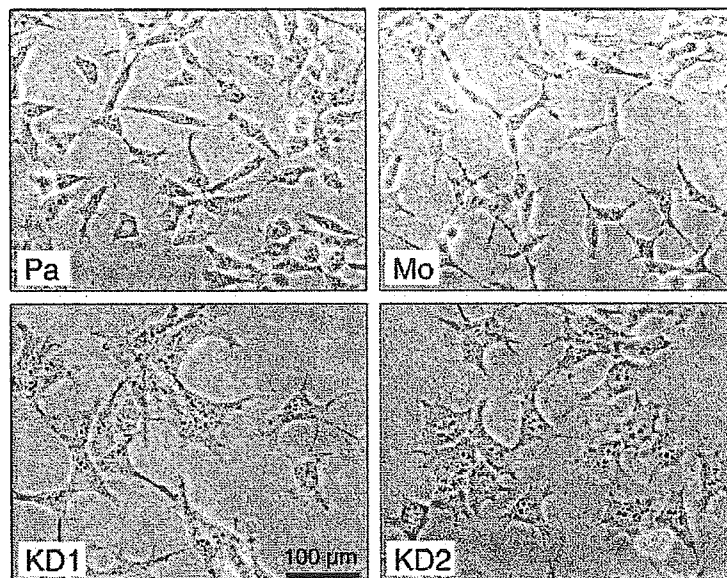


Fig. 4. Morphological changes in GnT-V-knockdown cells. Parent (Pa), mock (Mo), and GnT-V-knockdown CHP134 cells (KD1, KD2) were plated on culture dishes and incubated for 24 h in culture media. Cell shapes were observed by phase contrast microscopy.

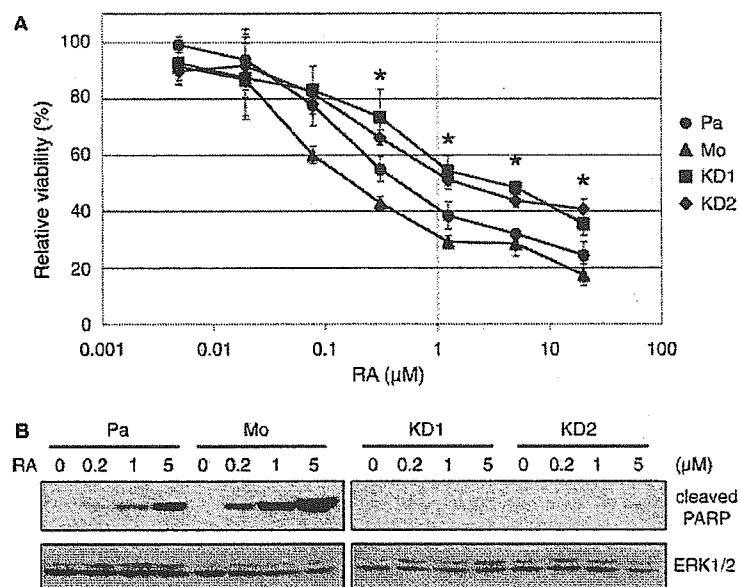


Fig. 5. Cell viabilities and PARP cleavage in retinoic acid-treated GnT-V-knockdown cells. Cell viabilities of parent (Pa), mock (Mo), and GnT-V-knockdown cells (KD1, KD2) were performed as described in Section 2 (A). Cells were treated with retinoic acid at the indicated concentrations for 3 days. * $P < 0.05$. (B) Western blot of cleaved PARP in a cell lysate using an anti-cleaved PARP antibody. Cells were harvested for analysis 24 h after retinoic acid-treatment.

restricted to regions comprised of several specialized epithelial cell layers and the neuroepithelium of the developing central nervous system [9]. On the other hand, GnT-IX is dominantly expressed in the human and mouse brain [7,23]. Thus, we attempted to examine the expressions of GnT-V and GnT-IX in primary NBL tissues.

The frequent gain of the chromosome 17q has been reported to be associated with a poor prognosis [24], and the preferential gain of the region from 17q22-qter indicated a dosage effect that provides a selective advantage to be aggressive NBLs [25].

The gene responsible for the selective advantage is unknown, but a candidate gene that is a member of the inhibitor of apoptosis proteins, survivin, which is mapped to 17q25, has been reported [14]. Although the GnT-IX gene is also mapped to 17q25 [7], an unequivocal correlation with prognosis was not observed in this study. Interestingly, a significant association between expression levels of GnT-V mRNA and the prognosis of 126 NBL patients was observed by real-time PCR analysis. Several human NBL cell lines also consistently express GnT-V. To understand the molecular mechanism associated with the

higher expression levels of GnT-V in the favorable prognosis of NBLs, we selected CHP134 cells as a cell model. Since the cell line is highly sensitive to retinoic acid-induced apoptosis [14,15], we compared the effects of retinoic acid on apoptosis between parent cells and GnT-V-knockdown cells.

In fact, GnT-V-knockdown cells showed a tendency to escape from retinoic acid-induced apoptosis, as confirmed by a cell viability assay and the extent of cleaved PARP, supporting the notion that a higher expression of GnT-V is correlated with a favorable prognosis of NBLs. It is noteworthy that a prominent morphological alteration with increased spreading was observed in the GnT-V-knockdown cells. The altered characteristic of GnT-V-knockdown CHP134 cells observed in this study is consistent with those of previous studies [3,18,26,27]. The overexpression of GnT-V enhances the metastatic potential in several cell types with reduced cell-matrix adhesion and increased motility [3,18,26]. Furthermore, GnT-V expression in human glioma cell line U-373 MG sensitizes these cells to drag-induced apoptosis [28]. Conversely, GnT-V null mouse embryonic fibroblasts exhibited an enhanced adhesion and spreading with associated reduced cell migration [27]. In addition, no significant effect of GnT-V overexpression was observed on apoptotic behavior in fibrosarcoma HT1080 cells, a fibroblast cell line, but a similar phenotypic change with regard to adhesion and migration has been reported [18]. In general, the adhesion of epithelial cells to extracellular matrices is weaker than that of fibroblast cells, and such adhesion is thought to be synergized with the signals of growth factor receptors for modulating cell proliferation and apoptosis. Therefore, we speculate that the GnT-V-induced decrease in cell adhesion could be a plausible factor responsible for the favorable prognosis in NBLs.

In conclusion, a correlation between higher expression levels of GnT-V with a favorable prognosis of NBL patients was found, and GnT-V may cause these tumors to regress by increasing their susceptibility to apoptosis.

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References

- [1] Hakomori, S. (2002) Glycosylation defining cancer malignancy: new wine in an old bottle. *Proc. Natl. Acad. Sci. USA* 99, 10231–10233.
- [2] Dennis, J.W., Laferte, S., Waghorne, C., Breitman, M.L. and Kerbel, R.S. (1987) Beta 1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* 236, 582–585.
- [3] Demetriou, M., Nabi, I.R., Coppolino, M., Dedhar, S. and Dennis, J.W. (1995) Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J. Cell. Biol.* 130, 383–392.
- [4] Granovsky, M., Fata, J., Pawling, J., Muller, W.J., Khokha, R. and Dennis, J.W. (2000) Suppression of tumor growth and metastasis in Mgat5-deficient mice. *Nat. Med.* 6, 306–312.
- [5] Saito, T., Miyoshi, E., Sasai, K., Nakano, N., Eguchi, H., Honke, K. and Taniguchi, N. (2002) A secreted type of beta 1,6-*N*-acetylglucosaminyltransferase V (GnT-V) induces tumor angiogenesis without mediation of glycosylation: a novel function of GnT-V distinct from the original glycosyltransferase activity. *J. Biol. Chem.* 277, 17002–17008.
- [6] Ihara, S., Miyoshi, E., Ko, J.H., Murata, K., Nakahara, S., Honke, K., Dickson, R.B., Lin, C.Y. and Taniguchi, N. (2002) Prometastatic effect of *N*-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding beta 1–6 GlcNAc branching. *J. Biol. Chem.* 277, 16960–16967.
- [7] Inamori, K., Endo, T., Ide, Y., Fujii, S., Gu, J., Honke, K. and Taniguchi, N. (2003) Molecular cloning and characterization of human GnT-IX, a novel {beta}1,6-*N*-acetylglucosaminyltransferase that is specifically expressed in the brain. *J. Biol. Chem.* 278, 43102–43109.
- [8] Kaneko, M., Alvarez-Manilla, G., Kamar, M., Lee, I., Lee, J.K., Troupe, K., Zhang, W., Osawa, M. and Pierce, M. (2003) A novel beta(1,6)-*N*-acetylglucosaminyltransferase V (GnT-VB). *FEBS Lett.* 554, 515–519.
- [9] Granovsky, M., Fode, C., Warren, C.E., Campbell, R.M., Marth, J.D., Pierce, M., Fregien, N. and Dennis, J.W. (1995) GlcNAc-transferase V and core 2 GlcNAc-transferase expression in the developing mouse embryo. *Glycobiology* 5, 797–806.
- [10] Brodeur, G.M. (2003) Neuroblastoma: biological insights into a clinical enigma. *Nat. Rev. Cancer* 3, 203–216.
- [11] Brodeur, G.M., Pritchard, J., Berthold, F., Carlsen, N.L., Castel, V., Castelberry, R.P., De Bernardi, B., Evans, A.E., Favrot, M. and Hedborg, F., et al. (1993) Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J. Clin. Oncol.* 11, 1466–1477.
- [12] Kato, C., Miyazaki, K., Nakagawa, A., Ohira, M., Nakamura, Y., Ozaki, T., Imai, T. and Nakagawara, A. (2004) Low expression of human tubulin tyrosine ligase and suppressed tubulin tyrosination/detyrosination cycle are associated with impaired neuronal differentiation in neuroblastomas with poor prognosis. *Int. J. Cancer* 112, 365–375.
- [13] Taniguchi, N., Nishikawa, A., Fujii, S. and Gu, J.G. (1989) Glycosyltransferase assays using pyridylaminated acceptors: *N*-acetylglucosaminyltransferase III, IV, and V. *Meth. Enzymol.* 179, 397–408.
- [14] Islam, A., Kageyama, H., Takada, N., Kawamoto, T., Takayasu, H., Isogai, E., Ohira, M., Hashizume, K., Kobayashi, H., Kaneko, Y. and Nakagawara, A. (2000) High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. *Oncogene* 19, 617–623.
- [15] Takada, N., Isogai, E., Kawamoto, T., Nakanishi, H., Todo, S. and Nakagawara, A. (2001) Retinoic acid-induced apoptosis of the CHP134 neuroblastoma cell line is associated with nuclear accumulation of p53 and is rescued by the GDNF/Ret signal. *Med. Pediatr. Oncol.* 36, 122–126.
- [16] Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M. and Lazebnik, Y.A., et al. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376, 37–43.
- [17] Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S. and Dixit, V.M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81, 801–809.
- [18] Guo, H.B., Lee, I., Kamar, M., Akiyama, S.K. and Pierce, M. (2002) Aberrant *N*-glycosylation of beta1 integrin causes reduced alpha5beta1 integrin clustering and stimulates cell migration. *Cancer Res.* 62, 6837–6845.
- [19] Fernandes, B., Sagman, U., Auger, M., Demetrio, M. and Dennis, J.W. (1991) Beta 1–6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. *Cancer Res.* 51, 718–723.
- [20] Seelentag, W.K., Li, W.P., Schmitz, S.F., Metzger, U., Aeberhard, P., Heitz, P.U. and Roth, J. (1998) Prognostic value of beta1,6-branched oligosaccharides in human colorectal carcinoma. *Cancer Res.* 58, 5559–5564.
- [21] Murata, K., Miyoshi, E., Kameyama, M., Ishikawa, O., Kabuto, T., Sasaki, Y., Hiratsuka, M., Ohgashi, H., Ishiguro, S., Ito, S., Honda, H., Takemura, F., Taniguchi, N. and Imaoka, S. (2000) Expression of *N*-acetylglucosaminyltransferase V in colorectal cancer correlates with metastasis and poor prognosis. *Clin. Cancer Res.* 6, 1772–1777.

- [22] Dosaka-Akita, H., Miyoshi, E., Suzuki, O., Itoh, T., Katoh, H. and Taniguchi, N. (2004) Expression of *N*-acetylglucosaminyltransferase V is associated with prognosis and histology in non-small cell lung cancers. *Clin. Cancer Res.* 10, 1773–1779.
- [23] Inamori, K., Mita, S., Gu, J., Mizuno-Horikawa, Y., Miyoshi, E., Dennis, J.W. and Taniguchi, N. (in press) Demonstration of the expression and the enzymatic activity of *N*-acetylglucosaminyltransferase IX in the mouse brain. *Biochim Biophys Acta*.
- [24] Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A.D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., Laureys, G., Speleman, F., Nicholson, J., Bernheim, A., Betts, D.R., Vandesompele, J. and Van Roy, N. (1999) Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N. Engl. J. Med.* 340, 1954–1961.
- [25] Lastowska, M., Cotterill, S., Bown, N., Cullinane, C., Variend, S., Lunec, J., Strachan, T., Pearson, A.D. and Jackson, M.S. (2002) Breakpoint position on 17q identifies the most aggressive neuroblastoma tumors. *Genes Chromosomes Cancer* 34, 428–436.
- [26] Yamamoto, H., Swoger, J., Greene, S., Saito, T., Hurh, J., Sweeley, C., Leestma, J., Mkrdichian, E., Cerullo, L., Nishikawa, A., Ihara, Y., Taniguchi, N. and Moskal, J.R. (2000) Beta1,6-*N*-acetylglucosamine-bearing *N*-glycans in human gliomas: implications for a role in regulating invasivity. *Cancer Res.* 60, 134–142.
- [27] Guo, H.B., Lee, I., Bryan, B.T. and Pierce, M. (2005) Deletion of mouse embryo fibroblast *N*-acetylglucosaminyltransferase V stimulates alpha5beta1 integrin expression mediated by the protein kinase C signaling pathway. *J. Biol. Chem.* 280, 8332–8342.
- [28] Dawson, G., Moskal, J.R. and Dawson, S.A. (2004) Transfection of 2,6 and 2,3-sialyltransferase genes and GlcNAc-transferase genes into human glioma cell line U-373 MG affects glycoconjugate expression and enhances cell death. *J. Neurochem.* 89, 1436–1444.



A review of DNA microarray analysis of human neuroblastomas

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Abstract

Neuroblastoma (NBL) is an enigmatic tumor with heterogeneous clinical behaviors including maturation, regression, and aggressive growth. Despite recent progress in therapeutic strategies against advanced NBL, long-term outcomes still remain very poor. The prediction of cancer prognosis is one of the most urgent demands to initiate the suitable treatment of NBL. Recent papers have demonstrated that cancers can be diagnosed on the basis of gene expression profiling. We have been proceeded NBL cDNA project to collect a large number of genes expressed in NBLs, to identify the genes differentially expressed between favorable and unfavorable NBLs, and to make an NBL-proper cDNA chip for large-scale analysis of NBL tumors. Computational analysis of gene expression data in NBLs identified many prognosis-related genes and provided a classifier to predict the patient prognosis with high efficiency. Conversion of these findings into better diagnosis and treatment is now underway. Thus, molecular profiling of NBL has become a feasible tool for clinical applications.

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Keywords: Neuroblastoma; Expression profile; Microarray; Diagnosis; Prognosis prediction; Differential expression

1. Introduction

Neuroblastoma (NBL) is one of the most frequent solid cancers in young children and has variable clinical and biological characteristics [1]. Favorable type of tumors frequently regress spontaneously, while unfavorable type of tumors are often resistant to intensive chemotherapy and lead patients to fatal

outcome. The poor prognosis of NBL patients depends on age at diagnosis (older than 12 months), advanced tumor stage (3 or 4), presence of *MYCN* amplification, low *TRKA* expression, unfavorable histology, diploidy, and chromosomal loss of 1p36 in tumors [2]. However, even these markers sometimes fail to classify the aggressiveness of tumors, especially for the intermediate type of NBL (stage 3 or 4 patients with single copy of *MYCN*). Moreover, some *MYCN*-amplified tumors can be distinguished by a better response to the combined treatment resulting in a better prognosis for the patient. Therefore, additional potent markers for

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Table 1
Genes whose expression is differential between neuroblastoma subsets and related to patient prognosis

Genes	Definition	Pattern ^a	Reference
<i>TRKA</i>	neurotrophic tyrosine kinase, receptor, type 1	F>UF	[3]
<i>CD44</i>	CD44 antigen	F>UF	[6]
<i>PTN</i>	Pleiotrophin	F>UF	[7]
<i>CDC10</i>	cell division cycle 10	F>UF	[10]
<i>HRAS</i>	v-Ha-ras Harvey rat sarcoma viral oncogene	F>UF	[9]
<i>XCE</i>	endothelin-converting enzyme-like 1	F>UF	[17]
<i>NLRR3</i>	neuronal leucine-rich repeat 3	F>UF	[18]
<i>TTL</i>	tubulin tyrosine ligase	F>UF	[19]
<i>BMCC1</i>	novel putative apoptosis-related gene with BCH domain	F>UF	
<i>FOG2</i>	Friend of GATA protein 2	F>UF	[16]
<i>NEDL1</i>	NEDD4-like ubiquitin ligase 1	F>UF	
<i>NEDL2</i>	NEDD4-like ubiquitin ligase 2	F>UF	
<i>GABARAP</i>	gamma-aminobutyric acid receptor-associated protein gene	F>UF	[20]
<i>GABA(A) family</i>	GABA(A) receptor subunit gene family	F>UF	[20]
<i>TRKB</i>	neurotrophic tyrosine kinase, receptor, type 2	F<UF	[4]
<i>hTERT</i>	telomerase reverse transcriptase	F<UF	[5]
<i>NM23A</i>	non-metastatic cells 1 (<i>NM23-H1</i>)	F<UF	[12]
<i>NM23B</i>	non-metastatic cells 2 (<i>NM23-H2</i>)	F<UF	[13]
<i>BIRC5</i>	baculoviral IAP repeat-containing 5 (survivin)	F<UF	[11]
<i>PPM1D</i>	protein phosphatase 1D	F<UF	[14]
<i>NLRR1</i>	neuronal leucine-rich repeat 1	F<UF	[18]
<i>LMO3</i>	LIM-only protein 3	F<UF	

^a F;favorable NBL, UF;unfavorable NBL.

predicting the NBL prognosis should be discovered to construct a more effective as well as less toxic therapeutic strategy. Recent works have demonstrated that cancers can be diagnosed on the basis of gene expression profiling using cDNA microarrays with calculation by computational algorithms. This process needs (1) mass collection or identification of genes expressed in NBLs, (2) construction of a DNA chip and analysis of tumor samples, (3) computational analysis of gene expression data and identification of prognosis-related genes, and (4) conversion of these findings into better diagnosis and treatment. In this review, we discuss the recent attempts of large-scale molecular profiling of NBL and their future applications to the clinic.

2. Prognostic markers for neuroblastoma

In addition to conventional prognostic markers such as age, INSS stage, *MYCN* copy number, histology, and DNA ploidy, expression levels of several genes have recently been added as new

indicators. They include inverse relationship of *TRKA* and *TRKB* expression [3,4], *telomerase* [5], *CD44* [6], *pleiotrophin* [7], *N-cadherin* [8], *H-RAS* [9], and *CDC10* [10] (Table 1). From the analyses of genomic aberrations occurred in NBLs, several candidate genes that exhibit overexpression in advanced NBL were identified such as *survivin* [11], *NM23-H1* and *NM23-H2* [12,13], and *PPM1D* [14]. These genes are located on chromosome 17q, which is known to be frequently increased chromosomal copies in advanced NBLs.

3. Identification of novel prognosis-related genes

Various genomic approaches have been used to identify differentially expressed genes among different tissue and tumor types, including differential hybridization screening, representational difference analysis (RDA), gene counting using cDNA libraries followed by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) screening, serial analysis of gene expression (SAGE), suppression subtractive

hybridization (SSH), and cDNA and oligonucleotide microarrays.

In order to collect a large number of genes expressed in various type of NBLs, we have constructed oligo-capping cDNA libraries from primary NBL tissues with different biological characteristics: the tumors with favorable (F; stage 1, single copy of *MYCN*, high *TrkA* expression) and unfavorable (UF; stage 3 or 4, amplification of *MYCN*, no expression of *TrkA*) characteristics and the stage 4 S tumor [15,16]. Ten thousands of clones in total were isolated from those libraries, which corresponded 5,340 independent genes, and approximately 40% of those were shown to contain novel sequences by database search [16]. To identify the genes expressed differentially between the F and UF subsets, all independent clones except housekeeping genes were subjected to semi-quantitative RT-PCR analysis using RNAs obtained from 16 F and 16 UF NBL tissues as templates. From this project, we have identified more than 500 genes differentially expressed between F and UF NBLs. These included many novel genes with unknown functions, including endothelin-converting enzyme-like 1 (*XCE/ECE1*) [17], neuronal leucine-rich repeat family members (*NLRR1* and *NLRR3*) [18], tubulin tyrosine ligase (*TTL*) [19], novel putative apoptosis-related gene with BCH domain (*BMCCI*, Machida et al., manuscript in preparation), a member of LIM-only protein (*LMO3*) (Aoyama et al., under submission), and NEDD4-like ubiquitin E3 ligase (*NEDL1* and *NEDL2*) (Miyazaki et al., manuscript in preparation) (Table 1). All these genes were analyzed by quantitative real-time RT-PCR method and confirmed to be strongly related to patient prognosis of NBLs. These genes are now being investigated by functional analysis.

Roberts et al. [20] have applied SSH technique to identify potential NBL biomarkers that may improve outcome prediction, and identified differential expression of members of the GABAergic gene family in NBL. They found that low levels of gamma-aminobutyric acid (GABA) receptor-associated protein (*GABARAP*) gene expression predict decreased survival, and that GABA(A) delta receptor subunit gene expression was predictive of a poor outcome among stage 4S patients.

4. Expression profiling of neuroblastomas by microarray approach

Recently, the DNA microarray method has been applied to comprehensively demonstrate the expression profiles of primary NBLs and cell lines (Table 2). The first microarray-based gene expression profiling study of NBL was reported by Khan et al. [21], who demonstrated that the small, round blue-cell tumors (SRBCTs), including NBL, rhabdomyosarcoma, non-Hodgkin lymphoma, and the Ewing family of tumors, which is often present diagnostic dilemmas in clinical practice, could be distinguished on the basis of their patterns of gene expression using artificial neural networks (ANNs). Among these SRBCTs, they showed that 6 of 7 test samples with NBL were classified correctly by using 93 unique genes. Among the 93 classifier genes, 15 genes were highly and specifically expressed in NBLs. This finding is very important when diagnostic tool is going to apply the gene expression data, because it is the first point to be confirmed whether the tumor, which is to be examined, is NBL or not.

Subsequent microarray studies have facilitated class separation of differentiating NBL tumors from poorly differentiated tumors and of high-risk tumors from low-risk tumors. Yamanaka et al. [22] examined 14 NBLs by 23,040 cDNAs microarray, and identified 78 genes whose expression levels were significantly different between differentiating NBLs and poorly differentiated NBLs. The 78 genes included those associated with cell maturation and apoptosis; 15 genes that were up-regulated in stage 4 tumors included those encoding cell adhesion molecules and cytoskeleton proteins. Berwanger et al. [23] examined expression profiles from 94 primary NBL specimens using a 4,608 cDNA human unigene chip. They found 24 significant genes differentially expressed between stage 1 ($n=19$) and stage 4 ($n=21$) *MYCN* non-amplified tumors. Interestingly, a significant percentage of the 24 genes encoded those involved in signaling through the nonreceptor tyrosine kinase Fyn and the actin cytoskeleton. These genes were coordinately down-regulated in advanced stage NBL, both in *MYCN* amplified and nonamplified tumors (Table 2). They also showed that expression of *FYN* predicts long term survival of NBL patients, independently of *MYCN* amplification. Takita et al. [24] performed DNA microarray analysis on 20

Table 2
Differentially expressed genes identified by gene expression profiling using microarray

	Genes identified	Genes on microarray	Sample number	Category
NBL diagnosis				
Khan et al. [21]	15 genes	6,567cDNAs	16 NBLs	For SRBCTs diagnosis <i>DPYSL4, CDH2, AFIQ, CRMP1, KIF3C, GAP43, MAP1B, RCV1, SFRP1, GATA2, PFN2, FHL1</i> (highly and specifically expressed in NBLs)
Expression profiling				
Yamanaka et al. [22]	78 genes	23,040 cDNAs	14 NBLs	Differentiating NBLs vs. poorly differentiated NBLs <i>ITGE, SYP, OLIG2, MADH2, DFFB, CASP8, CASP9</i> (up-regulated in differentiating NBLs) <i>CLDN5, CCND1, NFKBIL2</i> (up-regulated in poorly differentiated NBLs)
Berwanger et al. [23]	36 genes	4,608 cDNAs	94 NBLs	Stage 1 vs. stage 4 <i>MYCN</i> non-amplified tumors <i>FYN, AFAP, CTNNA1, NRCAM, tropomodulin, MARCKS</i> (down-regulated in advanced stage NBL)
Takita et al. [24]	3 genes	1,700 genes	20 NBLs	Stage 1 vs. stage 4 <i>BIRC3, CDKN2D</i> (up-regulated in the early-stage group) <i>SMARCD3</i> (down-regulated in the early-stage group)
Hiyama et al. [25]	123 genes	6,272 cDNAs	20 NBLs	Unfavorable vs. favorable (regressing and maturing) 43 genes including <i>MYCN, hTERT, NME1, CCND1, CCNE1, E1, BIRC5, BIRC1</i> (up-regulated in unfavorable NBLs) 80 genes (up-regulated in favorable NBLs) <i>CD44, IGF2, TRKA, ANKI</i> (highly expressed in maturing NBLs) <i>CASP8, CASP9, TNFSF10, NGFA, GDF10</i> (highly expressed in regressing NBLs)
McArdle et al. [26]		14,500 genes	20 NBLs	Differentially expressed in the 11q-, <i>MYCN</i> non-amplified and hyperdiploid subtypes of NBL
Janoueix-Lerosey et al. [27]		320 genes (on 1p35-36)	43 NBLs	1p loss vs. 1p-normal <i>CDC42, VAMP3, CLSTN1, GNBI, STMN1, RPA2, RBAF600, FBXO6, MAD2L2</i> (decreased expression in NBLs with 1p deletion)
Prognosis prediction				
Wei et al. [28]	19 genes	42,578 cDNA	56 NBLs	To develop an accurate predictor of survival for patient with NBL <i>DLK1, PRSS3, ARC, SLIT3, MYCN, JPH1</i> (up-regulated in the poor-outcome group) <i>ARHI, CNRI, CD44, ROBO2, BTBD3, KLRC3</i> (down-regulated in the poor-outcome group)
Ohira et al. [29]	70 genes	5,340 cDNAs	136 NBLs	To develop an accurate predictor of survival for patient with NBL

primary tumors (stage 1 versus 4) and identified that the expression of *BIRC3* and *CDKN2D* genes were significantly higher in the early-stage group than in the advanced-stage group and that the expression of the *SMARCD3* gene was significantly reduced in the early-stage group. The *BIRC3*, *CDKN2D*, and *SMARCD3* genes have been reported to be associated with apoptosis, cell cycles, and the transcriptional activator, respectively. Hiyama et al. [25] analyzed 20 NBLs with 6272 cDNAs microarray, and revealed that 43 genes,

including *MYCN*, *hTERT*, *NM23-H1*, cell cycle regulatory protein-coding genes (*CCND1*, *CCNE1*, *E1*), and apoptosis-escape genes (*BIRC5*, *BIRC1*) were highly expressed in unfavorable neuroblastomas, while another 80 genes, including neuronal differentiating genes and apoptotic inducing genes (*CD44*, *IGF2*, *TRKA*, *ANKI* in maturing NBLs, *CASP8*, *CASP9*, *TNFSF10*, *NGFA*, *GDF10* in regressing NBLs) were detected as highly expressed in favorable tumors.

There have been several studies by focusing the certain genomic aberrations and corresponding gene expression profiles. McArdle et al. [26] identified transcripts that are differentially expressed in the 11q-, *MYCN* nonamplified and hyperdiploid subtypes of NBL. Janoueix-Lerosey et al. [27] compared the expression profiles between the tumors with 1p loss and those with normal 1p status and identified the genes with decreased expression in NBLs with 1p deletion (Table 2).

5. Microarray-based system for predicting prognosis of neuroblastoma patients

We now have in our hands the increasing number of information for genes which can distinguish prognosis of the patient with NBL as described above. These data should subsequently be integrated and organized to construct a simple prediction system which is practical for the clinic. Such efforts are now ongoing.

Wei et al. [28] have performed gene expression profiling of 56 NBLs using cDNA microarrays containing 42,578 cDNA clones and used artificial neural networks (ANNs) to develop an accurate predictor of survival for each individual patient with NBL. ANN-based prognosis prediction has been accomplished by using expression levels of only 19 genes including *MYCN* and *CD44*. In addition, these 19 predictor genes were able to additionally classify high-risk patients into two subgroups according to their survival status. Among these predictor genes, *DLK1*, *PRSS3*, *ARC*, *SLIT3*, *MYCN* and *JPH1* were up-regulated in the poor-outcome group included, whereas *ARHI*, *CNRI*, *CD44*, *ROBO2*, *BTBD3* and *KLRC3* were down-regulated.

We also have constructed an in-house, microceramic pump-based ink-jet-printed cDNA microarray carrying 5,340 genes obtained from primary NBL cDNA libraries and applied it for the analysis of 136 tumors. A probabilistic output computational analysis using learning samples has selected 70 genes which constructed a classifier for patient outcome, and provided a correct prognosis of test samples with high efficiency [29]. Of clinical interest, Kaplan-Meier analysis indicated that the classifier can divide significantly 5-year survivals of NBL patients, even for the intermediate risk type. Furthermore, our

microarray prediction exhibited the best balance between sensitivity and specificity among prognostic factors including *MYCN* amplification and *TrkA* expression.

These findings provide evidence of a gene expression signature that can predict prognosis independent of currently known risk factors and could assist physicians in the individual management of patients with high-risk NBL. Such gene expression-based diagnosis system should be highly accurate and reproducible, as well as simple, easy to analyze, and with low cost. Based on this, we subsequently made a mini-chip carrying top-ranked 200 genes for clinical use. We are now investigating its (1) reproducibility from the original 5,340 genes chip, (2) potential of predicting prognosis for the newly added test samples, (3) potential of predicting prognosis when whole experimental process (RNA isolation, sample labeling, hybridization, and calculation of the probability of patient survival by using the classifier) is conducted in an independent laboratory. After confirming these, the microarray system will come to prove its practical use to be feasible in the clinic to predict the prognosis of the patient with NBL.

6. Conclusion

Using the gene expression profiling, we are now able to distinguish a group of high-risk patients with high efficiency who will not respond to conventional therapy and therefore require alternative treatment strategies. Although further prospective studies will be necessary, we may also be able to reduce the toxicity of treatment regime for the patients who have been predicted to survive according to gene expression profile. Recently, in addition to the gene expression profiling, certain genome aberrations as well as epigenetic alterations have been reported to be strongly related to the patient prognosis with NBL. Abe et al. [30] indicated that poor NBLs suffer the increased methylation pressure in their tumor genome, and that the methylation of certain CpG island, such as *Protocadherin beta* family, can predict poor NBL prognosis with high sensitivity. Furthermore, screening of prognosis-related proteins secreted into serum of NBL patients has been started by many groups. Combination of such independent systems to predict

prognosis will further improve the accuracy of the diagnostic system for NBL.

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References

- [1] R.P. Bolande, The neurocristopathies: a unifying concept of disease arising in neural crest maldevelopment, *Hum. Pathol.* 5 (1974) 409–429.
- [2] J.L. Weinstein, H.M. Katzenstein, S.L. Cohn, Advances in the diagnosis and treatment of neuroblastoma, *Oncologist* 8 (2003) 278–292.
- [3] A. Nakagawara, M. Arima-Nakagawara, N.J. Scavarda, C.G. Azar, A.B. Cantor, G.M. Brodeur, Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma, *N. Engl. J. Med.* 328 (1993) 847–854.
- [4] A. Nakagawara, C.G. Azar, N.J. Scavarda, G.M. Brodeur, Expression and function of TRK-B and BDNF in human neuroblastomas, *Mol. Cell Biol.* 14 (1994) 759–767.
- [5] E. Hiyama, K. Hiyama, T. Yokoyama, Y. Matsuura, M.A. Piatyszek, J.W. Shay, Correlating telomerase activity levels with human neuroblastoma outcomes, *Nat. Med.* 1 (1995) 249–255.
- [6] M.C. Favrot, V. Combaret, C. Lasset, CD44—a new prognostic marker for neuroblastoma, *N. Engl. J. Med.* 1993; 329.
- [7] A. Nakagawara, J. Milbrandt, T. Muramatsu, T.F. Deuel, H. Zhao, A. Cnaan, G.M. Brodeur, Differential expression of pleiotrophin and midkine in advanced neuroblastomas, *Cancer Res.* 55 (1995) 1792–1797.
- [8] R. Shimono, S. Matsubara, H. Takamatsu, T. Fukushima, M. Ozawa, The expression of cadherins in human neuroblastoma cell lines and clinical tumors, *Anticancer Res.* 20 (2000) 917–923.
- [9] T. Tanaka, T. Sugimoto, T. Sawada, Prognostic discrimination among neuroblastomas according to Ha-ras/trk A gene expression: a comparison of the profiles of neuroblastomas detected clinically and those detected through mass screening, *Cancer* 83 (1998) 1626–1633.
- [10] T. Nagata, Y. Takahashi, S. Asai, Y. Ishii, H. Mugishima, T. Suzuki, et al., The high level of hCDC10 gene expression in neuroblastoma may be associated with favorable characteristics of the tumor, *J. Surg. Res.* 92 (2000) 267–275.
- [11] A. Islam, H. Kageyama, N. Takada, T. Kawamoto, H. Takayasu, E. Isogai, et al., High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma, *Oncogene* 19 (2000) 617–623.
- [12] N. Hailat, D.R. Keim, R.F. Melhem, X.X. Zhu, C. Eckerskorn, G.M. Brodeur, et al., High levels of p19/nm23 protein in neuroblastoma are associated with advanced stage disease and with N-myc gene amplification, *J. Clin. Invest.* 88 (1991) 341–345.
- [13] A. Leone, R.C. Seeger, C.M. Hong, Y.Y. Hu, M.J. Arboleda, G.M. Brodeur, et al., Evidence for nm23 RNA overexpression, DNA amplification and mutation in aggressive childhood neuroblastomas, *Oncogene* 8 (1993) 855–865.
- [14] F. Saito-Ohara, I. Imoto, J. Inoue, H. Hosoi, A. Nakagawara, T. Sugimoto, J. Inazawa, PPM1D is a potential target for 17q gain in neuroblastoma, *Cancer Res.* 63 (2003) 1876–1883.
- [15] M. Ohira, A. Morohashi, Y. Nakamura, E. Isogai, K. Furuya, S. Hamano, et al., Neuroblastoma oligo-capping cDNA project: toward the understanding of the genesis and biology of neuroblastoma, *Cancer Lett.* 197 (2003) 63–68.
- [16] M. Ohira, A. Morohashi, H. Inuzuka, T. Shishikura, T. Kawamoto, H. Kageyama, et al., Expression profiling and characterization of 4200 genes cloned from primary neuroblastomas: identification of 305 genes differentially expressed between favorable and unfavorable subsets, *Oncogene* 22 (2003) 5525–5536.
- [17] T. Kawamoto, M. Ohira, S. Hamano, T. Hori, A. Nakagawara, High expression of the novel endothelin-converting enzyme genes, Nbla03145/ECEL1alpha and beta, is associated with favorable prognosis in human neuroblastomas, *Int. J. Oncol.* 22 (2003) 815–822.
- [18] S. Hamano, M. Ohira, E. Isogai, K. Nakada, A. Nakagawara, Identification of novel human neuronal leucine-rich repeat (hNLRR) family genes and inverse association of expression of Nbla10449/hNLRR-1 and Nbla10677/hNLRR-3 with the prognosis of primary neuroblastomas, *Int. J. Oncol.* 24 (2004) 1457–1466.
- [19] C. Kato, K. Miyazaki, A. Nakagawa, M. Ohira, Y. Nakamura, T. Ozaki, et al., Low expression of human tubulin tyrosine ligase and suppressed tubulin tyrosination/detyrosination cycle are associated with impaired neuronal differentiation in neuroblastomas with poor prognosis, *Int. J. Cancer* 112 (2004) 365–375.
- [20] S.S. Roberts, M. Mori, P. Pattee, J. Lapidus, R. Mathews, J.P. O'Malley, et al., GABAergic system gene expression predicts clinical outcome in patients with neuroblastoma, *J. Clin. Oncol.* 22 (2004) 4127–4134.

- [21] J. Khan, J.S. Wei, M. Ringner, L.H. Saal, M. Ladanyi, F. Westermann, et al., Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks, *Nat. Med.* 7 (2001) 673–679.
- [22] Y. Yamanaka, Y. Hamazaki, Y. Sato, K. Ito, K. Watanabe, T. Heike, et al., Maturational sequence of neuroblastoma revealed by molecular analysis on cDNA microarrays, *Int. J. Oncol.* 21 (2002) 803–807.
- [23] B. Berwanger, O. Hartmann, E. Bergmann, S. Bernard, D. Nielsen, M. Krause, et al., Loss of a FYN-regulated differentiation and growth arrest pathway in advanced stage neuroblastoma, *Cancer Cell* 2 (2002) 377–386.
- [24] J. Takita, M. Ishii, S. Tsutsumi, Y. Tanaka, K. Kato, Y. Toyoda, et al., Gene expression profiling and identification of novel prognostic marker genes in neuroblastoma, *Genes Chromosomes Cancer* 40 (2004) 120–132.
- [25] E. Hiyama, K. Hiyama, H. Yamaoka, T. Sueda, C.P. Reynolds, T. Yokoyama, Expression profiling of favorable and unfavorable neuroblastomas, *Pediatr. Surg. Int.* 20 (2004) 33–38.
- [26] L. McArdle, M. McDermott, R. Purcell, D. Grehan, A. O'Meara, F. Breatnach, et al., Oligonucleotide microarray analysis of gene expression in neuroblastoma displaying loss of chromosome 11q, *Carcinogenesis* 25 (2004) 1599–1609.
- [27] I. Janoueix-Lerosey, E. Novikov, M. Monteiro, N. Gruel, G. Schleiermacher, B. Lorigod, et al., Gene expression profiling of 1p35-36 genes in neuroblastoma, *Oncogene* 23 (2004) 5912–5922.
- [28] J.S. Wei, B.T. Greer, F. Westermann, S.M. Steinberg, C.G. Son, Q.R. Chen, et al., Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma, *Cancer Res.* 64 (2004) 6883–6891.
- [29] M. Ohira, S. Oba, Y. Nakamura, E. Isogai, S. Kaneko, A. Nakagawa, et al., Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate-risk neuroblastomas, *Cancer Cell* 7 (2005) 337–350.
- [30] M. Abe, M. Ohira, A. Kaneda, Y. Yagi, S. Yamamoto, Y. Kitano, et al., CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas, *Cancer Res.* 65 (2005) 828–834.

Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas

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Summary

To predict the prognosis of neuroblastoma patients and choose a better therapeutic protocol, we developed a cDNA microarray carrying 5340 genes obtained from primary neuroblastomas and examined 136 tumor samples. We made a probabilistic output statistical classifier that provided a high accuracy in prognosis prediction (89% at 5 years) and a highly reliable method to validate it. Kaplan-Meier analysis indicated that the patients in an intermediate group defined by existing markers are divided by microarray into two further groups with 5 year survivals for 36% and 89% of patients ($p < 10^{-4}$), i.e., with unfavorably and favorably predicted neuroblastomas, respectively. According to these results, we developed a gene subset chip for a clinical tool, for which our classifier exhibited 88% prediction accuracy.

Introduction

Neuroblastoma is one of the most common solid tumors in children and originates from the sympathoadrenal lineage of the neural crest (Bolande, 1974). Its clinical behaviors are heterogeneous. The tumor, when developed in infants, frequently regresses spontaneously by inducing differentiation and/or programmed cell death. When developed in children over 1 year of age, however, the tumor is often aggressive and acquires resistance to intensive chemotherapy. Although recent progress in therapeutic strategies against advanced neuroblastoma has improved patient survival, long-term outcomes still remain very poor. Furthermore, part of neuroblastomas categorized to the intermediate group (stage 3 or 4 tumors that possess a single copy of the *MYCN* gene) often recurs after complete response to initial therapy. Such differences in the final outcomes of the tumor are considered presumably attributable to differences in genetic and biological abnormalities, which are reflected in the gene and protein expression profiles of the tumor.

The prediction of cancer prognosis is one of the most urgent demands to initiate the treatment of neuroblastoma. As expected from the natural course of neuroblastoma, patient age at diagnosis (over or under 1 year of age) is an important prognostic factor (Evans et al., 1971). Disease stage is also a powerful indicator for neuroblastoma prognosis (Brodeur et al., 1993). Moreover, recent advances in basic research have discovered several molecular markers that are useful in clinical practice, including amplification of the *MYCN* oncogene (Schwab et al., 1983; Brodeur et al., 1984), DNA ploidy (Look et al., 1984; Look et al., 1991), deletion of chromosome 1p (Brodeur et al., 1988), and *TtkA* expression (Nakagawara et al., 1992; Nakagawara et al., 1993). Other indicators also include *telomerase* (Hiyama et al., 1995), *CD44* (Favrot et al., 1993), *pleiotrophin* (Nakagawara et al., 1995), *N-cadherin* (Shimono et al., 2000), *CDC10* (Nagata et al., 2000), and *Fyn* (Berwanger et al., 2002). However, the combinations thereof still frequently fail to predict patient outcome. In the post-genome sequence era, therefore, the advent of new diagnostic tools has been ex-

SIGNIFICANCE

Neuroblastoma is an enigmatic tumor with heterogeneous clinical behaviors including maturation, regression, and growth. Despite recent improvements in the cure rate of many pediatric tumors, the prognosis of advanced neuroblastoma is still poor. In addition, it is usually difficult to predict the prognosis of the intermediate risk group in advanced stages without *MYCN* amplification. Through our supervised machine learning and highly reliable statistical validation procedure with the 5 year prognosis of the patients, we established a simple, low-cost microarray system carrying top-ranked genes, which exhibited high accuracy (88%) to predict the neuroblastoma prognosis and is highly feasible as a clinical tool.

pected. Recently, the DNA microarray method, applied to comprehensively demonstrate expression profiles of primary neuroblastomas and cell lines, has already identified the following: (1) differences in gene expression between favorable and unfavorable subsets (Yamanaka et al., 2002; Berwanger et al., 2002); and (2) differences in gene expression that occur during retinoic acid-induced neuronal differentiation (Ueda, 2001). However, a study to predict neuroblastoma prognosis with a microarray using a large number of neuroblastoma samples has never been reported. We have recently isolated 5500 genes from the cDNA libraries, which were generated from primary neuroblastomas, part of which has previously been reported (Ohira et al., 2003a; Ohira et al., 2003b). In this study, to identify genes strongly associated with neuroblastoma prognosis and to apply them to make a really practical cDNA microarray for neuroblastoma diagnosis, we constructed an in-house, ink-jet-printed cDNA microarray carrying 5340 genes proper to neuroblastoma and applied it to analyze 136 samples. After selecting genes significantly related to patient prognosis, we made a mini-chip carrying 200 top-ranked genes to apply for the clinic.

There have been many attempts to predict cancer outcome using microarray. A reliable prediction for outcomes of cancer patients naturally demands its reproducibility, and it is quite important to use sound and highly reliable statistical methodologies; a complete crossvalidation analysis without introducing any information leakage and an independent test using new samples are necessary. As Ntzani and Ioannidis (2003) pointed out, however, such a careful methodology has often been ignored in most microarray studies. We here developed a supervised classification method without any information leakage as a statistic tool and demonstrated that the probabilistic output of the analysis defines the molecular signature of neuroblastoma to predict its prognosis. Although the construction of the statistical tool was based on one of the most reliable statistical tests, we also consulted a validation test for an independent experiment examining 50 samples (whose RNAs were prepared in an independent laboratory) by using the mini-chip. The high performance for the outcome prediction by the mini-chip system suggests the high feasibility of developing a clinical tool based on molecular signature.

Results

Neuroblastoma proper cDNA microarray

The whole scheme of our study is summarized in Figure 1. We first constructed a neuroblastoma proper cDNA microarray harboring the spots of 5340 genes on a slide glass by using a ceramics-based ink-jet printing system (the 5340 genes system). This in-house cDNA microarray appeared to have overcome the previous problems caused by pin-spotting, e.g., uneven quantity or shape of individual spots on an array. Ten micrograms each of the total RNA extracted from 136 frozen tissues of primary neuroblastomas were labeled with Cy3 dye. As a common reference, the mixture of the total RNA obtained from four neuroblastoma cell lines with a single copy of *MYCN* (NB69, NBLS, SK-N-AS, and SH-SY5Y) was labeled with Cy5 dye.

We first evaluated the quality of our cDNA microarray, the 5340 genes system. The log Cy3/Cy5 fluorescence ratio of

each gene spot was normalized to eliminate intensity-dependent biases. Since the 5340 genes array contains 260 duplicated or multiplicated genes, the expression ratio of such a duplicated gene was represented by the average of multiple spots. Based on estimation performance for missing values (see the Supplemental Data available with this article online) and on reproduction variance of the duplicated genes, the standard deviation for the log ratio of a single gene was sufficiently small, ranging between about 0.2 and about 0.3 (Figure S1A). The scatter plots of the log Cy3/Cy5 fluorescence ratio between duplicated gene spots in the 136 experiments and those between repeated experiments also indicated high reproducibility of spotting and experiment (Figures S1B and S1C). These suggest that the production of and experiments by our cDNA microarray are highly reproducible.

Supervised classification

To develop a statistical tool that predicts the prognosis of a new patient with neuroblastoma, we introduced a supervised classification. In the development, we used 136 neuroblastomas, randomly selected tumor samples from the neuroblastoma tissue bank, consisting of 41 stage 1 tumors, 22 stage 2 tumors, 33 stage 3 tumors, 28 stage 4 tumors, and 12 stage 4s tumors. The follow-up duration ranged between 3 and 241 months (median, 56 months, mean, 57.3 months) after diagnosis. The left panel in Figure 2 compiles summary information of each sample, including survival time and important prognosis markers (see Experimental Procedures for details). Since variations in follow-up duration generated noises in the supervised classification, we used patient outcome (dead or alive) at 5 years after diagnosis as the target label to be predicted. Since the outcomes of 40 of 136 samples were unknown at 5 years after diagnosis, data for 96 remaining samples were used subsequently. When we were interested in short-term outcome prediction, the target label was set at 2 years after diagnosis, for which purpose 126 samples out of the 136 samples were used.

We constructed the weighted voting as a supervised classifier after important genes were selected according to pairwise *F* scores. To estimate the prediction accuracy for new data, we consulted leave two out (LTO) analysis, which obtains almost unbiased estimation of prediction accuracy for new data while avoiding overestimation due to information leakage (Figure S2A). Although it is known that the prediction accuracy of a supervised classifier depends on the number of genes to be used (Figure S3), the LTO procedure enables us to optimize it without introducing information leakage, by using a sample left out at the outer loop of the double-loop procedure (see Experimental Procedures). The crossvalidation accuracy for the 5 year prognosis prediction was as high as 88.5% (sensitivity of 86.7% and specificity of 89.4%) (Table 1, "Whole cases"). In the LTO analysis, we selected genes and constructed the corresponding classifier individually for the outcome prediction of each sample. The average number of the selected genes, *n*, was 30.7. If we applied the same procedure to the short-term (2 year) prediction, the accuracy, sensitivity, and specificity were 89.8%, 88.0%, and 90.2%, respectively (data not shown).

Construction of a probabilistic output

According to the LTO analysis, we can obtain weighted vote values and the corresponding survival rates. After approximat-

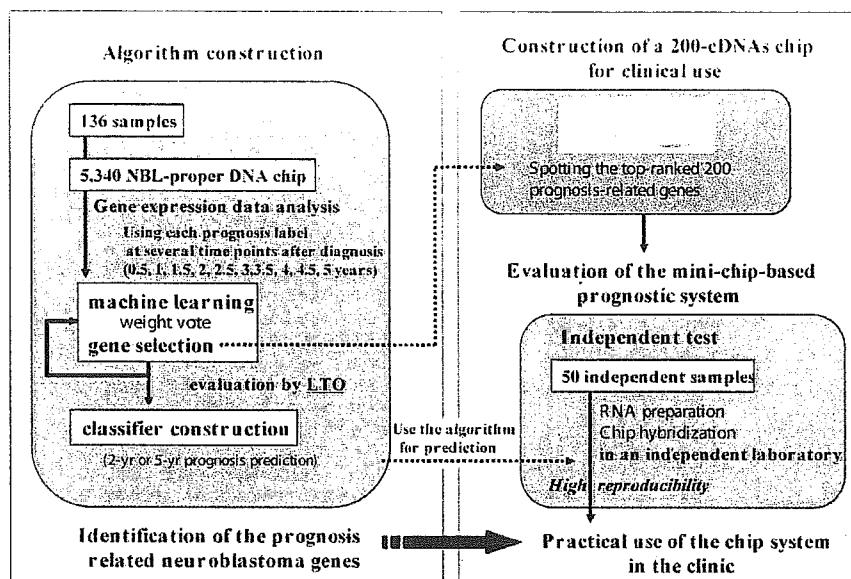


Figure 1. Schematic diagram of this study

ing a nonlinear transformation from weighted vote values to the survival rates, the transformation outputs the reliability of each sample's outcome prediction as a probabilistic output, posterior probability. We suppose each posterior probability, a real number between 0 and 1, corresponds to the expected 5 year survival rate. The right upper panel of Figure 2 shows the predictions for the 136 samples as posterior probabilities. Most of the samples alive at 5 years after diagnosis (blue mark) are found to have posterior values near 1, while most of the dead samples (red mark) have those near 0. It is known that it is difficult to predict the prognosis of neuroblastoma patients of the intermediate risk group (the type II subset: stage 3 or 4, without amplification of *MYCN*), denoted by green area. The posterior values are likely to take intermediate values near 0.5; however, their binarization after being separated by threshold 0.5 shows good accordance with the actual outcome. Frequencies of posterior values for alive and dead samples are shown in the right middle panel. The rate of alive samples among the whole samples, which denotes the actual survival rate, is plotted against each posterior value in the right bottom panel in Figure 2; this panel shows the good correspondence between the posterior value and the survival rate.

Probabilistic outputs are considered to be advantageously useful as compared with conventional binary outputs when used in making a clinical assessment and may be considered identical to them if establishing an appropriate threshold value. The real-valued posterior can be used for categorization into arbitrary number of groups. For example, dividing the posterior values into three by setting thresholds 0.3 and 0.7, we obtain three groups whose survival curves are significantly different from each other; this tertiary categorization provides another definition of intermediate risk group based only on expression patterns (Figure S4).

Comparing the survival curves

Figure 3A shows survival curves for favorable and unfavorable patients predicted by the classifier with a binary threshold (0.5).

The 5 year survival rate for the former ($n = 98$) was as good as 94%, while that for the latter ($n = 38$) was as poor as 33% ($p < 10^{-5}$). When 70 sporadic neuroblastomas were evaluated after excluding the tumors found by mass screening, the 5 year survival rate for the former ($n = 40$) was 85%, while that for the latter ($n = 30$) was 20% ($p < 10^{-5}$) (Figure 3B). To further evaluate the efficiency of our system, we calculated the posterior value for the intermediate subset of neuroblastoma (type II), whose prognosis is usually difficult to predict. As shown in Figure 3C, the survival curves were significantly categorized into two groups. The 5 year survival rate of patients who were predicted as favorable was 89%, while that for unfavorable patients was 36% ($p = 0.000067$). Since the age at diagnosis (≥ 1 year) is currently used as a poor prognostic factor for the type II tumors, we examined the ability of the classifier for the older patients with type II tumors. Even for such patients whose prognosis is difficult to predict, the survival rate (45%) of all 18 patients was divided solely by gene expression into the group with favorable prognosis ($n = 10$; 73%) and that with poor outcome ($n = 8$; 13%) (Figure 3D). In addition, if the intermediate risk group was further separated into stage 3 tumor group and stage 4 tumor group, the posterior value was significantly related to the survival, especially for stage 3 tumors (Figure S5). These results suggest that the posterior value obtained by our statistical analysis highly efficaciously allows the classification of patient outcomes, even when the tumor is of the intermediate type.

We further compared our results to existing prognosis markers in Table 1 and found that the supervised microarray analysis showed the best sensitivity-specificity balance among the prognostic factors for predicting the outcome of neuroblastoma. When the classifier is combined with the age at diagnosis, the disease stage (stage 1, 2, or 4s versus stage 3 or 4) and the *MYCN* amplification, accuracy, sensitivity, and specificity increased up to 95.8%, 93.3%, and 97.0%, respectively. Although the currently used markers (age, stage, and *MYCN*)

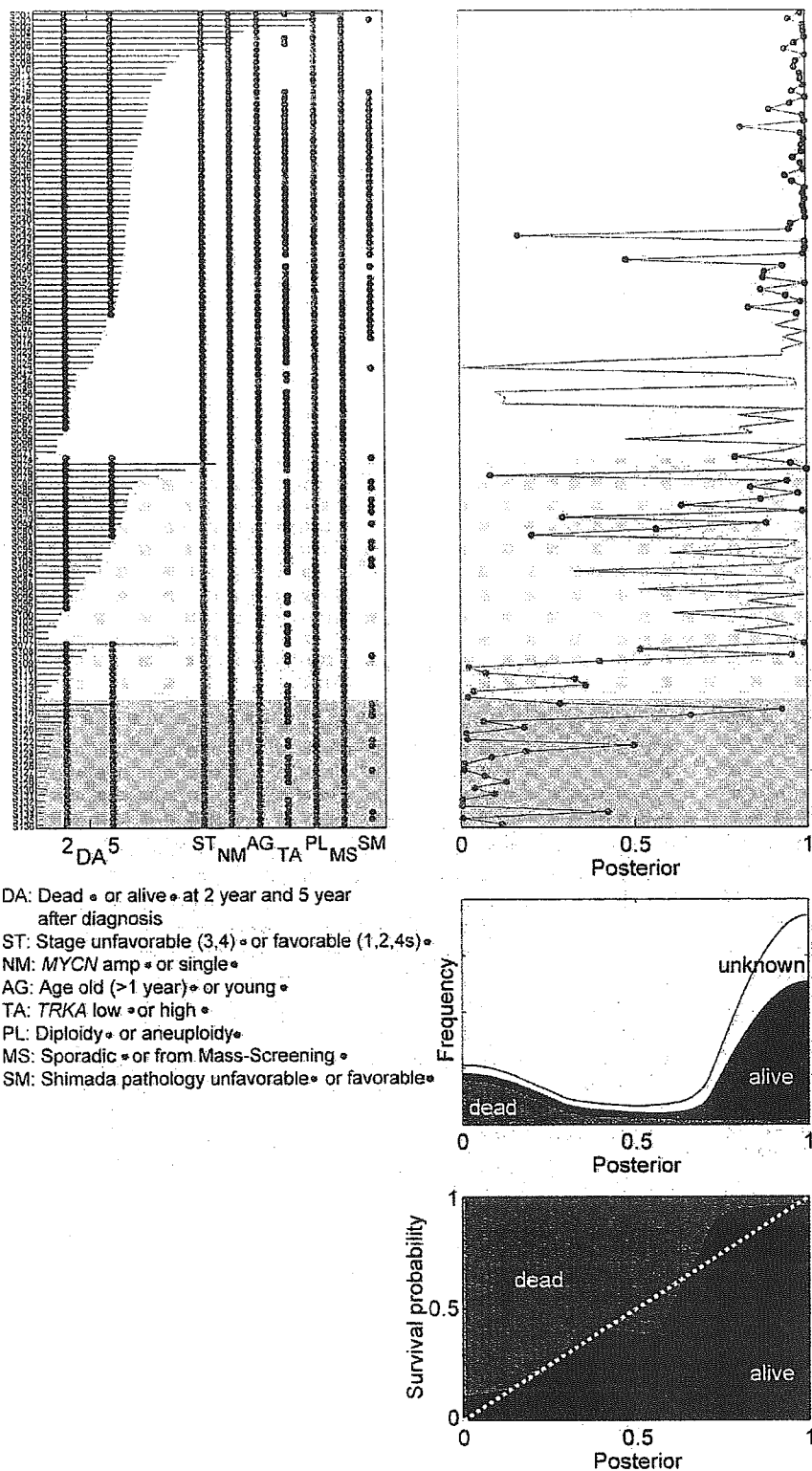


Figure 2. Posterior probability of survival at 5 years

Posterior probability of survival at 5 years for 136 training data samples, output by the leave two out (LTO) crossvalidation without any information leakage. Left panel: Neuroblastoma samples. A red or blue horizontal line denotes survival period after diagnosis for a dead or alive patient, respectively. Red and blue marks denote various clinical properties of patients; see text below the panel for detailed explanation. Background colors show groups determined by stage and MYCN amplification status: red, type III, with MYCN amplification; green, type II, with single copy of MYCN at unfavorable stage (3 or 4); and blue, type I, with single copy of MYCN and at favorable stage (1, 2, or 4s). Right upper panel: The LTO crossvalidated prediction (posterior) for each patient; a red or a blue mark denotes that the patient is dead or alive at 5 years, respectively. Right middle panel: Cumulative smooth histogram of posterior probabilities for patients of dead (red), alive (blue), and unknown (white) at 5 years after diagnosis. Right lower panel: The horizontal and vertical axes denote the posterior and the empirical probability of 5 year survival, i.e., the ratio of the smooth histogram values between dead and alive patients, shown in the middle panel, respectively. Because the border between dead and alive samples is close to the white broken line ($x = y$), the posterior can be regarded as a 5 year survival chance rate.

Table 1. Accuracy of each marker for prognosis prediction (5 years after diagnosis)

	Whole cases				Sporadic cases		Intermediate and old age ^a	
	n	accuracy	sensitivity	specificity	n	accuracy	n	accuracy
Microarray classifier	136	89%	87%	89%	56	82%	14	86%
Age (less than 1 year old)	136	81%	83%	80%	56	71%	14	64%
Stages (1, 2, and 4s)	136	83%	97%	77%	56	84%	14	64%
Shimada classification (unfavorable)	62	87%	78%	89%	25	72%	(n < 10)	—
Hyperdiploidy (aneuploidy)	62	72%	67%	73%	27	56%	(n < 10)	—
MYCN amplification	136	89%	67%	99%	56	80%	14	36%
Microarray + age + stages + MYCN*	136	96%	93%	97%	56	93%	14	86%

Sensitivity/specificity is the rate of unfavorably/favorably predicted samples, i.e., LTO posterior $<0.5/>0.5$, among actually unfavorable/favorable samples. Microarray classifier, supervised classification based on the microarray data. *By this classifier, all patients with the MYCN amplification are predicted as unfavorable, and all patients with a single copy of MYCN and at stage 1, 2, or 4s are predicted as favorable. In the remaining intermediate samples (with a single copy of MYCN and at stage 3 or 4), the patients with age <1 year are predicted as favorable, and the microarray predictions are applied for those with age >1 year.

^aAge at diagnosis >1 year.

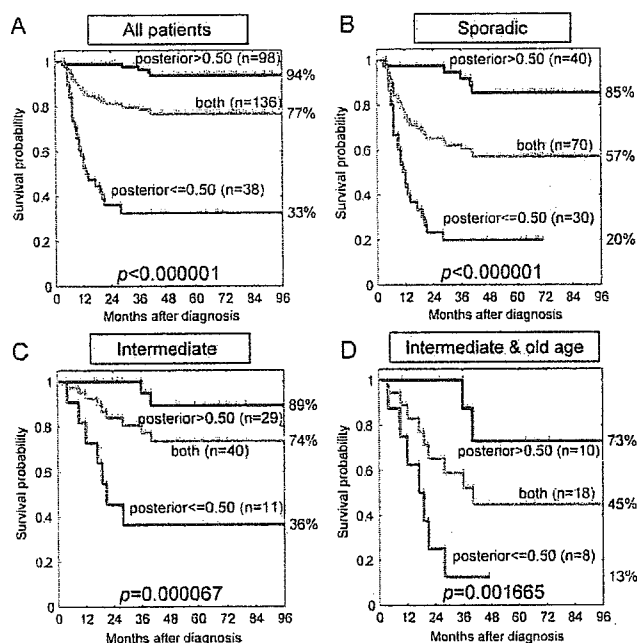


Figure 3. Disease-free survival of patients who were stratified based on the gene expression patterns

For each of the four figures, whole objective patients (green) are divided into favorable (blue) or unfavorable (red) based on the posterior values with threshold 0.5, which are calculated from gene expression patterns, and statistical features of their survival times are denoted by the Kaplan-Meier survival curves. The differences of the survival curves between the favorable (blue) and unfavorable (red) groups are evaluated by p values of the log rank test.

A and B: Survival analysis of whole and sporadic patients, respectively, divided by the supervised classifier based on microarray data.

C and D: Survival analysis of patients in the intermediate risk group with different definitions, divided by the supervised classifier. The intermediate risk group shown in (C) is defined as MYCN single and stage 3 or 4 (type II), and that in (D) is defined as MYCN single, stage 3 or 4, and older than 1 year of age.

also showed good potential to predict generally but less than the microarray, these exhibited only 64% accuracy of prediction for the type II tumors with ≥ 1 year of age (Table 1). Together with the results of survival analysis, the microarray classifier is revealed to be a powerful predictor to classify such group of neuroblastomas (86% accuracy; Table 1).

Practical application of 200 cDNAs microarray and independent test

For the practical use in the clinic, a cDNA microarray system that contains cDNA spots of a relatively small number and hence is easy to treat is expected. According to our gene selection based on the pairwise F score, the numbers of genes that were appropriate for the 5 year and 2 year prognosis prediction for all available samples were 10 and 70, respectively. In order for the system to reserve the applicability to short-term and long-term outcome prediction simultaneously, we selected 200 top-ranked genes according to the pairwise F scores in the 2 year prediction, because the 2 year prediction required larger variety of genes, and then made a smaller cDNA microarray system carrying the 200 genes. The newly designed microarray system (the mini-chip system) was evaluated by being hybridized with 5 μ g total RNA obtained from 50 independent test samples. To preserve the independence of experimental procedure, these RNAs were prepared and hybridized in a different laboratory from the original experiments of 136 samples with the 5340 genes system (see Experimental Procedures). Although the weight values in the weighted voting classifier were determined by the 5340 genes system without any information leakage from the 50 independent samples, the result was as good as that obtained by the 5340 cDNA microarray analysis (90% [45/50] for 2 year, and 87.8% [43/50] for 5 year prognosis prediction; Figure 4B). This test validated not only the prediction robustness of our classifier constructed by the 5340 genes system, but also the construction procedure of the mini-chip system according to our gene selection based on pairwise F scores. When we reconstructed another supervised classifier by applying the LTO analysis to the 50 samples measured by the mini-chip system, the accuracy of the 5 year prediction was 91.8% (45/49) (Figure 4C). These results suggest

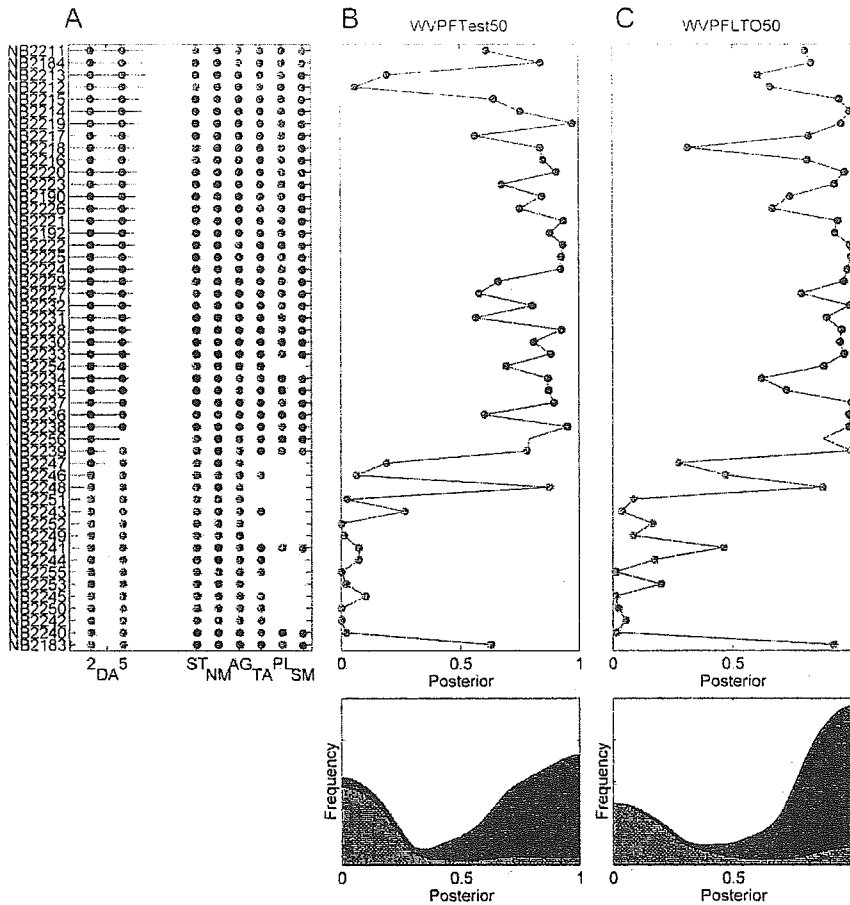


Figure 4. Posterior probability of survival at 5 years for test samples

Posterior probability of survival at 5 years for 50 independent test samples measured by newly developed 200 genes chip (the mini-chip system). Left panel: Neuroblastoma samples; see also Figure 2 legend. Center panel: Prediction results when the supervised classifier constructed from 96 training samples is applied to the 50 independent samples (independent test for the classifier's reproducibility). Right panel: LTO crossvalidation analysis using the new 50 samples (test for the procedure's reproducibility). Both tests do not introduce any information leakage. Lower panels: Smooth histograms of posterior probabilities for dead (red) and alive (blue) patients.

three things. (1) The supervised classifier obtained by the statistical analysis by the 5340 genes system is reproducible even if it is applied to the data measured by the reduced 200 genes system. Note that the 50 samples were completely new data for the classifier in this case. (2) Our procedure to construct a supervised classifier according to the LTO analysis is also reproducible, because the same procedure was successful in making another classifier with a high prediction accuracy when applied to the data taken by the mini-chip system. (3) A simple, low-cost microarray system, the mini-chip system, is highly feasible for predicting the prognosis of neuroblastoma.

Genes selected for prognosis prediction

To assess the relationship between the clinically defined subsets of neuroblastoma and the expression of 70 genes that were selected as top scored with 2 year prognosis according to the pairwise F score, we conducted an unsupervised clustering analysis (Figure 5). As expected, part of the type II (intermediate) tumors of patients with a poor prognosis showed an expression pattern that was similar to that of the type III (unfavorable) tumors, and many of them died. On the other hand, expression profiles of the remaining type II tumors seemed to be heterogeneous similarly to those of the type I (favorable)

tumors with a good outcome. Most of the tumors with highly expressed *TrkA* and hyperdiploidy, as well as tumors detected by mass screening, were included in the latter group. Table 2 shows a list of 41 genes that corresponded to the 70 top-scored genes and their p and q values (Storey and Tibshirani, 2003) in the log rank test, since we found that several genes were duplicated in the selected 70 genes. Based on the above clustering, these genes were categorized into two groups (group F and group UF; the gene groups strongly correlated with favorable and unfavorable prognosis, respectively) (Figure 5 and Table 2).

The genes in group F tended to show high levels of expression in the type I tumors, while those in group UF were highly expressed in the type III tumors. The former contained genes that were related to neuronal differentiation (*tubulin α* , *peripherin*, *neuromodulin* [*GAP43*], and *HMP19*) and genes that were related to catecholamine metabolism (*dopa decarboxylase* [*DDC*], *dopamine β -hydroxylase* [*DBH*], and *tyrosine hydroxylase* [*TH*]). On the other hand, the latter involved many members of genes that are related to protein synthesis (ribosomal protein genes such as *RPL18A*, *RPLP0*, *RPL5*, *RPL4*, and *RPL7A* as well as translation initiation and elongation factor genes *EEF1G* and *EIF3S5*) and genes that are related to me-

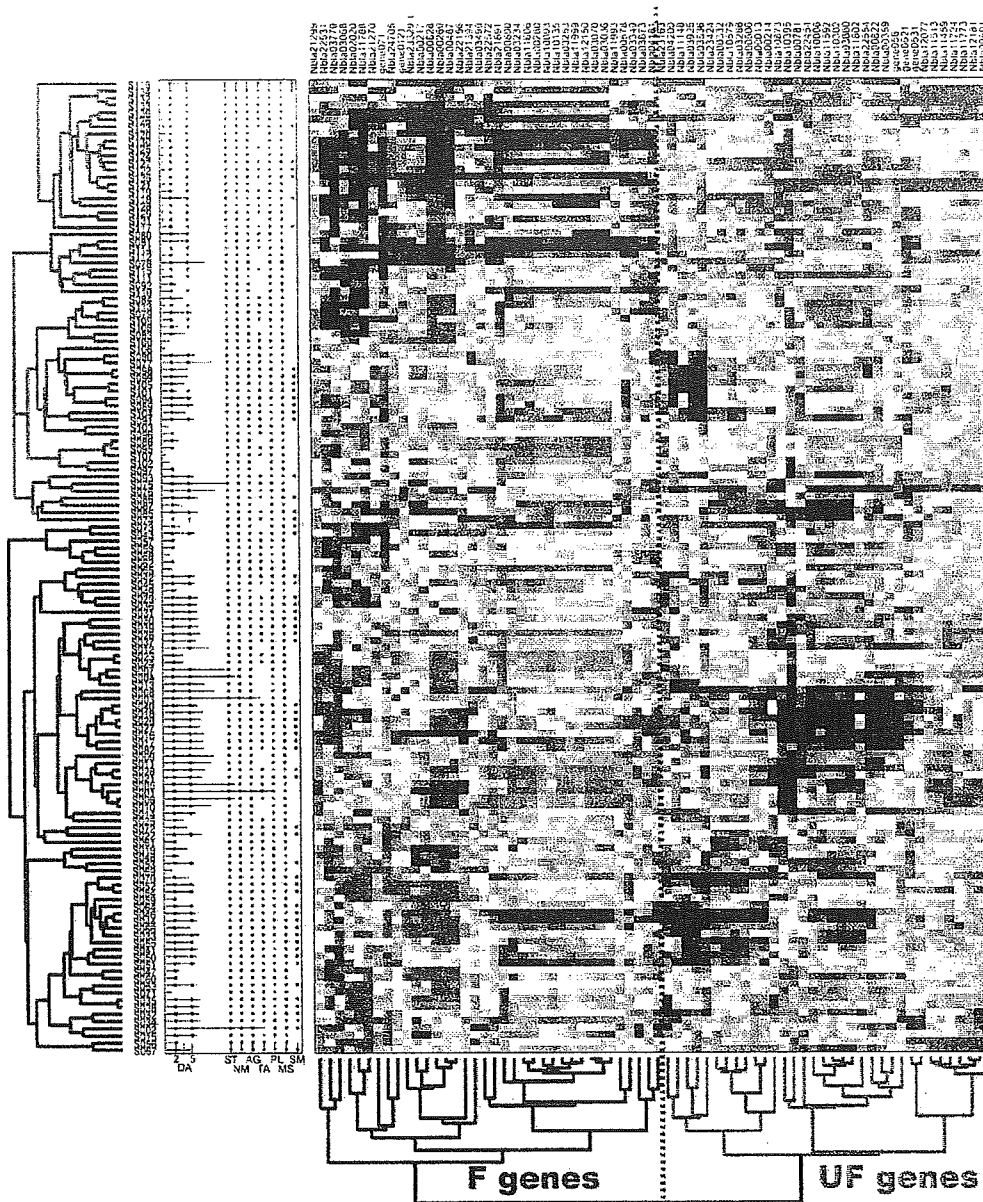


Figure 5. Expression profiles of 70 genes selected for predicting neuroblastoma prognosis at 2 years

Note that 10 genes for predicting prognosis at 5 years are also included in the 70 genes. The left and lower trees depict hierarchical clustering of the 136 neuroblastoma samples and the 70 genes selected in the present study, respectively. In the left tree, blue, green, and red colors denote "MYCN single and stage 1, 2 or 4s tumor" (type I, favorable), "MYCN single and stage 3, 4 tumor" (type II, intermediate), and "MYCN amplified tumor" (type III, unfavorable), respectively. The blue and red colors in the expression matrix show the high and low expression, respectively. A gene showing high expression level likely for unfavorable samples belongs to the group "UF" (red subtree in the lower tree), while one showing high expression likely for favorable samples belongs to the group "F" (blue subtree in the lower tree).

metabolism (*enolase 1* [*ENO1*] and *transketolase* [*TKT*]). The top 10 genes selected for the 5 year outcome prediction were *RPL18A*, *ENO1*, *EEF1G*, *TUBA3*, *GNB2L1*, *ARHGFE7*, *GCC2*, *DDX1* (duplicated), and *PRPH*. The *MYCN* gene was also a member of 70 genes (group UF) as expected; however, it was

outside of the top 10 genes for the 5 year label. Instead, *DDX1*, which is frequently coamplified with *MYCN* on chromosome 2p24, was a member of the top 10 genes (UF group) for both of the 2 year and 5 year labels. Confirmation of the differential expression of the selected genes was further conducted by

Table 2. Top-ranked genes used for prediction of 2 year and 5 year prognosis of neuroblastoma

	Spot name	Accession number	Gene code	Chromosome map	Pattern	Log rank p	q value
F group							
	Nbla11606	NM_006009	<i>TUBA3</i>	12q13.12	F > UF	0	0.000674
	Nbla00890	NM_003899	<i>ARHGEF7</i>	13q34	F > UF	0.000001	0.000743
	Nbla00260	NM_006082	<i>K-ALPHA-1</i>	12q13.12	F > UF	0.000003	0.000926
	Nbla21891	U87309	<i>VPS41</i>	7p14.1	F > UF	0.000006	0.001096
	Nbla03873	NM_006054	<i>RTN3</i>	11q13.1	F > UF	0.00001	0.001282
	Nbla11788	NM_006262	<i>PRPH</i>	12q13.12	F > UF	0.000017	0.001522
	Nbla10093	NM_000183	<i>HADHB</i>	2p23.3	F > UF	0.000018	0.001541
	Nbla22572	NM_000790	<i>DDC</i>	7p12.2	F > UF	0.000035	0.00213
	Nbla21270	NM_001915	<i>CYB561</i>	17q23.3	F > UF	0.00016	0.00495
	gene071	NM_000360	<i>TH</i>	11p15.5	F > UF	0.000787	0.012173
	Nbla03499	NM_002074	<i>GNB1</i>	1p36.33	F > UF	0.000795	0.012237
	Nbla04181	AK55112	<i>AK55112</i>	5q13.2	F > UF	0.001425	0.017462
	Nbla00487	AB075512	<i>C6orf134</i>	6p21.33	F > UF	0.002751	0.025273
	Nbla00269	NM_000787	<i>DBH</i>	9q34.2	F > UF	0.00362	0.030407
	Nbla22531	NM_002045	<i>GAP43</i>	3q13.31	F > UF	0.004394	0.034175
	Nbla22156	NM_014944	<i>CLSTN1</i>	1p36.22	F > UF	0.005233	0.038274
	Nbla00578	NM_006818	<i>AF1Q</i>	1q21.3	F > UF	0.009397	0.05354
	Nbla00217	NM_032638	<i>GATA2</i>	3q21.3	F > UF	0.010245	0.056301
	Nbla21394	NM_000743	<i>CHRNA3</i>	15q25.1	F > UF	0.072464	0.162629
	Nbla11993	NM_015980	<i>HMP19</i>	5q35.2	F > UF	0.204274	0.282486
UF group							
	Nbla00214	NM_000980	<i>RPL18A</i>	19p13.11	F < UF	0.000002	0.001107
	Nbla00013	NM_006098	<i>GNB2L1</i>	5q35.3	F < UF	0.000006	0.001051
	Nbla11459	NM_004939	<i>DDX1</i>	2p24.3	F < UF	0.000024	0.001795
	Nbla11148	NM_001002	<i>RPLP0</i>	12q24.23	F < UF	0.000049	0.002549
	Nbla00332	NM_001404	<i>EEF1G</i>	11q12.3	F < UF	0.000055	0.002696
	Nbla10395	NM_002593	<i>PCOLCE</i>	7q22.1	F < UF	0.000164	0.005009
	Nbla03286	NM_020198	<i>GK001</i>	17q23.3	F < UF	0.000175	0.005204
	Nbla23163	NM_003754	<i>EIF3S5</i>	11p15.4	F < UF	0.000341	0.007105
	Nbla10579	NM_181453	<i>GCC2</i>	2q12.3	F < UF	0.000962	0.01407
	Nbla00359	NM_003550	<i>MAD1L1</i>	7p22.3	F < UF	0.00112	0.01525
	gene052-1	NM_005378	<i>MYCN</i>	2p24.3	F < UF	0.001253	0.016367
	Nbla03925	NM_002295	<i>LAMR1</i>	3p22.2	F < UF	0.001773	0.01931
	Nbla23424	NM_001404	<i>EEF1G</i>	11q12.3	F < UF	0.003579	0.030326
	Nbla22554	NM_000687	<i>AHCY</i>	20q11.22	F < UF	0.003946	0.032409
	gene056	NM_000546	<i>TP53</i>	17p13.1	F < UF	0.004087	0.032829
	Nbla10873	NM_005762	<i>TRIM28</i>	19q13.43	F < UF	0.004984	0.037476
	Nbla00501	NM_000969	<i>RPL5</i>	1p22.1	F < UF	0.005786	0.04012
	Nbla10302	NM_001428	<i>ENO1</i>	1p36.23	F < UF	0.007702	0.048179
	Nbla04200	NM_000968	<i>RPL4</i>	15q22.31	F < UF	0.04097	0.120453
	Nbla03836	NM_000972	<i>RPL7A</i>	9q34.2	F < UF	0.048031	0.132345
	Nbla00781	NM_001064	<i>TKT</i>	3p21.1	F < UF	0.048075	0.132342

Although 70 clones were selected as important genes for the supervised classifier, duplicated and multiplicated clones are omitted in this table. The 41 genes are classified into two groups, "F > UF" and "F < UF," when the expression in favorable samples is higher than that in unfavorable samples, and vice versa, respectively. In each group, genes are sorted by log rank p values. The log rank p value for each gene was calculated by comparing survival curves of two patient groups, in which the expression of the gene is higher and lower, respectively, than the median over the samples. A "q value" of a gene denotes the estimated false discovery rate among the genes whose p value is the same or smaller than that of the gene, and is a p-like value while incorporating multiplicity of the statistical test.

using representative 16 favorable and 16 unfavorable tumor samples that were independent of the 136 samples used in the present analysis, by semiquantitative RT-PCR (Figure S6; refer also to Ohira et al., 2003a). We also conducted immunohistochemical analysis for peripherin antibody using tissue sections prepared from primary neuroblastoma with favorable and unfavorable histology, since peripherin gene is a member of the top 10 genes for both 2 year and 5 year outcome prediction (Table 2). Peripherin protein was positively detected in the cytoplasm of neuroblastic cells as well as neuritis in all three favorable histology tumors (Figure S7, FH&NA). Two unfavorable histology tumors with poorly differentiated subtype, regardless of *MYCN* status, showed sporadic staining (less than 20% of the

favorable histology tumor) for peripherin protein in neurites. Peripherin was completely negative in the unfavorable histology tumor of undifferentiated subtype (Figure S7, UF&NA). These results indicate the reliability of our gene selection. In the log rank test, p values of 18 of 20 genes in group F and of all 21 genes in group UF were less than 0.05 (Table 2), indicating that these 39 genes can be independent prognostic factors for primary neuroblastomas.

Discussion

Our study has disclosed the molecular signature of neuroblastoma that predicts patient outcomes by using RNA ob-

tained from 136 primary neuroblastomas. The highly reliable statistical analysis by using a neuroblastoma proper cDNA microarray harboring 5340 genes based on an electrically controlled ceramics-based ink-jet method led us to design a cDNA microarray system harboring 200 genes, which is applicable to short-term (2 year) and long-term (5 year) prognosis predictions for neuroblastoma.

Our study demonstrated that the supervised classifier produced by the 5340 genes system provided a high accuracy (88.5%) for the 5 year outcome prediction, with a good balance between sensitivity (86.7%) and specificity (89.4%). Although age at diagnosis, disease stage, *MYCN* amplification, and patients found by mass screening have been useful prognostic markers currently used at the bedside, most of them have either high sensitivity or high specificity (Table 1). The microarray analysis showed the best sensitivity-specificity balance among the prognostic factors for predicting the outcome of neuroblastoma. When the classifier is combined with the age at diagnosis, the disease stage (stage 1, 2, or 4s versus stage 3 or 4) and the *MYCN* amplification, accuracy, sensitivity, and specificity increased up to 95.8%, 93.3%, and 97.0%, respectively. Furthermore, the intermediate subset of neuroblastomas (type II), for which a long-term prognosis is usually difficult to make, was also categorized by microarray analysis into groups of patients with a favorable prognosis and those with an unfavorable prognosis. These successful results led us to produce a more practical tool at the bedside, the mini-chip system, whose accuracy, sensitivity, and specificity were 87.8%, 76.5%, and 93.8%, respectively, when the classifier constructed by the 5340 genes system was applied to 50 independent samples measured by the mini-chip system, and were 91.8%, 82.4% and 96.9%, respectively, when another classifier was constructed by applying the LTO procedure to the same data (Figure 4).

It is well recognized now that gene expression analyses for cancer prognosis prediction should pay close attention to the reproducibility of obtained results. A complete crossvalidation analysis without introducing any information leakage and an independent test using new samples are necessary. Although the determination of the appropriate number of genes used in supervised classifiers should be included in the validation procedure, it has often been ignored in most microarray studies. van 't Veer et al. (2002) applied the supervised classification to the breast cancer gene signature, which is predictive of a short interval to distant metastases in 78 patients who were initially devoid of local lymph node metastasis. Although their crossvalidation analysis without the validation of the number of genes correctly predicted the actual outcome of disease for 63 of 78 patients (80.7%), the accuracy was worse when a complete validation was applied (73.1%). This difference suggests that even small information leakage may lead to overestimation of the accuracy. Beer et al. (2002) applied the supervised classification to the outcome prediction of lung adenocarcinoma. Their statistical analysis was complete without any information leakage. They did not report the prediction accuracy, but we estimated the accuracy to be about 70% from the data in their paper and found that the prediction by their supervised classifier was not very superior to that by existing prognosis markers. Iizuka et al. (2003) applied the supervised classification

to the prediction of intrahepatic recurrence within 1 year after curative surgery for hepatocellular carcinoma patients. Although their predictor showed sufficiently high accuracy in an independent test with 27 samples, their crossvalidation procedure excluded the validation of the determination process of the number of optimum genes (steps 5 and 6 in their algorithm). The high crossvalidation accuracy of 100% may be an overestimation due to the information leakage.

According to the recent study that evaluated statistical methodologies used by microarray studies published between 1995 and April 2003, the three papers above were the only ones that reported both fairly sound crossvalidation analyses and independent tests (Ntzani and Ioannidis, 2003). Our LTO procedure includes the validation process of the number of genes used in the classifier and hence is a complete crossvalidation process. In addition, the obtained classifier was applied to the 50 independent samples that were measured by the reduced 200 genes system. This is a stronger test than usual independent tests but is important for the development of a practical system at the bedside. In addition, our LTO analysis achieved an almost unbiased estimation of the accuracy. Our crossvalidation analysis using the LTO procedure, the independent test of the classifier, and the validation of the procedure itself within a new experimental environment using the mini-chip system exhibited one of the most conservative and reliable statistical methodologies. In addition, our gene selection procedure according to the pairwise *F* score tries to extract correlation structures among genes, based on an idea similar to the exhaustive optimization method used in Iizuka et al. (2003), is beneficial in enhancing the applicability of the mini-chip system to various prediction problems, namely, short-term and long-term outcome predictions.

In addition to high accuracy, another advantage of our method is to provide a type of predictive information beyond the conventional binary prediction like favorable and unfavorable, which is ambiguous. The probabilistic output based on the hypothetical distribution obtained by the LTO analysis, the posterior probability, was found to show good accordance with actual survival rate (right bottom panel in Figure 2); this enables us to make a simple interpretation of the output: a patient with a posterior value of 0.8 has 80% chance for the 5 year survival, for example. Moreover, by calculating posterior probabilities for various future time points, a survival chance curve for each patient can be depicted (Figure 6). Although the follow-up period of patient "S057" is 2 years, and the patient is alive at this time, the individual survival chance curve says that his/her survival chance estimated from the gene expression pattern at diagnosis will get smaller than 50% at about 3 years after diagnosis. Such an individual survival chance curve can be used in choosing a suitable therapeutic protocol.

Another advantage of our method is that the probabilistic output is very stable in the presence of noise. Even when an artificial noise, whose variance is as large as the estimated noise variance of microarray, was added to expression profile data, prognosis prediction did not degrade very much (Figure S8). This robustness was confirmed when the noise variance went up to 1.0, which was sufficiently greater than the actual reproduction noise level of 0.4 (Figures S1A-S1C).