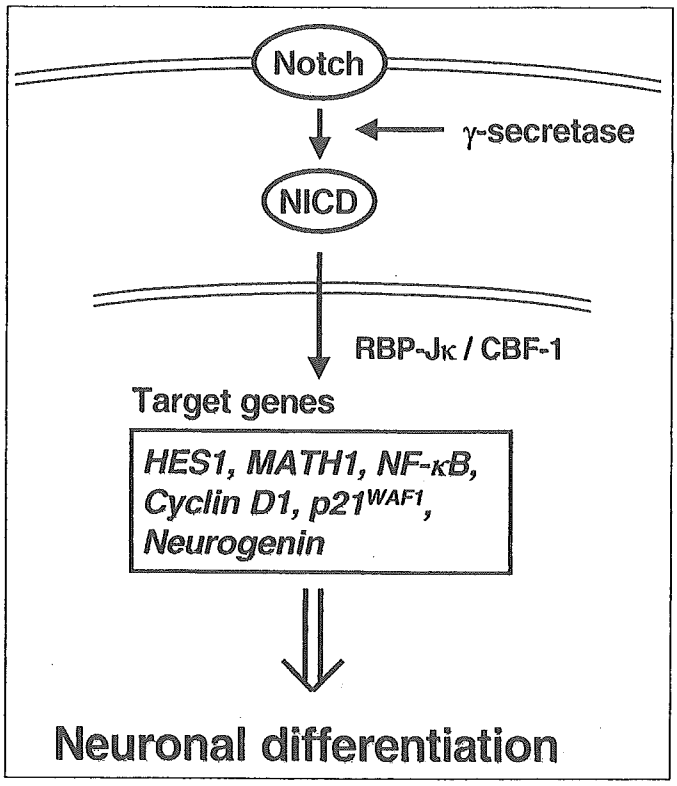


Figure 5.3

Notch signaling transactivates gene expression to induce neuronal differentiation. Binding of the ligand delta to its receptor notch triggers intramembrane proteolytic cleavage by γ-secretase. This results in the release of the notch intracellular domain (NICD), which then translocates to the nucleus where it associates with the CSL family of DNA binding proteins and transactivates gene expression. The target genes include *HES1, MATH1, NF-kB, cyclin D1, p21,* and *neurogenin. HES1* then inhibits transactivation of *MASH1* (*hASH1*)

sympathetic neurons, suggesting the important role of *MASH1* in sympathetic differentiation (Guillemot et al. 1993). *MASH1* is transiently induced during neural development to promote neuronal cell differentiation; however, high *hASH1* expression persists in neuroblastoma tumors and cell lines (Soderholm et al. 1999; Ichimiya et al. 2001). Retinoic acid treatment decreases the expression of *hASH1* and induces neurite extension (Ichimiya et al. 2001). *hASH1* also directly represses the expression of PACE4, a mammalian subtilin-like proprotein convertase that activates TGF-β-related proteins (e.g., BMPs) in neuroblastoma cell lines (Yoshida et al. 2001). The Notch signaling pathway also plays a key role during neuronal development (Axelson 2004). One of the important regulators of *hASH1* is a basic HLH protein, *HES1* (Fig. 5.3). *HES1* is regulated, at least in part, by Notch signaling and is induced at the transcription level. *HES1* directly binds to the promoter of *hASH1* and inhibits its transcriptional activation. A constitutively active form of Notch could block neurite extension during the induced differentiation of human neuroblastoma cells, possibly by inhibiting *hASH1* through the induction of *HES1* (Radtke and Raj 2003).

5.1.1.3 Phox2a and Phox2b

Phox2a and Phox2b are paired-like homeodomain transcription factors with complete conservation in their homeodomain. They are specifically expressed in noradrenergic neurons and activate the tyrosine hydroxylase and dopamine-β-hydroxylase genes (Schneider et al. 1999; Stanke et al. 1999; Ernberger 2000). While the expression of Phox2a is regulated by *MASH1*, Phox2b is not (Lo et al. 1999) (Fig. 5.4). The genetic disruption of either Phox2a or Phox2b gene de-monstrated that both genes are essential for the development of autonomic neural crest derivatives (Morin et al. 1997; Pattyn et al. 1999). Interestingly, Trochet et al. (2004) reported that the Phox2b gene was mutated in a family case of neuroblastoma and in a neuroblastoma patient with Hirschsprung's disease.

5.1.1.4 Id

Id proteins generally function as inhibitors of differentiation and as positive regulators of proliferation in neuronal development (Lavarone and Lasorella 2004). Id is a protein with the helix-loop-helix domain without a basic region and forms heterodimers with bHLH proteins, e.g., *MASH1* and *HES1* to inhibit their transactivation function (Massari and Murre 2000). In pediatric cancers, *MYC* oncoproteins and EWS-Ets fusion proteins are targeted to induce Id2 which in turn inhibits Rb and other target proteins including bHLH proteins, Ets and Pax. In neuroblastoma, *MYCN* has been shown to induce Id2 which stimulates cell proliferation by inhibiting Rb function (Lasorella et al. 2000).

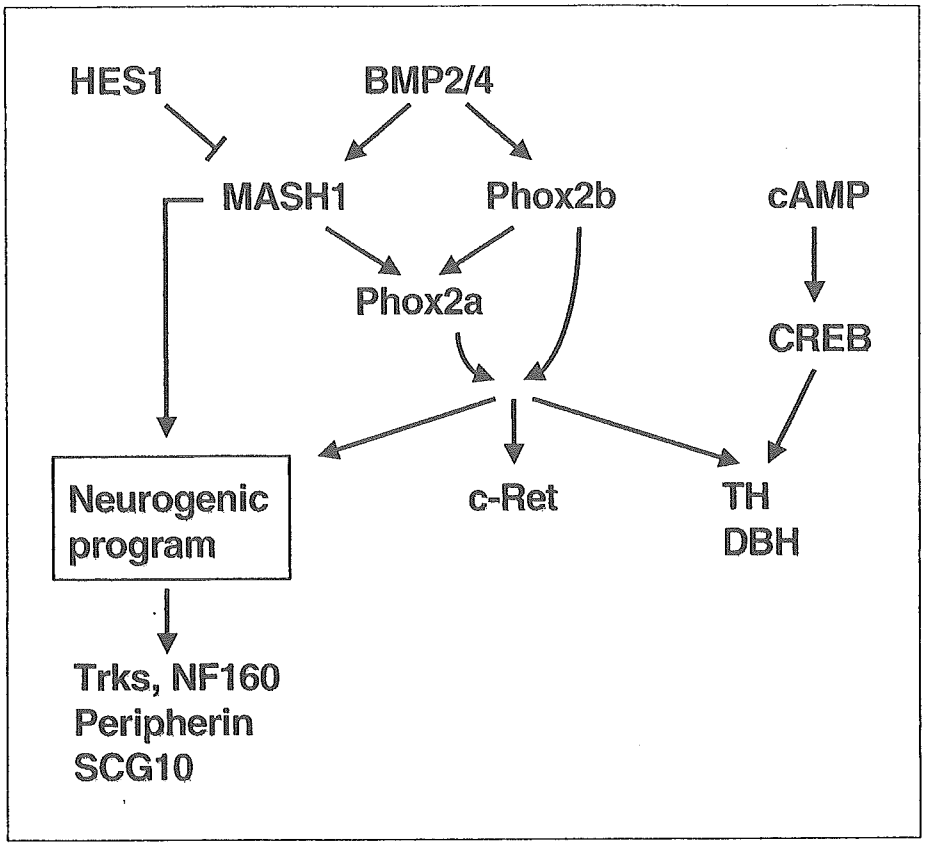


Figure 5.4

Regulatory network controlling sympathetic neuron development. BMP2 and BMP4 are required for the expression of *MASH1* and *Phox2b*. *HES1* induced by notch signaling inhibits expression of *MASH1*. *MASH1* and *Phox2b* are genetically upstream of *Phox2a*, and *Phox2b* is genetically upstream of *Gata3*. Expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) depends on *MASH1*, Phox2b, and Gata3. Cyclic AMP also controls expression of TH and DBH. Phox2a and Phox2b may affect induction or maintenance of *MASH1* expression. *MASH1*, Phox2a, and Phox2b regulate the downstream neurogenic program, leading to terminal differentiation of sympathetic neurons by inducing the genes, e.g., Trks, NF160, peripherin, and SCG10

5.1.1.5 *MYCN*

MYCN is a member of the group of *MYC*-box genes, and its product is a bHLH protein (Schwab et al. 2003). *MYCN* is transiently expressed during normal neural development and defines the direction of neuronal differentiation. *MYCN* is frequently amplified in advanced-stage neuroblastoma (Schwab et al. 1983, 1984; Brodeur et al. 1984; Seeger et al. 1985), and the biology of high-risk neuroblastoma is influenced by the subsequent overexpression of *MYCN* oncoprotein and its targets including telomerase and those functioning in ribosome biogenesis and protein synthesis (Mac et al. 2000; Boon et al. 2001).

5.2 Molecular Bases of Differentiation and Programmed Cell Death

5.2.1 Molecular Aspect of Spontaneous Regression

It is well known that some subsets of neuroblastoma can regress spontaneously. One of the most important hints to understand the mechanism of spontaneous regression is age of the patient at the onset of neuroblastoma. Regression rarely occurs when the tumor is found in patients over 1 year of age. The dramatic regression of the stage 4s tumor after its rapid growth usually occurs within 6 months after birth; therefore, it is plausible that epigenetic regulations, timed with the development of sympathetic neurons, might also control neuroblastoma regression. It is well known that massive death of sympathetic neurons is induced during the perinatal period – a process called developmentally regulated neuronal programmed cell death following deprivation of tar-

get tissue-derived neurotrophins (Oppenheim 1991). This same death mechanism appears to be conserved in primary neuroblastomas found in infants, leading to the induction of their spontaneous regression (Nakagawara 1998b).

5.2.2 Neurotrophic Factors and Their Receptors

5.2.2.1 Neurotrophins and Their Receptors in Neuroblastoma

The neurotrophin family of growth factors consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5; Huang and Reichardt 2003). The corresponding high-affinity neurotrophin receptors with tyrosine kinase activity have been identified as TrkA, TrkB, and TrkC (Snider 1994) (Fig. 5.5 a, b). TrkA is a preferred receptor for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower-affinity neurotrophin receptor $p75^{NTR}$, a member of the tumor necrosis factor receptor (TNFR)/Fas family (Snider 1994). The targeted disruption of neurotrophins and their receptors has demonstrated that NGF/TrkA signaling supports the survival and differentiation of sympathetic and sensory neurons responsive to temperature and pain, while BDNF/TrkB, NT-4/TrkB, and NT-3/TrkC signaling supports those of sensory neurons responsive to tactile stimuli and motor and sensory neurons responsive to limb movement and position, respectively (Klein 1994). These results suggest that neural development and maintenance of the neural network are spatiotemporally controlled by neurotrophin signaling with or without some redundancy in both peripheral and central nervous systems.

In neuroblastoma, high levels of TrkA are expressed in subsets of tumors with good prognosis, often showing spontaneous regression (Nakagawara et al. 1992, 1993; Suzuki et al. 1993; Kogner et al. 1993). Such tumors usually occur in patients under 1 year of age, and their DNA ploidy is aneuploid. A very limited amount of NGF may be supplied from stromal cells, e.g., Schwannian cells and fibroblasts, which at least partly regulate the differentiation and programmed cell death of neuroblastoma cells (Nakagawara 1998a). On the other hand, TrkA expression is strongly downregulated in tumors with aggressive behavior that usually possess amplification of the *MYCN* oncogene and allelic loss of chromosome 1p36 (Nakagawara et al. 1992, 1993). TrkB is preferentially expressed in aggressive neuroblastomas together with its preferred ligands BDNF and NT-4/5 which stimulate in an autocrine/paracrine manner, conferring an enhanced malignant phenotype to the tumor cells (Nakagawara et al. 1994; Matsumoto et al. 1995). TrkC is expressed in favorable neuroblastomas at variable levels (Yamashiro et al. 1996), but its preferred ligand, NT-3, is nearly undetectable by RT-PCR in primary neuroblastomas (Nakagawara 1998a); thus, in regressing neuroblastomas, tumor cells expressing the TrkA receptor may be dependent on a limited amount of NGF supplied from stromal cell. In the presence of NGF the cells mature, whereas they will die in the absence of this ligand (Nakagawara 1998a,b); however, in clinically aggressive neuroblastomas, the TrkA is downregulated and the downstream signaling cascades are disturbed, and these cells utilize the BDNF or NT-4/TrkB autocrine system for efficient growth. Neurotrophin signaling may also regulate tumor metastasis (Matsumoto et al. 1995), proliferation (Matsumoto et al. 1995), and angiogenesis (Canete et al. 2000). The role of $p75^{NTR}$ in neuroblastoma is unclear. The $p75^{NTR}$ receptor is expressed in both neuroblastoma cell lines (Azar et al. 1990) and primary neuroblastomas (Nakagawara et al. 1993). Interestingly, the expression levels of $p75^{NTR}$ mRNA are significantly higher in favorable neuroblastomas (stages 1, 2 and 4s) as compared with the advanced stage tumors, especially those with *MYCN* amplification (Nakagawara et al. 1993).

5.2.2.2 Neurotrophin Signaling in Neuroblastoma

In a rat pheochromocytoma cell line PC12, differentiation signals by NGF may be mediated through the tyrosine phosphorylation of the Trk receptor and through the subsequent activation of Shc/Grb2/SOS, Ras, Raf, MEK, and ERKs, while survival signals in the same cells may be transduced through the direct

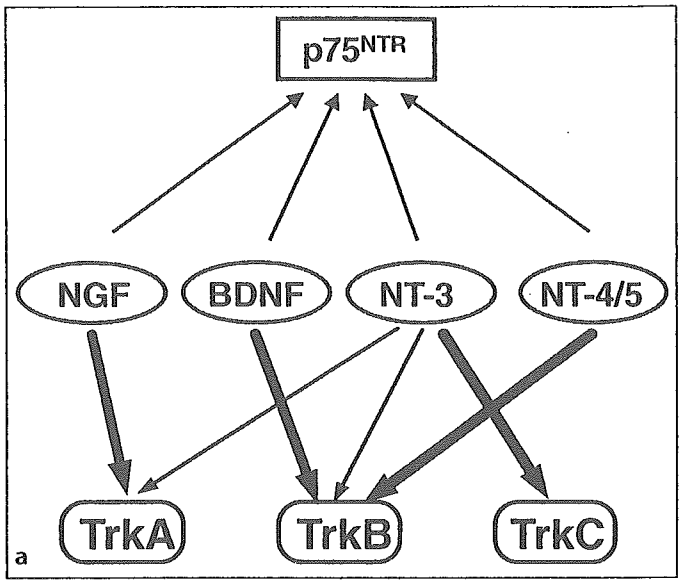
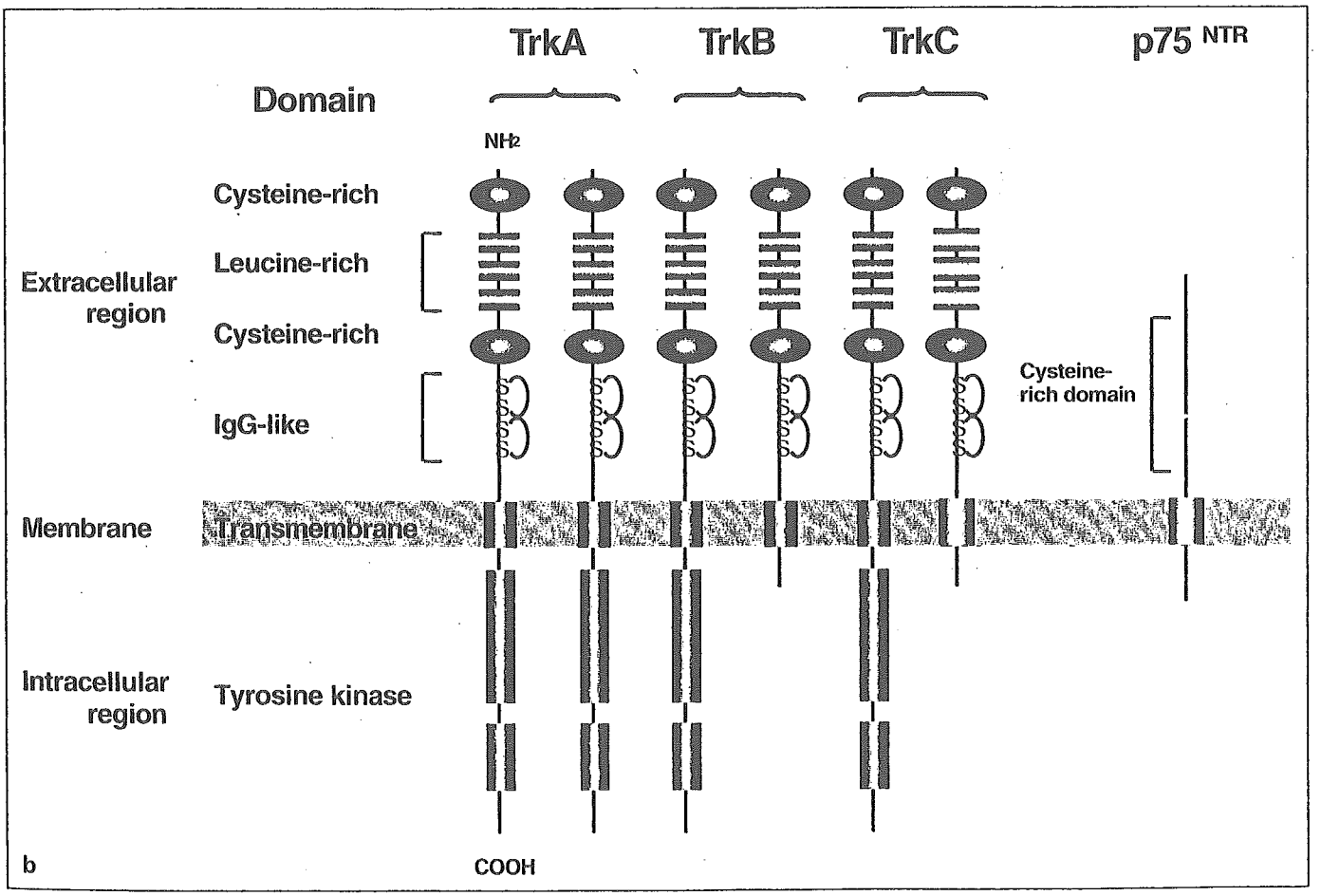


Figure 5.5 a, b

Neurotrophins and their receptors. a TrkA is a preferred high-affinity receptor for NGF, TrkB for BDNF, and NT-4/5, and TrkC for NT-3. All of the neurotrophins also bind similarly to a lower affinity neurotrophin receptor $p75^{NTR}$. b The structures of neurotrophin family receptors. The extracellular domains of TrkA, TrkB, and TrkC have high structural similarity. The intracellular domain of Trks possesses tyrosine kinase activity. TrkB and TrkC receptors have truncated forms which lack the tyrosine kinase domain. The low-affinity receptor, $p75^{NTR}$, has a short intracellular region containing the death domain, and belongs to the Fas/TNFR family of the receptors



activation of PI3-kinase which in turn activates downstream molecules, e.g., Akt and Bad (Klesse and Parada 1999). On the other hand, in normal sympathetic neurons, the activation of PI3-kinase is mediated not by the tyrosine phosphorylation of the receptor but by the Ras activation which promotes neuronal survival, suggesting that the Trk intracellular signaling pathway might be deregulated in cancer cells. This is also the case in neuroblastoma. In the neuroblastoma cell lines with a single copy of *MYCN*, NGF can induce differentiation when exogenous TrkA is overexpressed (Eggert et al. 2000). In the cell lines with *MYCN* amplification, however, the NGF-stimulated TrkA receptors which were overexpressed cannot normally activate downstream signaling molecules, resulting in unresponsiveness to the ligand. Furthermore, it is surprising that BDNF/TrkB signaling appears to be functioning in the same cells by promoting survival (Nakagawara et al. 1994; Hishiki et al. 1998), although the signaling pathway might be different from that of sympathetic neurons (Klesse and Parada 1999).

5.2.2.3 GDNF Family Receptors

Neurotrophic factors of the glial cell line-derived neurotrophic factor (GDNF) family, which include GDNF, artemin and neurturin, are secreted by neuroblastoma cells as well as stromal cells and activate their receptor complex composed of Ret tyrosine kinase and the GFRα co-receptors expressed in neuroblastoma cells (Hishiki et al. 1998; Ichikawa et al. 2004). In contrast to NGF/TrkA and BDNF/TrkB, however, the GDNF/Ret/GFRα autocrine system is functioning in both favorable and unfavorable neuroblastomas to enhance the survival and differentiation of tumor cells (Hishiki et al. 1998).

5.2.2.4 Other Factors and Receptors

Neuroblastoma cells express other growth factors and receptors. Both pleiotrophin (PTN) and midkine (MK) are factors in the same family with neurotrophic function (Kadomatsu et al. 1990; Li et al. 1990; Kadomatsu and Muramatsu 2004). PTN is expressed significantly at high levels in favorable neuroblastomas, while MK is highly expressed in almost all neuroblastomas with a tendency to be expressed at high levels in tumors in advanced stages (Nakagawara et al. 1995). Neuroblastoma also expresses many other receptors, e.g., fibroblast growth factor receptor (FGFR; Schweigerer et al. 1991), insulin-like growth factor (IGFR; El-Badry et al. 1991), DCC (deleted in colon cancer) (Reale et al. 1996), and neuronal leucine-rich repeat receptors (NLRRs; Hamano et al. 2004), as well as a novel plasma membrane enzyme ECEL1, which is significantly highly expressed in favorable neuroblastomas (Kawamoto et al. 2003). The biological significance of these factors and receptors in neuroblastoma are not currently known.

5.2.3 Functional Role of p53 Family Genes

Recent lines of evidence suggest that both the p53 tumor suppressor protein and its related protein p73 are involved in the induction of programmed cell death and growth arrest in neuronal cells (Pozniak et al. 2000). p73 is a recently identified candidate tumor suppressor gene mapped to chromosome 1p36.2, a frequently deleted region in many human cancers including neuroblastoma and oligodendroglioma (Ichimiya et al. 1999; Billon et al. 2004). In cultured neonatal sympathetic neurons, p53 protein levels are increased in response to NGF withdrawal as well as $p75^{NTR}$ activation, and it functions downstream of c-Jun NH_2-terminal kinase (JNK) and upstream of Bax to induce apoptosis (Aloyz et al. 1998) (Fig. 5.6). Indeed, in $p53^{-/-}$ mice, naturally occurring sympathetic neuron death is inhibited. Pozniak et al. (2000) have also reported that p73 is primarily present in developing neurons as ΔNp73, an NH_2-terminally truncated isoform, whose level is decreased when sympathetic neurons undergo apoptosis after NGF withdrawal, and that p53 becomes activated to be pro-apoptotic. In contrast to the truncated form of p73, full-length p73 has induced neuronal differentiation in a mouse neuroblastoma cell line N1E115 (Laurenzi et al. 2000). These data suggest that the neuronal apoptosis induced by NGF withdrawal is at least partly regulated by a reciprocal balance between levels of pro-apoptotic p53 and anti-apoptotic ΔNp73.

Figure 5.6

A model of signaling pathway for survival and death in sympathetic neurons regulated by NGF. NGF depletion may induce activation of JNK/p53 pathway which could be modified by p73/ΔNp73 regulatory system. $p75^{NTR}$ activation, which sends signals of both survival and death, may also regulate downstream p53/p73/ΔNp73 pathway

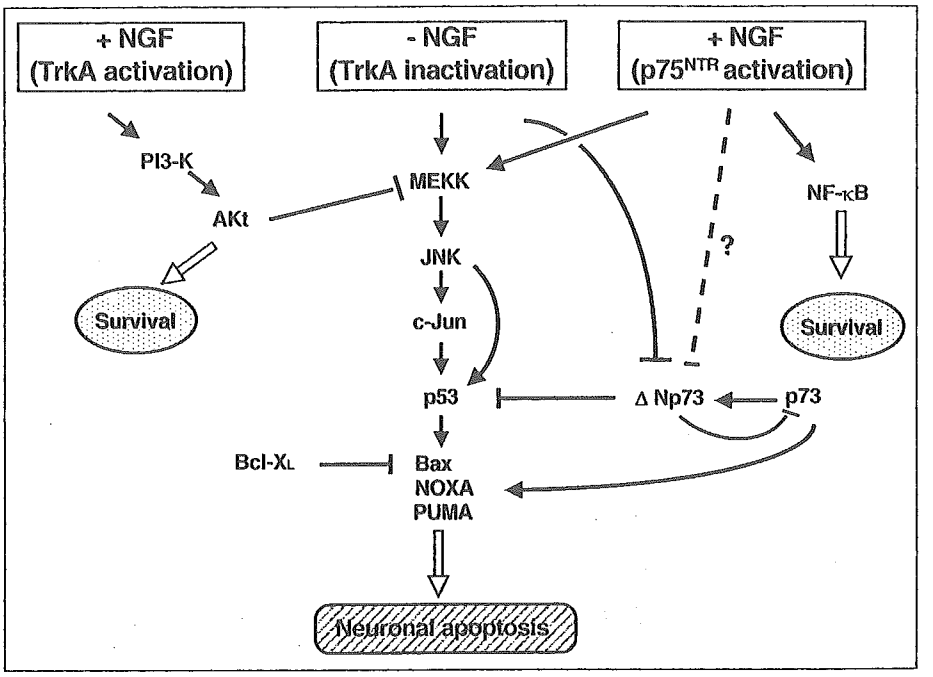
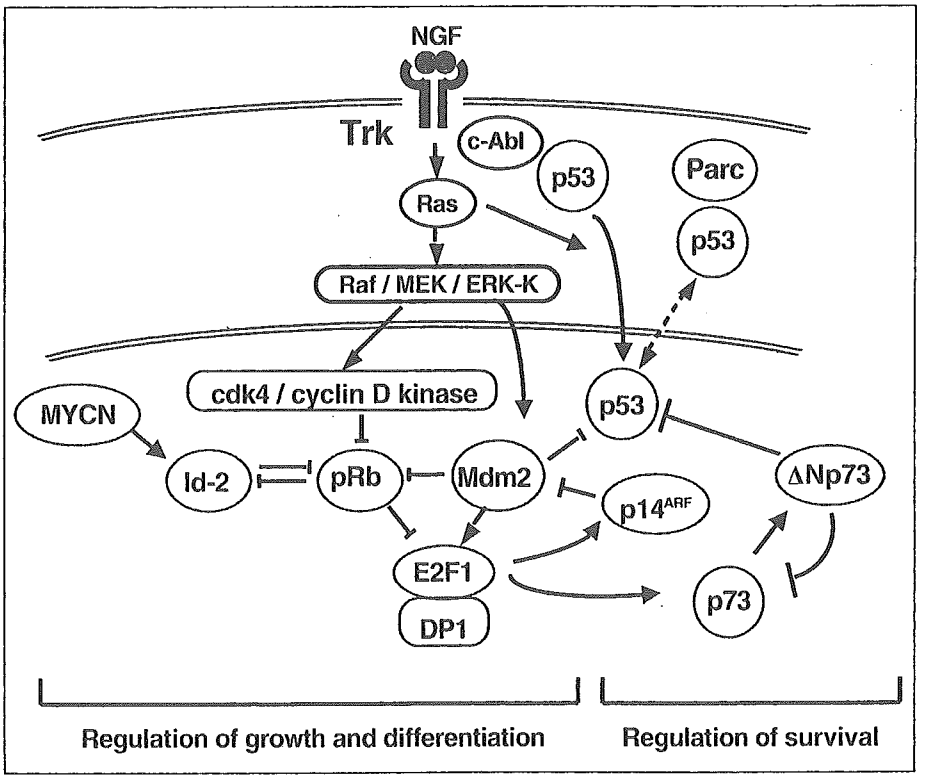


Figure 5.7

A possible signaling pathway regulating growth, differentiation and survival in neuroblastoma cells or sympathetic neurons. The NGF-triggered autophosphorylation of TrkA tyrosine kinase receptor induces activation of Ras/MAPK pathway, which in turn regulates nuclear pRB and Mdm2. In some poor-outcome neuroblastomas, p53, which is shuttling between cytosol and nucleus, is trapped in the cytosol by Parc, an anchoring protein of p53. *MYCN* induces expression of *Id-2* whose protein product in turn inhibits pRB. E2F1 negatively regulated by pRB directly induces expression of p73. p73 is regulated by ΔNp73 in a negative autoregulatory manner (Nakagawa et al. 2002), and ΔNp73 also inhibits p53



The importance of p53 and p73 has also been emphasized by the important observation that, in cultured neuroblastoma and other cancer cells, p73 directly transactivates the ΔNp73 gene by binding to its promoter after treating the cells with genotoxic reagents, e.g., cisplatin (Nakagawa et al. 2002). The induced ΔNp73 protein in turn interacts with either wild-type p53 or TAp73 and inhibits their proapoptotic function; thus, ΔNp73 can act as an oncogene and as an inhibitor of wild-type p53 and TAp73. The presence of this autoinhibitory feedback loop among p53, TAp73, and ΔNp73 may at least in part explain why there is no mutation of the p73 gene in cancers.

p53 is associated with TrkA via the proto-oncogene product c-Abl as an adaptor or bridging molecule, suggesting that it may also play a role in Trk signaling (Yano et al. 2000) (Fig. 5.7). The activation of Ras by NGF stimulation of the TrkA receptor induces p53 nuclear translocation and growth arrest in PC12 cells (Hughes et al. 2000). The c-Ha-Ras gene could be a target of p53, and protein products induce a positive feedback loop by activating $p14^{ARF}$ which counteracts the negative feedback loop mediated by mdm2 (Deguin-Chambon et al. 2000). These observations strongly suggest that p53 and p73 tumor suppressors function in neurotrophin signaling and modulate the growth, differentiation, and apoptosis of neurons.

In neuroblastoma and some other human cancers, wild type p53 is often localized in the cytoplasm (Moll et al. 1995). Although the regulatory mechanism of cellular localization of p53 and p73 is still unknown, activated Ras in NGF/TrkA signaling stimulates the nuclear translocation of p53 and leads to growth arrest by the induction of $p21^{WAF1}$ in PC12 cells (Hughes et al. 2000). Furthermore, some fractions of recurrent neuroblastomas and neuroblastoma cell lines acquire mutation of the p53 gene (Tweddle et al. 2001).

5.2.4 Apoptotic Signals in Neuroblastoma

To date, the spontaneous regression of neuroblastoma, has occurred only *in vivo*. Although this makes the analysis difficult, there are some important reports. An anti-apoptotic protein, Bcl-2, is expressed in primary neuroblastomas and neuroblastoma cell lines. The expression levels of Bcl-2 and $Bcl\text{-}X_L$ are high in aggressive tumor cells but are low in regressing cells (Ikeda et al. 1995; Ikegaki et al. 1995). Caspase-1 and caspase-3 are expressed at significantly higher levels in favorable neuroblastomas (Nakagawara et al. 1997), and caspase-8 is silenced in aggressive neuroblastomas by the methylation of its promoter as one of mechanisms (Teitz et al. 2000). Silencing of caspase-8 is observed in 25–35% of primary neuroblastomas with a high frequency in more aggressive tumors (Teitz et al. 2000; Eggert et al. 2001; van Noesel et al. 2003). Survivin, a member of the inhibitors of apoptosis protein (IAP), is mapped to the long arm of chromosome 17. In neuroblastoma, survivin is highly expressed in high-risk tumors, and its overexpression inhibits cellular apoptosis (Islam et al. 2000). Kitanaka et al. (2002) have recently reported an interesting observation that "autophagy" may be involved in the regression of neuroblastoma cells.

5.3 Conclusions

Development of neuroblastoma may be triggered by a genetic event(s) that leads to chromosome and/or the genomic DNA abnormalities such as amplification of the *MYCN* gene and deletions or gains in chromosomal regions including 1p, 11q, and 17q. Together with other epigenetic mechanisms of gene activation or gene silencing, they affect gene and protein expression which in turn deregulate cellular signaling. In neuroblastoma the normal biology of developing neuronal cells and cancer biology appear to overlap. A further understanding of the mechanisms involved in the transformation of progenitors or the stem cells into neuroblastoma with significant cellular heterogeneity may provide clues for the development of novel therapeutic strategies for this often aggressive lethal disease.

Acknowledgements. I thank M. Ohira and T. Ozaki for reading the manuscript. I also thank K. Yagyu for preparing the figures.

References

Aloyz RS et al. (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol 143:1691–1703

Altaba ARi, Stecca B, Sanchez P (2004) Hedgehog-Gli signaling in brain tumors: stem cells and paradevelopmental programs in cancer. Cancer Lett 204:145–157

Axelson H (2004) The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1. Cancer Lett 204:171–178

Azar CG, Scavarda NJ, Reynolds P, Brodeur GM (1990) Multiple defects of the nerve growth factor receptor in human neuroblastomas. Cell Growth Differ 1:421–428

Ball DW (2004) Achaete-scute homolog-1 and Notch in lung neuroendocrine development and cancer. Cancer Lett 159–169

Billon N et al. (2004) Roles for p53 and p73 during oligodendrocyte development. Development 131:1211–1220

Boon K et al. (2001) N-*MYC* enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. EMBO J 20:1383–1393

Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of N-*MYC* in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124

Canete A, Navarro S, Bermudez J, Pellin A, Castel V, Llombart-Bosch A (2000) Angiogenesis in neuroblastoma: relationship to survival and other prognostic factors in a cohort of neuroblastoma patients. J Clin Oncol 18:2789–2791

Deguin-Chambon V, Vacher M, Jullien M, May E, Bourdon JC (2000) Direct transactivation of c-Ha-Ras gene by p53: evidence for its involvement in p53 transactivation activity and p53-mediated apoptosis. Oncogene 19:5831–5841

Eggert A et al. (2000) Molecular dissection of TrkA signal transduction pathways mediating differentiation in human neuroblastoma cells. Oncogene 19:2043–2051

Eggert A et al. (2001) Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Cancer Res 61:1314–1319

El-Badry OM, Helman LJ, Chatten J, Steinberg SM, Evans AE, Israel MA (1991) Insulin-like growth factor II-mediated proliferation of human neuroblastoma. J Clin Invest 87: 648–657

Enomoto H et al. (1994) Identification of human DAN gene, mapping to the putative neuroblastoma tumor suppressor locus. Oncogene 9:2785–2791

Ernsberger U (2000) Evidence for an evolutionary conserved role of bone morphogenetic protein growth factors and phox2 transcription factors during noradrenergic differentiation of sympathetic neurons. Induction of a putative synexpression group of neurotransmitter-synthesizing enzymes. Eur J Biochem 267:6976–6981

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463–476

Hamano S, Ohira M, Isogai E, Nakada K, Nakagawara A (2004) 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 24:1457–1466

Hishiki T et al. (1998) Glial cell line-derived neurotrophic factor/neurturin-induced differentiation and its enhancement by retinoic acid in primary human neuroblastomas expressing c-Ret, GFR-1 and GFR-2. Cancer Res 58:2158–2165

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642

Huber K, Combs S, Ernsberger U, Kalcheim C, Unsicker K (2002) Generation of neuroendocrine chromaffin cells from sympathoadrenal progenitors: beyond the glucocorticoid hypothesis. Ann NY Acad Sci 971:554–559

Hughes AL, Gollapudi L, Sladek TL, Neet KE (2000) Mediation of nerve growth factor-driven cell cycle arrest in PC12 cells by p53: simultaneous differentiation and proliferation subsequent to p53 functional inactivation. J Biol Chem 275: 37829–37837

Iavarone A, Lasorella A (2004) Id proteins in neural cancer. Cancer Lett 204:189–196

Ichikawa M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. Cancer Lett 204:197–211

Ichimiya S et al (1999) p73 at chromosome 1p36.3 is lost in advanced stage neuroblastoma but its mutation is infrequent. Oncogene 18:1061–1066

Ichimiya S, Nimura Y, Seki N, Ozaki T, Nagase T, Nakagawara A (2001) Down-regulation of *hASH1* is associated with the retinoic acid-induced differentiation of human neuroblastoma cell lines. Med Pediatr Oncol 36:132–134

Ikeda H et al. (1995) Bcl-2 oncoprotein expression and apoptosis in neuroblastoma. J Pediatr Surg 30:805–808

Ikegaki N, Katsumata M, Tsujimoto Y, Nakagawara A, Brodeur GM (1995) Relationship between bcl-2 and *MYC* gene expression in human neuroblastoma. Cancer Lett 91:161–168

Islam A et al (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

Kadomatsu K, Muramatsu T (2004) Midkine and pleiotrophin in neural development and cancer. Cancer Lett 204:127–143

Kadomatsu K, Huang R-P, Suganuma T, Murata F, Muramatsu T (1990) A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis. J Cell Biol 110:607–616

Kawamoto T, Ohira M, Hamano S, Hori T, Nakagawara A (2003) High expression of the novel endothelin-converting enzyme genes, Nblao3145/ECEL1alpha and beta is associated with favorable prognosis in human neuroblastomas. Int J Oncol 22:815–822

Kitanaka C et al (2002) Increased Ras expression and caspase-independent neuroblastoma cell death: possible mechanism of spontaneous neuroblastoma regression. J Natl Cancer Inst 94:358–368

Klein R (1994) Role of neurotrophins in mouse neuronal development. FASEB J 8:738–744

Klesse LJ, Parada LF (1999) Trks: signal transduction and intracellular pathways. Microsc Res Tech 45:210–216

Kogner P, Barbany G, Dominici C, Castello MA, Raschella G, Persson H (1993) Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res 53:2044–2050

Lasorella A, Noseda M, Beyna M, Yokota Y, Iavarone A (2000) Id2 is a retinoblastoma protein target and mediates signalling by *MYC* oncoproteins. Nature 407:592–598

Laurenzi VD et al. (2000) Induction of neuronal differentiation by p73 in a neuroblastoma cell line. J Biol Chem 275: 15226–15231

Li Y-S et al. (1990) Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. Science 250:1690–1694

Lo L, Morin X, Brunet JF, Anderson DJ (1999) Specification of neurotransmitter identity by Phox2 proteins in neural crest stem cells. Neuron 22:693–705

Mac SM, D'Cunha CA, Farnham PJ (2000) Direct recruitment of N-*MYC* to target gene promoters. Mol Carcinogr 29: 76–86

Massari ME, Murre C (2000) Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms. Mol Cell Biol 20:429–440

Matsumoto K, Wada RK, Yamashiro JM, Kaplan DR, Thiele CJ (1995) Expression of brain-derived neurotrophic factor and $p145^{TrkB}$ affects survival, differentiation, and invasiveness of human neuroblastoma cells. Cancer Res 55:1798–1806

Moll UM, LaQuaglia M, Benard J, Riou G (1995) Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc Natl Acad Sci USA 92:4407–4411

Morin X, Cremer H, Hirsch MR, Kapur RP, Goridis C, Brunet JF (1997) Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. Neuron 18:411–423

Nakagawa T et al (2002) Autoinhibitory regulation of p73 by ΔNp73 to modulate cell survival and death through p73-specific target element within the ΔNp73 promoter. Mol Cell Biol 22:2575–2585

Nakagawara A (1998a) The NGF story and neuroblastoma. Med Pediatr Oncol 31:113–115

Nakagawara A (1998b) Molecular basis of spontaneous regression of neuroblastoma: role of neurotrophic signals and genetic abnormalities. Hum Cell 11:115–124

Nakagawara A (2001) Trk receptor tyrosine kinases: a bridge between cancer and neural development. Cancer Lett 169:107–114

Nakagawara A (2004) Neural crest development and neuroblastoma: the genetic and biological link. In: Aloe L, Calza L (eds) NGF and related molecules in health and disease. Elsevier, Amsterdam, pp 233–242

Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM (1992) Inverse relationship between trk expression and N-*MYC* amplification in human neuroblastomas. Cancer Res 52:1364–1368

Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM (1993) Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl J Med 328:847–854

Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM (1994) Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol 14:759–767

Nakagawara A et al. (1995) Differential expression of pleiotrophin and midkine in advanced neuroblastomas. Cancer Res 55:1792–1797

Nakagawara A et al. (1997) High levels of expression and nuclear localization of ICE and CPP32 in favorable human neuroblastomas. Cancer Res 57:4578–4584

Nakamura Y, Ozaki T, Koseki H, Nakagawara A, Sakiyama S (2003) Accumulation of $p27^{KIP1}$ is associated with BMP2-mediated growth arrest and neuronal differentiation of human neuroblastoma-derived cell lines. Biochem Biophys Res Commun 307:206–213

Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453–501

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399:366–370

Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD (2000) An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science 289:304–306

Radtke F, Raj K (2003) The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 3:756–767

Reale MA et al. (1996) Loss of DCC expression in neuroblastoma is associated with disease dissemination. Clin Cancer Res 2:1097–1102

Rodriguez-Leon J, Merino R, Macias D, Ganan Y, Santesteban E, Hurle JM (1999) Retinoic acid regulates programmed cell death through BMP signalling. Nat Cell Biol 1:125–126

Schneider C, Wicht H, Enderich J, Wegner M, Rohrer H (1999) Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. Neuron 24:861–870

Schwab M et al. (1983) Amplified DNA with limited homology to *MYC* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305:245–248

Schwab M et al (1984) Chromosome localization in normal human cells and neuroblastomas of a gene related to c-*MYC*. Nature 308:288–291

Schwab M et al. (2003) Neuroblastoma: biology and molecular and chromosomal pathology. Lancet Oncol 4:472–480

Schweigerer L, Ledoux D, Fleischmann G, Barritault D (1991) Enhanced *MYCN* oncogene expression in human neuroblastoma cells is associated with altered FGF receptor expression and cellular growth response to basic FGF. Biochem Biophys Res Commun 179:1449–1454

Seeger RC et al. (1985) Association of multiple copies of the N-*MYC* oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111–1116

Shinbo J et al. (2002) p73-dependent expression of DAN during cisplatin-induced cell death and osteoblast differentiation. Biochem Biophys Res Commun 295:501–507

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627–638

Soderholm H et al. (1999) Human achaete-scute homologue 1 (HASH-1) is downregulated in differentiating neuroblastoma cells. Biochem Biophys Res Commun 256:557–563

Stanke M, Junghans D, Geissen M, Goridis C, Ernsberger U, Rohrer H (1999) The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. Development 126:4087–4094

Suzuki T, Bogenmann E, Shimada H, Stram D, Seeger RC (1993) Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. J Natl Cancer Inst 85:377–384

Teitz T et al (2000) Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of *MYCN*. Nat Med 6:529–535

Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL (1998) The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev 12:3320–3324

Trochet D et al (2004) Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet 74:761–764

Tweddle DA, Malcolm AJ, Bown N, Pearson AD, Lunec J (2001) Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line. Cancer Res 61:8–13

Van Noesel MM, Pieters R, Voute PA, Versteeg R (2003) The N-*MYC* paradox: N-*MYC* overexpression in neuroblastomas is associated with sensitivity as well as resistance to apoptosis. Cancer Lett 197:165–172

Yamashiro D, Nakagawara A, Ikegaki N, Liu X, Brodeur GM (1996) Expression of TRK-C in favorable human neuroblastomas. Oncogene 12:37-41

Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 404:99–103

Yano H, Cong F, Birge RB, Goff SP, Chao MV (2000) Association of the Abl tyrosine kinase with the Trk nerve growth factor receptor. J Neurosci Res 59:356–364

Yoshida I, Koide S, Nakagawara A, Tsuji A, Matsuda Y (2001) Proprotein convertase PACE4 is a target gene of the basic helix-loop-helix transcription factor hASH-1. Biochem J 360:683–689

CpG Island Methylator Phenotype Is a Strong Determinant of Poor Prognosis in Neuroblastomas

Masanobu Abe,[1,2] Miki Ohira,[3] Atsushi Kaneda,[1] Yukiko Yagi,[1] Seiichiro Yamamoto,[4] Yoshihiro Kitano,[5] Tsuyoshi Takato,[2] Akira Nakagawara,[3] and Toshikazu Ushijima[1]

[1]Carcinogenesis Division, National Cancer Center Research Institute; [2]Department of Oral and Maxillo Facial Surgery, University of Tokyo Graduate School of Medicine; [3]Biochemistry Division, Chiba Cancer Center Research Institute; [4]Information Division, Research Center for Cancer Prevention and Screening, National Cancer Center; and [5]Department of Pediatric Surgery, National Center for Child Health and Development, Tokyo, Japan

Abstract

Neuroblastoma, one of the most common pediatric solid tumors, is characterized by two extreme disease courses, spontaneous regression and life-threatening progression. Here, we conducted a genome-wide search for differences in DNA methylation that distinguish between neuroblastomas of the two types. Three CpG islands (CGI) and two groups of CGIs were found to be methylated specifically in neuroblastomas with a poor prognosis. By quantitative analysis of 140 independent cases, methylation of all the five CGI (groups) was shown to be closely associated with each other, conforming to the CpG island methylator phenotype (CIMP) concept. The presence of CIMP was sensitively detected by methylation of the *PCDHB* CGIs and associated with significantly poor survival (hazard ratio, 22.1; 95% confidence interval, 5.3-93.4; $P < 0.0001$). Almost all cases with N-*myc* amplification (37 of 38 cases) exhibited CIMP. Even in 102 cases without N-*myc* amplification, the presence of CIMP (30 cases) strongly predicted poor survival (hazard ratio, 12.4; 95% confidence interval, 2.6-58.9; $P = 0.002$). Methylation of *PCDHB* CGIs, located in their gene bodies, did not suppress gene expression or induce histone modifications. However, CIMP was significantly associated with methylation of promoter CGIs of the *RASSF1A* and *BLU* tumor suppressor genes. The results showed that neuroblastomas with CIMP have a poor prognosis and suggested induction of silencing of important genes as an underlying mechanism. (Cancer Res 2005; 65(3): 828-34)

Introduction

Epigenetic abnormalities, especially alterations in DNA methylation, are intimately involved in development of various human tumors (1). Aberrant methylation of promoter CpG islands (CGI) causes inactivation of tumor suppressor genes. Genomic instability is caused by genomic hypomethylation and is associated with hypermethylation (2, 3). Identification of epigenetic abnormalities in human cancers is expected to lead not only to discovery of novel disease mechanisms but also to development of new diagnostic markers. Therefore, we previously developed a genome-wide scanning method, methylation-sensitive representational difference analysis (MS-RDA), for detecting differences in DNA methylation (4, 5). This technique analyzes unmethylated, CpG-rich regions of the genome and has already identified genes silenced in human lung, stomach, breast, and pancreatic cancers (6–9).

Neuroblastoma derived from primitive cells of the sympathetic nervous system is one of the most common solid tumors in childhood, characterized by two extreme disease courses, spontaneous regression, and life-threatening progression (10, 11). The clinical outcome is associated with disease stage, age at diagnosis, histologic classification, N-*myc* amplification, DNA ploidy, and *TrkA* overexpression (10–12). These characteristics are therefore used to classify cases into low-, intermediate-, and high-risk groups. However, especially in the cases with intermediate risk, prediction of prognosis and therapeutic decision-making are still difficult, and development of new markers is an urgent priority. Moreover, the molecular bases underlying the two distinct clinical courses are still unknown, and their clarification is needed to allow development of novel therapeutics.

In the present study, considering the major involvement of epigenetic machinery in embryonic development (13, 14), we searched for differences in DNA methylation between neuroblastomas with a good prognosis and counterparts with a poor prognosis by MS-RDA.

Materials and Methods

Tissue Samples and Cell Lines. Tumor samples were obtained from 145 nonrecurrent cases between 1995 and 1999 and were used under approval of institutional review boards. The mean age at initial diagnosis was 27 months (range, 0-216 months). Their clinical stages were determined according to the International Neuroblastoma Staging System, and 40, 17, 20, 60, and 8 cases belonged to stages I, II, III, IV, and IVS, respectively. Normal adrenal medulla tissue was collected from a case undergoing nephrectomy for a renal cancer. Neuroblastoma cell lines were obtained from the American Type Culture Collection (Manassas, VA), the Japanese Collection of Research Bioresources (Tokyo, Japan), and the RIKEN Bio Resource Center (Tsukuba, Japan). GANB was established by A.N. and normal human bronchial epithelial cells were purchased from Cambrex (East Rutherford, NJ). High molecular weight DNA and total RNA were extracted as previously described (7). Total RNAs of brain and adrenal glands were purchased from Clontech (Palo Alto, CA).

MS-RDA and Database Search. MS-RDA was done as previously described (4, 5). Genomic DNA of primary neuroblastomas with a good prognosis (cases 92, 98, 104, 112, and 148) and neuroblastoma cell lines established from cases with a poor prognosis (CHP134, IMR32, GANB, NGP, and TGW) were digested with *Hpa*II, and then two pooled DNA samples were prepared. Although use of cell lines is highly recommended for MS-RDA (5), no cell lines were available for neuroblastomas with a good prognosis, and therefore we used the primary samples. To isolate CGIs that were hypermethylated in the latter, the cell line pool was used as the tester, and the primary tumor pool as the driver. MS-RDA in the opposite direction

Note: Supplementary data for this article are available at Cancer Research online (http://cancerres.aacrjournals.org/).

Requests for reprints: Toshikazu Ushijima, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Phone: 133-547-5240; Fax: 135-565-1753; E-mail: tushijim@ncc.go.jp.

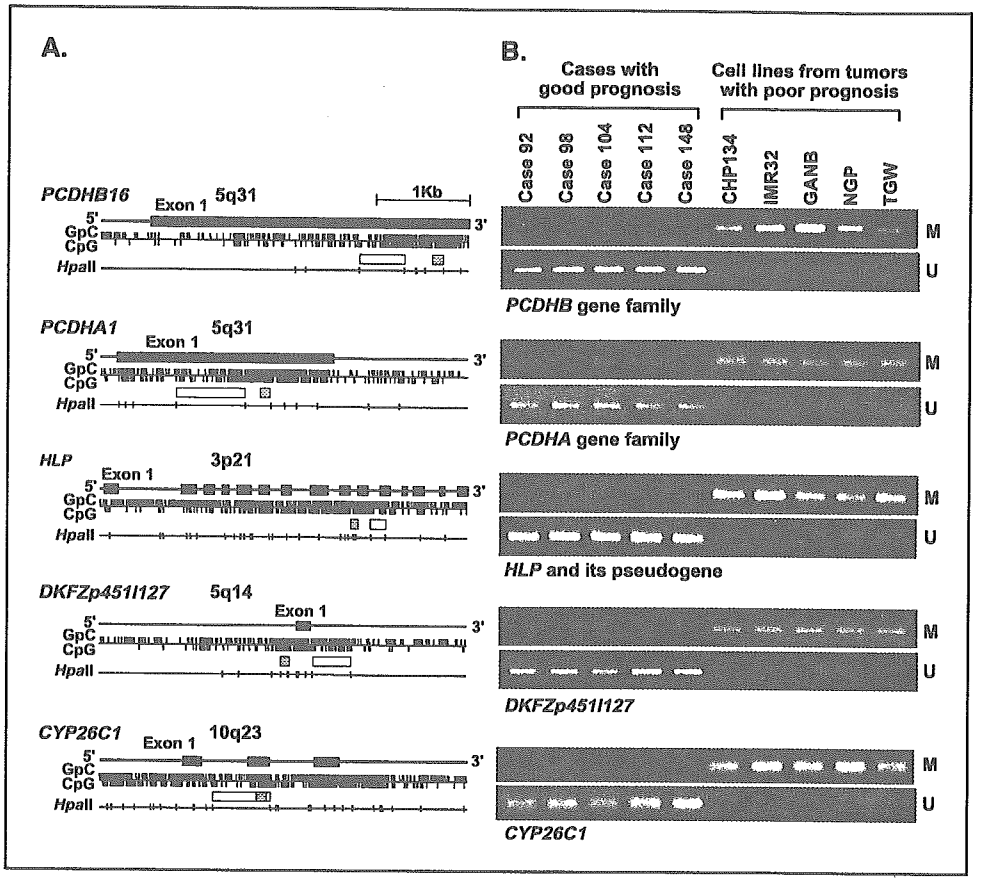


Figure 1. Five CGIs isolated by MS-RDA and their methylation statuses in the samples used for MS-RDA. *A*, genomic structures of the five CGIs. GpC, CpG, and *HpaII* recognition sites (5′-CCGG-3′) are shown by ticks. *Closed boxes*, exons; *open boxes*, clones isolated by MS-RDA; *shaded boxes*, regions analyzed by MSP. *B*, methylation statuses analyzed by MSP. *M*, MSP using primers specific to methylated DNA; *U*, MSP using primers specific to unmethylated DNA. All the five CGIs were found to be differentially methylated between the two groups used for MS-RDA.

was also done. For each series of MS-RDA, 96 clones were analyzed for redundancy, and nonredundant clones were sequenced. Their genomic origins were examined using BLASTN software http://www.ncbi.nlm.nih.gov/BLAST/).

Sodium Bisulfite Modification and Methylation-Specific PCR. One microgram of DNA underwent sodtlbium bisulfite modification (15), and was suspended in 20 μL of TE buffer. For methylation-specific PCR (MSP), 1 μL of the solution was used for PCR with primers specific to methylated or unmethylated sequences. Using DNA from normal human bronchial epithelial and DNA methylated with *Sss*I methylase, annealing temperatures specific for methylated and unmethylated primers were determined. Quantitative MSP was done separately for methylated DNA molecules and for unmethylated DNA molecules. Standard DNA was prepared by cloning PCR products amplified by methylated and unmethylated primers into a vector, respectively. The numbers of methylated and unmethylated molecules in a test sample were determined by comparing their amplification with those of standard samples containing 10 to 10^6 molecules. The "methylation index" was calculated as the fraction of methylated molecules in the total DNA molecules (no. methylated molecules + no. unmethylated molecules). Each sample was analyzed twice, blind to clinical information, and high reproducibility was confirmed (correlation coefficient = 0.98).

The *protocadherin* β (*PCDHB*) family consists of 16 genes with single exons and three pseudogenes on 5q31, and their CGIs are located in the gene bodies. MSP primers were designed to recognize 17 of the 19 members (all except for the *PCDHB1* gene and the *PCDHB19* pseudogene). The *protocadherin* α (*PCDHA*) family consists of 15 genes and one pseudogene having unique first exons and shared exons 2 to 4 on 5q31, and their CGIs are located in exon 1. MSP primers were designed to recognize 13 of the 16 members (all except for the *PCDHAC1* and *PCDHAC2* genes and the *PCDHA14* pseudogene). The *hepatocyte growth factor-like protein* (*HLP/MSP/MST1*) gene is highly homologous to *macrophage stimulating, pseudogene 9* (*MSTP9*), and MSP primers were designed to recognize both of these. For *DKFZp451I127*, *FLJ37440*, *Zinc finger protein 297* (*ZNF297*), and *Cytochrome p450 CYP26C1* (*CYP26C1*), MSP primers were designed to recognize each of them specifically. The primers and PCR conditions are shown in Supplementary Table 1.

Semiquantitative and Quantitative Reverse Transcription-PCR. cDNA was synthesized from 3 μg of total RNA treated with DNase using a Superscript II kit (Invitrogen Co., Carlsbad, CA). For semiquantitative reverse transcription-PCR (*PCDHB1-PCDHB15*), multiple cycles of PCR were tested for each gene, and numbers giving a wide dynamic range were determined. The primers and PCR conditions are shown in Supplementary Table 2. For quantitative reverse transcription-PCR (*PCDHB16*), the number of cDNA molecules was determined by quantitative PCR, as in quantitative MSP, and the copy number was normalized to that of *GAPDH*.

Chromatin Immunoprecipitation Assay. From 1×10^6 cells, DNA/histone complexes were immunoprecipitated, and DNA was eluted in 30 μL of TE after reversing cross-linking. Copy numbers of DNA molecules of the *PCDHB16* exon, *RASSF1A* promoter, and *GAPDH* promoter in 1 L of the eluate were determined by quantitative PCR (primer sequences in Supplementary Table 3), and normalized to the copy numbers in the input. Anti-acetyl-histone H3 antibody (AcH3) and anti-dimethylated-histone H3 (lysine 9; MetH3K9) were purchased from Cell Signalling (Beverly, MA).

Statistical Analysis. Associations between methylation levels among CGI groups were examined using the Pearson correlation coefficient and Fisher's exact test. Survival time was measured from the date of initial diagnosis to the date of death or last contact. Kaplan-Meier analysis and log-rank tests were done to compare survival between the groups defined by methylation levels. Hazard ratio (HR) between groups and dose-response relationships between methylation levels and survival were estimated by the Cox proportional hazard model. Kaplan-Meier curves were drawn with the help of Aabel software (Gigawiz. Ltd. Co., Tulsa, OK) and other analyses were conducted using SAS version 8.2 (SAS Institute, Inc., Cary, NC).

Results

Genome-Scanning for Differentially Methylated CpG Islands. MS-RDA was done using five primary neuroblastomas with a good prognosis and five neuroblastoma cell lines established from cases with a poor prognosis. Seven DNA fragments, derived from CGIs of *PCDHB16*, *PCDHA1*, *HLP*, *DKFZp451I127*, *FLJ37440*, *ZNF297*, and *CYP26C1*, were isolated as methylated in the latter samples. No DNA fragments were isolated as methylated in the former samples. Methylation statuses of (i) 17 CGIs of the *PCDHB* family (detailed structure in Supplementary Fig. 1), (ii) 13 CGIs of the *PCDHA* family, (iii) *HLP* and its pseudogene, and (iv) other four unique CGIs were examined by MSP. This revealed that the *PCDHB* family (5q31), the *PCDHA* family (5q31), *HLP* (3p21) and its pseudogene (1p36), *DKFZp451I127* (5q14), and *CYP26C1* (10q23) were specifically methylated in the latter samples (Fig. 1*A* and *B*).

Close Association between Methylation and Poor Prognosis in 140 Independent Primary Samples. To analyze the significance of the differential methylation of the above five CGI (groups) in primary neuroblastomas, 140 primary samples, all different from the initial five samples, were analyzed by quantitative MSP. When distributions of methylation indices were analyzed (Fig. 2), a clear bimodal distribution was observed for (i) the CGI group in the *PCDHB* family (17 CGIs), (ii) the CGIs of *HLP* and its pseudogene, and (iii) the *CYP26C1* CGI. The results thus indicated that the cases could be classified into two groups, one with high methylation and the other with low methylation. The dose-response relationships between high *PCDHB* methylation and poor prognosis were analyzed by the Cox proportional model using the methylation index as a continuous value, and the association was confirmed with a trend $P < 0.0001$. Normal adrenal medulla had a methylation index of 4%.

According to the bimodal distribution, the effect of high methylation was assessed by dichotomous groups. For the *PCDHB* family, cutoff values of 30%, 40%, 50%, 60%, 70%, and 80% were tested, and HRs of 16.8 [95% confidence interval (95% CI), 4.0-70.9], 22.1 (95% CI, 5.3-93.4; Fig. 3), 13.1 (95% CI, 4.5-37.9), 9.1 (95% CI, 3.8-23.4), 7.0 (95% CI, 3.1-15.8), and 7.8 (95% CI, 3.4-17.6), respectively, were obtained ($P < 0.001$ for all cutoff values). This showed that cases can be classified into two groups with distinct prognoses, and we adopted a cutoff value of 40%, which gave the highest HR, for convenience in the following analysis.

The dose-response relationships were also confirmed for other four CGI (groups), *PCDHA* ($P = 0.004$), *HLP* ($P < 0.0001$), *DKFZp451I127* ($P = 0.02$), and *CYP26C1* ($P < 0.0001$). Cutoff values were similarly tested, and those for *PCDHA*, *HLP*, *DKFZp451I127*, and *CYP26C1* were set at 80%, 10%, 20%, and 70%, respectively, with HRs of 5.7 (95%CI, 1.4-24.0; $P = 0.07$), 21.7 (95% CI, 5.1-91.4; $P < 0.0001$), 3.2 (95% CI, 1.0-10.5; $P = 0.045$), and 8.7 (95% CI, 4.1-18.1; $P < 0.0001$), respectively (Fig. 3).

Existence of the CpG Island Methylator Phenotype in Neuroblastomas. Methylation of the different CGI (groups) had shown close associations with each other (Table 1). When correlation was analyzed as a continuous value, Pearson correlation coefficients between *PCDHB* and *PCDHA*, *HLP*, *DKFZp451I127* and *CYP26C1* were 0.55, 0.70, 0.26 and 0.77, respectively. This showed that multiple CGIs were simultaneously methylated in

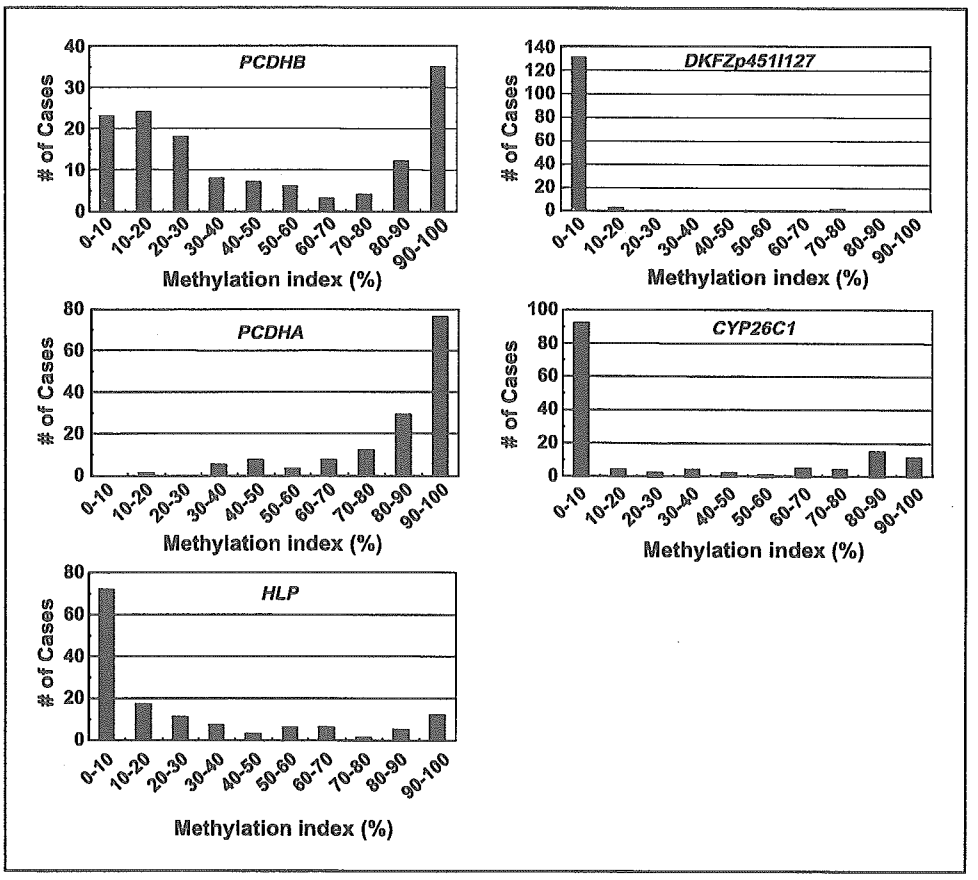


Figure 2. The distribution of methylation indices among the 140 cases analyzed: (*i*) 17 CGIs of the *PCDHB* family, (*ii*) 13 CGIs of the *PCDHA* family, (*iii*) CGIs of *HLP* and its pseudogene, (*iv*) *DKFZp451I127*, and (*v*) *CYP26C1*.

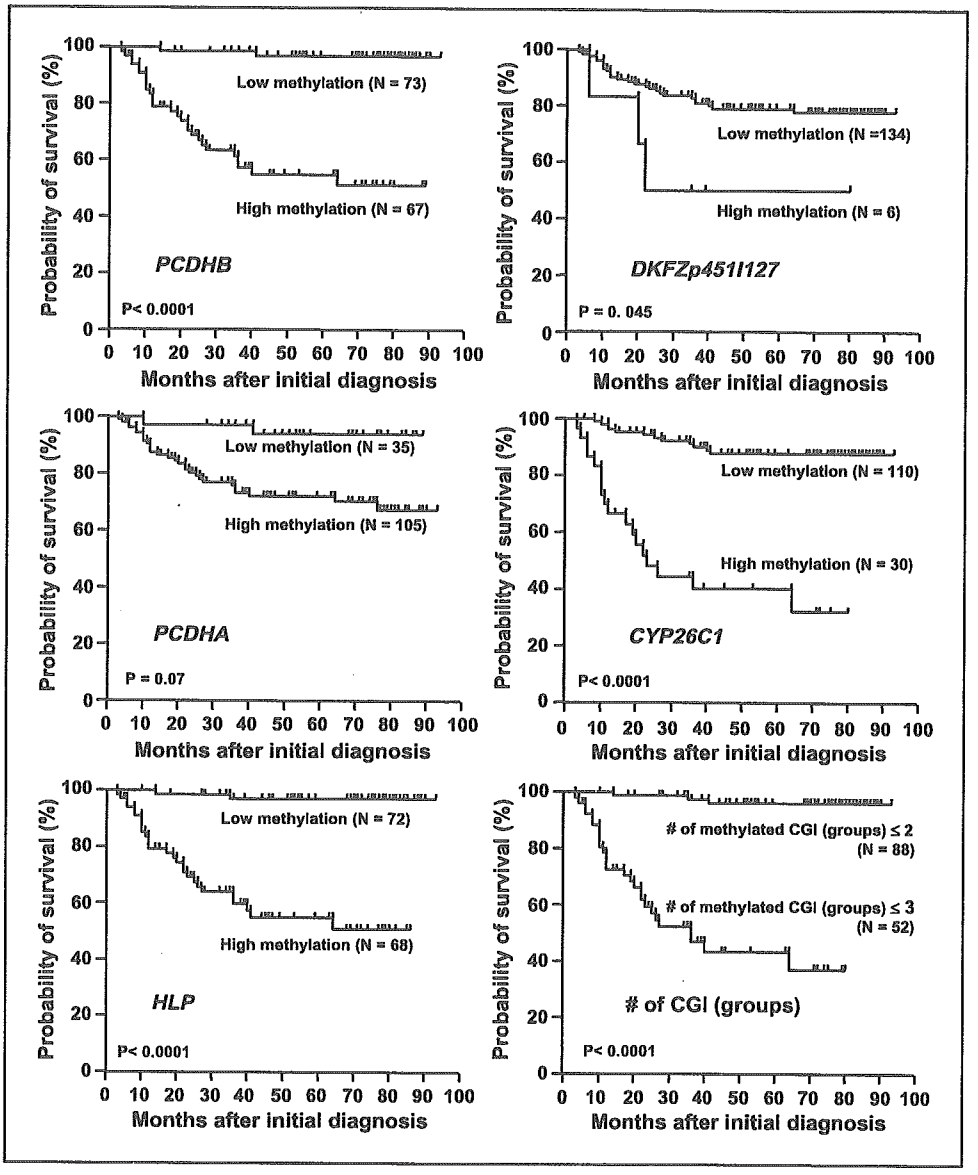


Figure 3. Predictive powers of methylation of the five CGI (groups) identified, and their multiple methylation: (*i*) 17 CGIs of the *PCDHB* family, (*ii*) 13 CGIs of the *PCDHA* family, (*iii*) CGIs of *HLP* and its pseudogene, (*iv*) *DKFZp451I127*, (*v*) *CYP26C1*, and (*vi*) methylation of three of these or more were analyzed by the Kaplan-Meier method using 140 primary samples. The *PCDHB* family, *HLP*, *DKFZp451I127*, *CYP26C1*, and methylation of multiple CGI (groups) had significant influence on survival.

neuroblastomas with a poor prognosis (Supplementary Fig. 2*A*). The simultaneous methylation of (i) 17 CGIs of the *PCDHB* family, (ii) 13 CGIs of the *PCDHA* family, (iii) CGIs of *HLP* and its pseudogene, (iv) *DKFZp451I127* CGI, and (v) *CYP26C1* CGI conformed with the concept of the CpG island methylator phenotype (CIMP; ref. 16).

Associations between CIMP and poor prognosis were examined by defining CIMP as cases with methylation of two CGI (groups) or more, those with three or more, those with four or five, and those with five. When CIMP was defined as cases with methylation of three CGI (groups) or more, the largest association with poor prognosis was observed, with a HR of 25.4 (95% CI, 7.6-84.5; Fig. 3). However, the HR (22.1) given by 17 CGIs of the *PCDHB* gene family approximated to this, and the *PCDHB* methylation level closely correlated with the number of methylated CGI (groups; Supplementary Fig. 2*B*). Therefore, for simplicity of analysis, we defined CIMP in neuroblastomas on the basis of high methylation of the *PCDHB* family, tentatively with a cutoff value of 40%.

Predictive Power of CIMP, Compared with Known Prognostic Factors. Univariate analyses showed that N-*myc* amplification, low *TrkA* expression, DNA diploidy, and an age no younger than 1 year gave HRs of 9.5 (95% CI, 4.4-20.5), 3.9 (95% CI, 1.7-9.3), 4.2 (95% CI, 1.65-10.8), and 12.3 (95% CI, 3.7-41.7). Cases were stratified by these known factors (Table 2). In those without N-*myc* amplification, CIMP also showed an influence with a HR of 12.4 (95% CI, 2.6-58.9), but almost all cases with N-*myc* amplification (37 of the 38 cases) showed CIMP. It was suggested that cases with N-*myc* amplification were contained in the cases with CIMP. CIMP was independent from *TrkA* overexpression, DNA ploidy, and age at diagnosis. Stage seemed to be a stronger prognostic factor. Notably, even when limited to cases in stages III and IV without N-*myc* amplification, which are classified into the intermediate risk group and clinically important, CIMP gave a HR of 4.8 (95% CI, 1.0-23.0; $P = 0.048$).

Multivariate analyses were finally done taking all the five known prognostic factors into account. Although CIMP gave a HR of 5.0 (95% CI, 0.47-52.7), it was not significant ($P = 0.18$), possibly due to limitation in the number of cases.

Table 1. Association between the *PCDHB* methylation and methylation of other CGIs

Variables	Methylation level of *PCDHB* family gene		*P**
	High (≥40%)	Low (<40%)	
No. cases (n = 140)	67	73	
Methylation of CGIs outside promoter regions (n = 140)			
PCDHA gene family (exon 1)†	65/67	41/73	<0.0001
HLP (exons 2-13)‡	52/67	16/73	<0.0001
CYP26C1 (exon 2)§	30/67	0/73	<0.0001
p41Arc (intron 8)	1/67	1/73	0.48
SIM2 (exon 2)	0/67	0/73	
Methylation of CGIs in promoter regions (n = 140)			
DKFZp451I127‖	6/67	0/73	0.011
RASSF1A	51/67	10/73	<0.0001
BLU	25/67	3/73	<0.0001
p16	0/67	0/73	
hMLH1	0/67	0/73	
PCDHB1	0/67	0/73	
TAF7	0/67	0/73	
p41Arc	0/67	0/73	
SIM2	0/67	0/73	

*Fisher's exact test.
†Boundaries for high methylation and low methylation of *PCDHA* gene family were set at 80% of the methylation index.
‡Boundaries for high methylation and low methylation of *HLP* were set at 10% of the methylation index.
§Boundaries for high methylation and low methylation of *CYP26C1* were set at 70% of the methylation index.
‖Boundaries for high methylation and low methylation of *DKFZp451I127* were set at 20% of the methylation index.

Effects of PCDHB Methylation on Gene Expression and Chromatin Structure. The CGIs of the *PCDHB* family were located in their gene bodies, whose methylation generally does not block gene transcription (17). The actual effects of methylation on expression were examined for 16 genes of the *PCDHB* family using 10 primary neuroblastomas with low methylation and five primary neuroblastomas with high methylation. The methylation was not associated with loss of expression (a representative result is shown in Fig. 4*A*). The effect of methylation of the *PCDHB16* CGI on the histone modification was further examined by chromatin immunoprecipitation assay. It was found that DNA methylation of the *PCDHB16* CGI did not induce histone H3 lysine 9 methylation or histone H3 deacetylation (data not shown).

Association between CIMP and Promoter Methylation. High methylation of *PCDHB* CGIs, a sensitive surrogate marker of CIMP in neuroblastomas, did not repress gene expression or induce histone modification. This indicated that CIMP is involved in the poor prognosis of neuroblastomas by causing methylation of promoter CGIs, although it is known that promoter CGIs are resistant to *de novo* methylation (18, 19).

Among the five CGI (groups) identified in this study, only that of *DKFZp451I127* was located in a promoter region. Although its methylation was infrequent, the methylation was observed only in neuroblastomas with CIMP (Table 1), and was associated with expression loss (Fig. 4*B*). To make the association clearer, methylation statuses were analyzed for eight additional CGIs in promoter regions. It was shown that methylation of promoter CGIs of *RASSF1A* (3p21) and *BLU* (3p21) was far more frequently observed in neuroblastomas with CIMP (Table 1, $P < 0.0001$). At the same time, there was a preference for CGIs affected by CIMP among CGIs in promoter regions, and also among those outside promoter regions (Table 2).

Discussion

Extensive methylation of multiple CGIs, conforming with the concept of CIMP, was here found specifically present in neuroblastomas with a poor prognosis and could be sensitively detected by focusing on the *PCDHB* family. *PCDHB* methylation did not suppress gene expression or induce histone modification. However, CIMP was associated with promoter methylation of *RASSF1A* and *BLU* genes and one of the mechanisms underlying the poor prognosis of neuroblastomas seemed to be silencing of these and possibly other tumor suppressor genes and genes important for differentiation.

CIMP was originally identified in colon cancers (16), but there has been some dispute over its presence (20). The clear correlation between CIMP and a poor prognosis found here for neuroblastomas was unequivocal and presumably reflects an intrinsic tendency for methylation of CGIs. This is because, first, neuroblastomas have a much shorter history than colon cancers, and the accumulated number of methylated CGIs in neuroblastomas is expected to parallel the speed of occurrence of

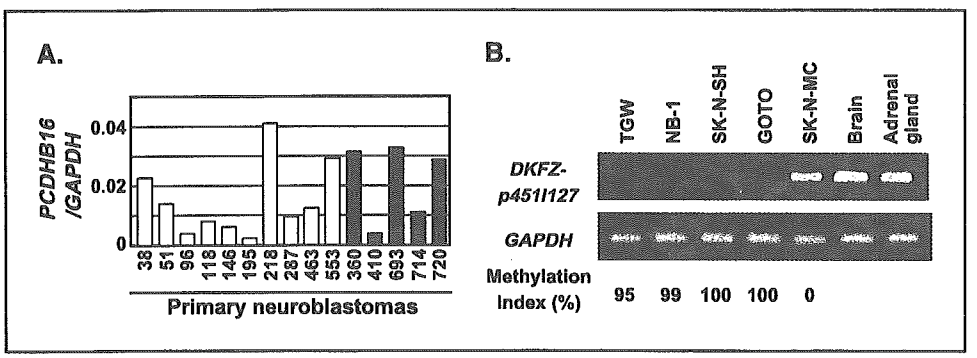


Figure 4. Effects of methylation of the *PCDHB* family and *DKFZp451I127* on gene expression. *A*, *PCDHB16* expression was analyzed by quantitative RT-PCR in 10 primary samples with low methylation (*open columns*) and five primary samples with high methylation (*closed columns*), and no difference was observed between the two groups. *B*, silencing of *DKFZp451I127* by methylation of its promoter CGI. The CGI was methylated in four cell lines, TGW, NB-1, SK-N-SH, and GOTO, whereas it was unmethylated in one cell line, SK-N-MC. *DKFZp451I127* was expressed in SK-N-MC, but not expressed at all in the four cell lines with the promoter methylation.

Table 2. HRs of death by *PCDHB* methylation status in subgroup of known prognostic factors

Stratified by		*PCDHB* methylation	No. cases	No. deaths	HR* (95% CI)	$P^{\dagger}$
Overall (n = 140)		High	67	1	22.1 (5.3-93.4)	< 0.0001
		Low	73	2	1	
N-*myc* amplification (n = 140)	No	High	30	8	12.4 (2.6-58.9)	0.002
		Low	72	2	1	
	Yes	High	37	20	NE	—
		Low	1	0		
TrkA overexpression (n = 130)	Yes	High	20	6	18.3 (2.2-152.6)	0.007
		Low	49	1	1	
	No	High	40	19	NE	
		Low	21	0		
DNA ploidy (n = 125)	Aneuploid	High	17	5	18.3 (2.1-156.7)	0.008
		Low	49	1	1	
	Diploid	High	38	17	NE	
		Low	21	0		
Clinical stages (n = 140)	Stages I, II, and IVS	High	8	0	NE	—
		Low	52	0		
	Stages III and IV	High	59	28	7.4 (1.8-31.3)	0.006
		Low	21	2	1	
Age at diagnosis (n = 140)	<1	High	11	3	NE	
	≥1	Low	59	0		
		High	56	25	4.5 (1.1-18.9)	0.043
		Low	14	2	1	

*HR of death for a case with high *PCDHB* methylation compared with a case with low methylation. NE shows not estimable due to no events in at least one category.
†Significance level for a high *PCDHB* methylation to low methylation using Cox proporitonal model.

methylation. Second, methylation of the *PCDHB* family did not affect gene expression, and there should have been no selection of cells with the *PCDHB* methylation, in contrast to the case of promoter methylation of tumor suppressor genes. Investigation into the mechanism of the intrinsic tendency for methylation of multiple CGIs is necessary. Furthermore, alleviation of the intrinsic tendency could block progression of neuroblastomas and have potential therapeutic value.

Among the six CGI (groups) outside promoter regions analyzed here, CIMP in neuroblastomas preferentially affected four CGI (groups); those of the *PCDHB* family, the *PCDHA* family, *HLP*, and *CYP26C1*. Unexpectedly, three CGIs that are known to be frequently methylated in human colon cancers with CIMP, MINT1, MINT2, and MINT17 (16) were not methylated in neuroblastoma cell lines (data not shown). Among the nine CGIs in promoter regions analyzed, CIMP in neuroblastomas affected only three, those of *RASSF1A*, *BLU*, and *DKFZp451I127*. The nine CGIs were selected based upon previous reports as tumor suppressor genes (*RASSF1A*, *BLU*, *p16*, and *hMLH1*; refs. 21–23), the chromosomal location flanking the *PCDHB* family (*PCDHB1* and *TAF7*), our previous report on the fidelity in inheriting methylation patterns (*p41Arc* and *SIM2*; ref. 19), and the findings here (*DKFZp451I127*). Because gene expression and possibly chromatin structures affect the frequency of *de novo* methylation (24, 25), the available data suggest that CGIs useful to sensitively detect CIMP might vary according to the tumor type.

The influence of CIMP on prognosis was here found to be comparable to that of the currently most reliable marker, N-*myc* amplification, and stronger than *TrkA* overexpression and DNA ploidy on univariate analysis. Subgroup analysis showed that the influence was independent of *TrkA* overexpression, DNA ploidy and age at diagnosis and CIMP had influence even in cases without N-*myc* amplification and in advanced stages. These points strongly indicated CIMP to be a promising new prognostic marker. However, the cutoff values adopted here are tentative, and the HRs obtained could have been overestimated. A validation study using independent samples is necessary for further evaluation. The fact that cases with CIMP contained almost all the cases with N-*myc* amplification suggested that a common molecular mechanism caused both alterations, or that CIMP may lead to N-*myc*

amplification. Whatever the case, the findings might provide clues to molecular mechanisms of neuroblastoma development.

In summary, the present study showed that CIMP is present specifically in neuroblastomas with poor prognosis and that can be sensitively detected by focusing on *PCDHB* methylation. CIMP seems to be a promising new prognostic marker, and its evaluation and investigations into the mechanisms underlying CIMP in neuroblastomas seem warranted.

Acknowledgments

Received 7/27/2004; revised 11/14/2004; accepted 11/24/2004.

Grant support: Grant-in-aid for the Third-term Cancer Control Strategy Program from the Ministry of Health, Labour, and Welfare, Japan and Research Resident Fellowship from the Foundation for Promotion of Cancer Research (M. Abe).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. E. Okochi-Takada and G. S. Goldberg for critical reading of the article and the institutions for participation in the collection of clinical materials.

References

1. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
2. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature 1998;395:89–93.
3. Kondo Y, Kanai Y, Sakamoto M, et al. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis-A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. Hepatology 2000;32:970–9.
4. Ushijima T, Morimura K, Hosoya Y, et al. Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. Proc Natl Acad Sci U S A 1997;94:2284–9.
5. Kaneda A, Takai D, Kaminishi M, Okochi E, Ushijima T. Methylation-sensitive representational difference analysis and its application to cancer research. Ann N Y Acad Sci 2003;983:131–41.
6. Takai D, Yagi Y, Wakazono K, et al. Silencing of *HTR1B* and reduced expression of *EDN1* in human lung cancers, revealed by methylation-sensitive representational difference analysis. Oncogene 2001; 20:7505–13.
7. Kaneda A, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T. Identification of silencing of nine genes in human gastric cancers. Cancer Res 2002;62:6645–50.
8. Miyamoto K, Asada K, Fukutomi T, et al. Methylation-associated silencing of heparan sulfate *D-glucosaminyl 3-O-sulfotransferase-2* (*3-OST-2*) in human breast, colon, lung and pancreatic cancers. Oncogene 2003;22:274–80.
9. Hagihara A, Miyamoto K, Furuta J, et al. Identification of 27 5′ CpG islands aberrantly methylated and 13 genes silenced in human pancreatic cancers. Oncogene 2004;23:8705–10.
10. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev. Cancer 2003;3:203–16.
11. Schwab M, Westermann F, Hero B, Berthold F. Neuroblastoma: biology and molecular and chromosomal pathology. Lancet Oncol 2003;4:472–80.
12. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, et al. Association between high levels of expression of the *TRK* gene and favorable outcome in human neuroblastoma. N Engl J Med 1993;328:847–54.
13. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33:245–54.
14. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 2002;3:662–73.
15. Kaneda A, Kaminishi M, Sugimura T, Ushijima T. Decreased expression of the seven ARP2/3 complex genes in human gastric cancers. Cancer Lett 2004; 212:203–10.
16. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A 1999;96:8681–6.
17. Gonzalgo ML, Hayashida T, Bender CM, et al. The role of DNA methylation in expression of the *p19/p16* locus in human bladder cancer cell lines. Cancer Res 1998;58:1245–52.
18. Nguyen C, Liang G, Nguyen TT, et al. Susceptibility of nonpromoter CpG islands to *de novo* methylation in normal and neoplastic cells. J Natl Cancer Inst 2001; 93:1465–72.
19. Ushijima T, Watanabe N, Okochi E, et al. Fidelity of the methylation pattern and its variation in the genome. Genome Res 2003;13:868–74.
20. Yamashita K, Dai T, Dai Y, Yamamoto F, Perucho M. Genetics supersedes epigenetics in colon cancer phenotype. Cancer Cell 2003;4:121–31.
21. Agathanggelou A, Dallol A, Zochbauer-Muller S, et al. Epigenetic inactivation of the candidate 3p21.3 suppressor gene *BLU* in human cancers. Oncogene 2003; 22:1580–8.
22. Takita J, Hayashi Y, Nakajima T, et al. The *p16* (*CDKN2A*) gene is involved in the growth of neuroblastoma cells and its expression is associated with prognosis of neuroblastoma patients. Oncogene 1998; 17:3137–43.
23. Harada K, Toyooka S, Maitra A, et al. Aberrant promoter methylation and silencing of the *RASSF1A* gene in pediatric tumors and cell lines. Oncogene 2002; 21:4345–9.
24. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene *MAGE-A1* in tumor cells. Mol Cell Biol 2004;24:4781–90.
25. Richards EJ, Elgin SC. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell 2002;108:489–500.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters 228 (2005) 5–11
CANCER Letters
www.elsevier.com/locate/canlet

Mini-review

A review of DNA microarray analysis of human neuroblastomas

Miki Ohira[a], Shigeyuki Oba[b], Yoko Nakamura[a], Takahiro Hirata[c], Shin Ishii[b], Akira Nakagawara[a,*]

[a]*Division of Biochemistry, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuoh-ku, Chiba 260-8717, Japan*
[b]*Graduate School of Information Science, Nara Institute of Science and Technology, Ikoma 630-0192, Japan*
[c]*Hisamitsu Pharmaceutical Co. Inc., Tokyo 100-622, Japan*

Received 20 December 2004; accepted 12 January 2005

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.

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

* Corresponding author. Tel.: +81 43 264 5431; fax: +81 43 265 4459.
E-mail address: akiranak@chiba-cc.jp (A. Nakagawara).

0304-3835/$ - see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2005.01.054

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

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

[a] F;favorable NBL, UF;unfavorable NBL.

the patient. Therefore, additional potent markers for 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/ECEL1*) [17], neuronal leucine-rich repeat family members (*NLRR1* and *NLRR3*) [18], tubulin tyrosine ligase (*TTL*) [19], novel putative apoptosis-related gene with BCH domain (*BMCC1*, 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