Table 2. Reports of Telomerase Study in Neuroblastoma

Study	Summary of Data	Ref.
Kim *et al.* (n = 5)	Telomerase positive 5/5 (100%)	[108]
Hiyama *et al.* (n = 105)	Detectable telomerase 101/105 (96%) Stage I,II 51 (low 48, high 3) Stage III,IV 45 (low 25, high 19) Stage IVS 9 (low 5, high 1)	[110, 111]
Reynolds *et al.* (n = 150)	High hTR expression 60 (40%). Stage I = 7%, II = 18%, III = 38%; IV = 80%, IVS = 30%	[112, 134]
Brinkschmidt *et al.* (n = 14)	Telomerase positive Stage IVS 4/14 Telomerase positive Regressed Stage IVS 1/10	[79]
Poremba *et al.* (n = 16)	Telomerase positive 12/16 (75%)	[113]
Dockhorn-Dworniczak *et al.* (n = 77)	TRAP positive 22/77 (29%) TRAP positivity correlated with poor survival in stage IVS	[135]
Choi *et al.* (n = 106)	Telomerase positive 43/106 (41%), hTR positive 43/106 (41%). High telomerase activity or hTR correlated with poor prognosis	[136]
Isobe *et al.* (n = 16)	High hTERT expression correlated with unfavorable tumor	[137]
Krams *et al.* (n = 124)	Full-length hTERT (30/124) correlated with poor prognosis	[121]
Streutker *et al.* (n = 38)	Detectable telomerase (19/38, 50%) correlated with poor prognosis. Dead cases: undetectable 1/19, low 0/4, high 6/15	[138]
Nozaki *et al.* (n = 65)	High telomerase activity and low NTRK1 expression correlated with poor prognosis. The 5-year event-free survival rates:low 86.5%	[139]
Maitra *et al.* (n = 32)	High hTR expression 11 (40%) Advanced stages: 9/12, early stages: 2/8 stage 4S: 0/4, ganglioneuroblastoma/ ganglioneuroma: 0/8	[140]

the other hand, none of those tumors with low or undetectable telomerase activity have elongated telomeres and those with shortened telomere lengths may be the result of repeated replication without sufficient telomerase activity. Alternative telomere lengthening (ALT), which is an alternative mechanism to elongate telomere in some immortal culture cells, has not been reported in primary neuroblastoma. Indeed, most stage 4S tumors examined showed shortened telomeres relative to normal tissue, suggesting that telomere shortening with low or absent telomerase activity may be a factor in promoting the spontaneous regression of the tumors seen in some patients.

Most patients whose tumors showed low or undetectable telomerase activity were younger than 1 year old at diagnosis, while high telomerase activity was also associated with advanced stages. More than one half of patients with high telomerase activity died of disease [111]. Amplification of the *MYCN* gene and 1p loss were usually detectable in the tumors with high telomerase activity, but rarely in the tumors with low or undetectable activity [111]. While most tumors with undetectable or low telomerase activity showed high expression of *NTRK1* and *HRAS*, more than one half of those tumors with high telomerase activity showed no expression of these genes. Thus, high telomerase activity correlates with molecular biological features of neuroblastoma that predict aggressive clinical behavior while low telomerase activity is associated with tumor biological features known to be associated with less-aggressive tumors (Table 3).

In human germ cells, telomerase is expressed to maintain telomere length, while in embryonal somatic tissues telomerase is likely repressed gradually before birth as cells differentiate [116]. Neuroblastoma is likely derived from primitive cells that originate in the embryonal neural crest, either from multipotent stem cells or from more differentiated neuroblasts. In fetal adrenal glands, neuroblasts increase in number and size until 14-20 weeks of gestational age and then regress [117, 118]. Adrenal gland tissues of fetuses at 16 and 18 weeks of gestational age exhibited low telomerase activity. As shown in Fig. (4), neuroblastomas could acquire telomerase activity from a failure to repress telomerase activity during development (i.e. retention of telomerase activity in pre-neoplastic neuroblasts). Alternatively, reactivation of telomerase could occur in the tumor cells, perhaps as a consequence of other genetic alterations. High telomerase activity seen in many aggressive neuroblastomas is likely to be due to reactivation of telomerase by the tumor during progression. The fact that most of these aggressive tumors are found in children older than one year is also compatible with

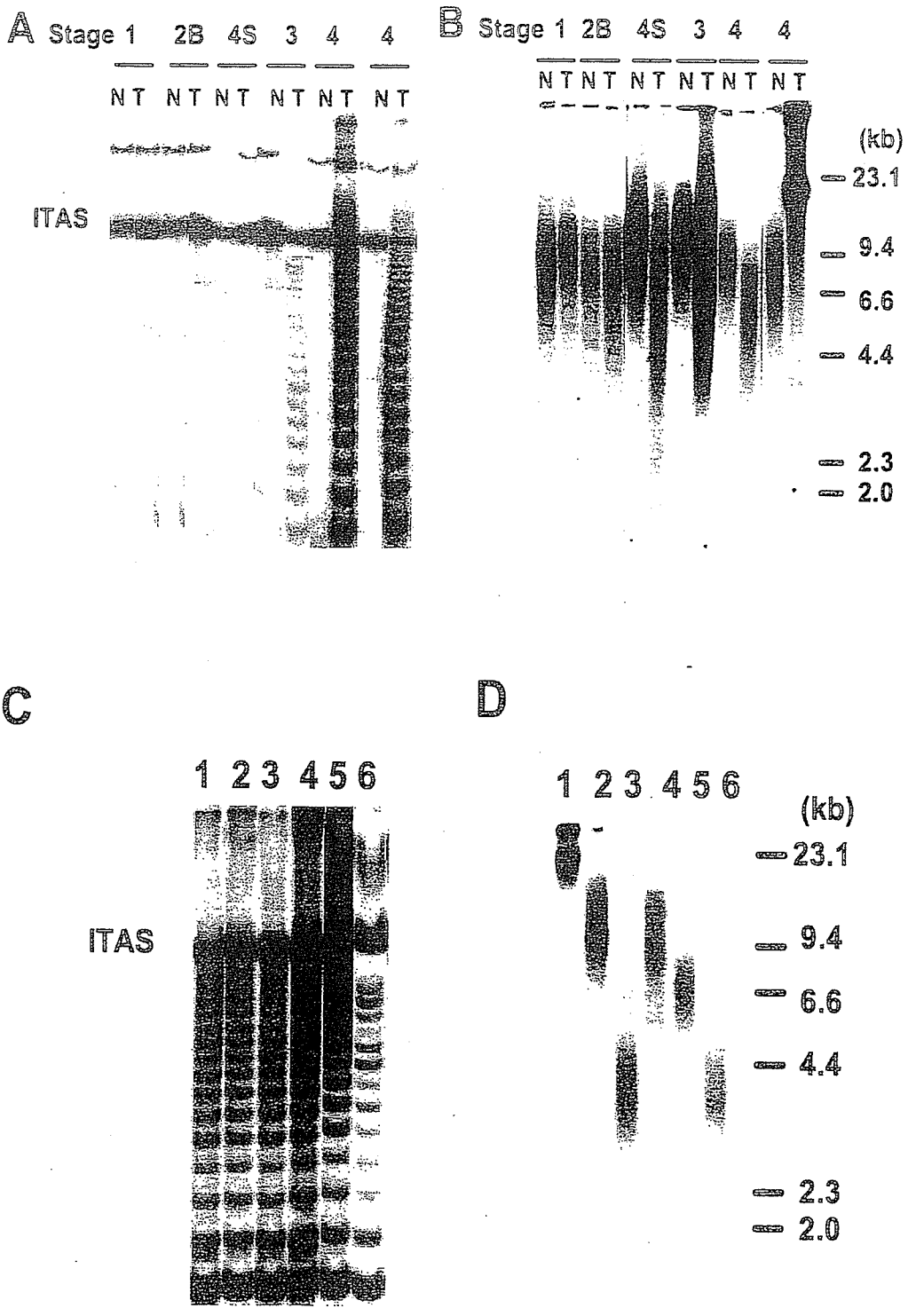

Fig. (3). Telomerase activity and telomere length in neuroblastoma specimens (A, B) and in neuroblastoma cell lines (C, D) N, normal adrenal gland; T, neuroblastoma tissue. (A) Telomerase activity was measured by TRAP assay in 6 representative neuroblastoma samples with each normal adrenal gland tissue. Stages were classified according to International Neuroblastoma Staging System (INSS). No noncancerous adrenal gland tissues showed telomerase activity. In neuroblastoma tissues, cases with stage 1, 2B, 4S tumors showed no detectable activity, while stage 3 case showed low activity and 2 stage 4 cases showed high activity. (B) Telomere length was measured by Southern blot analysis in 6 representative neuroblastoma samples. In cases of stage 1, 2B and 3, telomere length in noncancerous tissues was undistinguishable from that of each neuroblastoma sample. In stage 4S case, telomere length was shorter than that of noncancerous tissue. In this case, the tumor did not have telomerase activity, suggesting that telomere shortening may occur with regression of this tumor. However, in two stage 4 cases, tumor samples showed shorter and longer telomere, respectively. Since telomerase was activated in these tumors, telomere lengths were stabilized in these lengths. (C) Telomerase activity was measured by TRAP assay in 6 representative neuroblastoma cell lines. All showed high levels of telomerase activity. (D) Telomere length was measured by Southern blot analysis in 6 representative neuroblastoma cell lines. Since telomerase was activated in these cell lines, telomere lengths were stabilized in various lengths.

Table 3. Correlation Between Telomerase Activity and Other Prognosis-Associated Factors in Neuroblastoma Cases [110, 111]

Telomerase activity*		undetectable (n = 9)	low (n = 107)	high (n = 35)
Stage	I, II (n = 72)	2	66	6
	III, IV (n = 63)	1	34	28
	IV-S (n = 14)	6	7	1
MYCN amplification		0	0	24
1p LOH¶ / informative cases		0/6	4/66	18/26
NTRK1 expression		4	77	8

According to serial dilution analysis of telomerase assay, telomerase positive samples were classified into two groups: high telomerase activity (e.g., a TRAP signal retained using 100 times diluted extracts containing 0.06 μg of protein) and low telomerase activity (e.g. a TRAP signal was detectable using the extract containing 6 μg of protein but not using 100-times diluted extracts containing 0.06 μg of protein).

¶1p LOH: loss of heterozygosity in short arm of chromosome 1

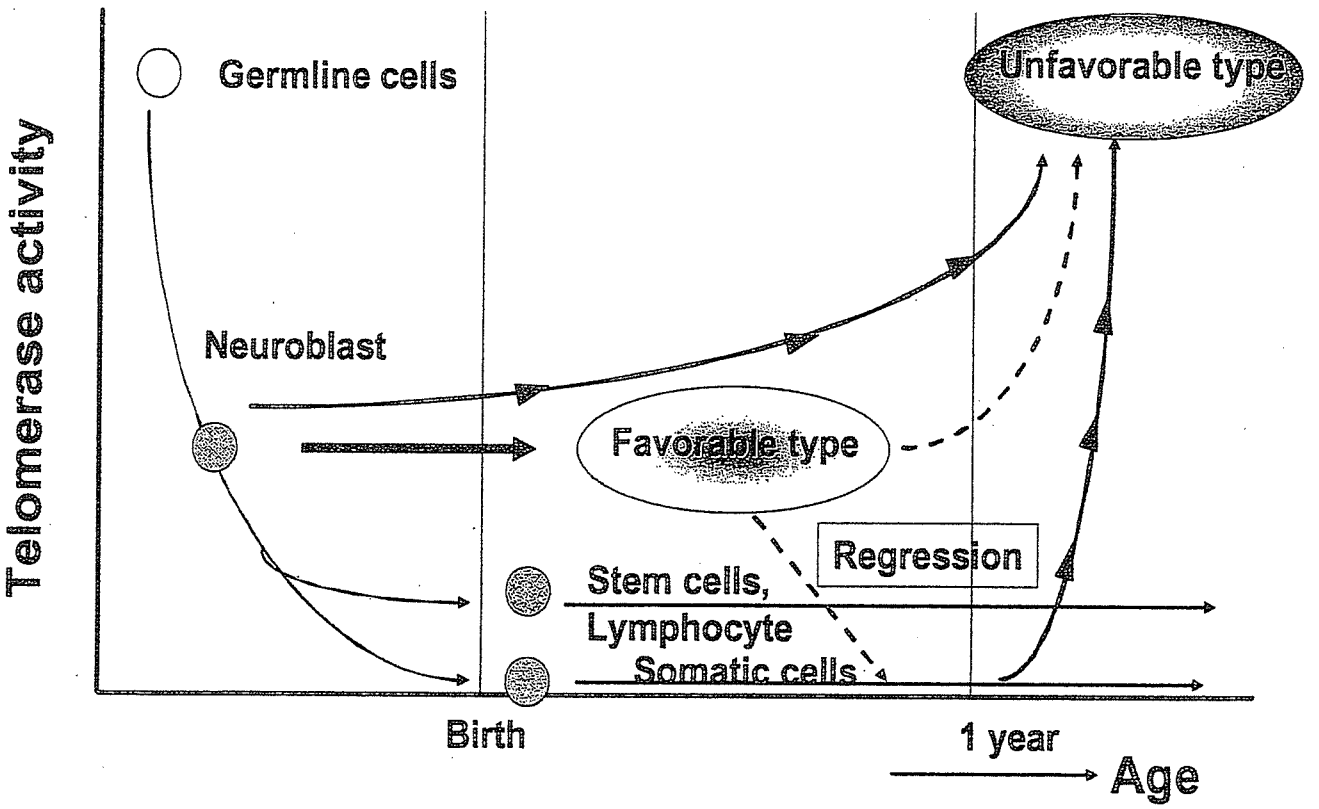

Fig. (4). Hypothesis of telomerase activity and neuroblastoma development. Normal neuroblasts, which develop from germline cells, have low telomerase activity at fetal development. Telomerase activity is repressed in normal neuroblasts and other somatic cells except for stem cells and lymphocytes before birth. Favorable neuroblastoma is developed from the neuroblasts retaining the expression of fetal levels of telomerase activity. Most of these tumors occur in young infants and some of them regress concomitant with a loss of telomerase activity. Telomere lengths of these tumors are indistinguishable or shorten from those of normal tissues. On the other hand, unfavorable tumors occur with telomerase reactivation. In these tumors, other genetic aberrations such as *MYCN* amplification promote cell division and shortening of telomere. Critical shortening of telomere causes chromosomal instability which contributes to the accumulation of additional genetic alterations and telomerase reactivation. This reactivation stabilize telomeres at various length in tumor cells capable of indefinite proliferation. These tumors occur in elder children and frequently show poor prognosis. Thus, telomerase expression may be required as a critical step in the multigenetic process of tumorigenesis, and two different pathways may exist for the development of neuroblastoma.

the hypothesis that such tumor cells likely have emerged after accumulating several genetic changes during the course of multiple cell divisions. Favorable neuroblastomas appear to share many features of neuroblasts. Most of these favorable tumors occur in infants (< one year old) and appear to have few genetic aberrations as described previous sections. In some of these cases, of which stage 4S patients may be the best example, a lack of telomerase activity may be related to

regression of the tumors. In such tumors the levels of telomerase activity are likely insufficient to maintain telomere length, so that as the tumors continue to proliferate, the telomeres shorten, eventually resulting in senescence or cell death. Thus, a failure to maintain telomeres may be one mechanism by which some neuroblastomas undergo spontaneous regression.

Human telomerase activity is associated with the expression of two major components: human telomerase RNA (hTR) [104] and human telomerase reverse transcriptase (hTERT) [119]. Recent studies have targeted the expression of these two components as surrogates of telomerase activity and discussed the feasibility of their quantitative evaluation. Since hTR is expressed at low level even in cells without telomerase activity [120], detection of *hTERT* mRNA expression is believed to be a more reliable marker for existing cancer cells in neuroblastoma [121-123]. However, the existence of splicing variants of *hTERT* mRNA that do not produce telomerase activity [124] is also problematic in detection of *hTERT* mRNA as a surrogate of telomerase activity.

In situ hybridization (ISH) of telomerase components (hTR and *hTERT* mRNA) and hTERT immunohistochemistry (IHC) are applicable to fixed cells [125-127]. However, hTR is detectable at low level in cells without telomerase activity, and it does not always represent telomerase activation. On the other hand, hTERT is the catalytic component of human telomerase and its expression levels appear to parallel with those of telomerase activity. Thus, ISH of *hTERT* mRNA and hTERT IHC are preferable to evaluate telomerase activation [125, 126, 128]. The expression of hTERT appears to be heterogeneously distributed in adult cancer tissues and the distribution shows regional variability in some cases. By contrast, in neuroblastoma, the level of hTERT expression in each cell differed between the unfavorable tumors with high telomerase activity and the favorable ones with low telomerase activity. The levels of hTERT expression may reflect the differentiating process of these tumor cells [125]. The detail analysis of hTERT, hTR, and telomerase associated proteins should contribute to clarify the biology of neuroblastoma in future.

Telomerase activity might also provide a therapeutic target against malignant cells, including neuroblastoma. Agents that inhibit telomerase activity which are under development include reverse transcriptase inhibitors [129], and antisense strategies (oligonucleotide, peptide nucleic acids (PNA) [130]) or ribozymes [131] against hTR. Since telomerase activity is repressed in most human somatic tissues, toxicity of agents targeting for telomerase will be minimal. The antitelomerase agent GRN163, which is a 13-mer oligonucleotide complementary to the template region of the human telomerase RNA subunit hTR, inhibits growth of glioblastoma *in vivo* [132]. This finding supports further development of this compound as a potential anticancer agent for neuroblastoma. However, such a therapeutic strategy may be limited by the length of therapy required if telomere shortening is required to trigger tumor cell death, and by alternative, non-telomerase mechanisms for telomere maintenance [133]. The successful application of such therapy will require a thorough understanding of the biology of telomeres and telomerase in both normal and neuroblastoma cells.

PERSPECTIVES

Neuroblastoma, despite many advances in the understanding of its biological heterogeneity and developmental molecular pathways, has remained serious in young children. Basic research and clinical efforts will lead to an understanding of the molecular pathways governing both progression and spontaneous regression of neuroblastoma. Neuroblatoma mass-screening project revealed that more than half of infant neuroblastomas regress or mature. These events should provide the platform from which new diagnostic tools can be developed and from which new types of therapies for individual patient can be attempted. Recently, genome-wide genetic aberrations and gene expression profiles are able to be identified by microarray analysis. More precise definition of the molecular changes in neuroblastomas may allow for more specific therapies with subsequent improvements in overall rates and quality of cure.

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Clinical significance of serum NM23-H1 protein in neuroblastoma

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We have previously reported that *NM23* genes are overexpressed in various hematological malignancies and that serum NM23-H1 protein levels are useful for predicting patient outcomes. In this study we assessed the clinical implications of serum NM23-H1 protein on neuroblastoma. We examined serum NM23-H1 protein levels in 217 patients with neuroblastoma, including 131 found by mass-screening and 86 found clinically by an enzyme-linked immunosorbent assay, and determined the association between levels of this protein, and known prognostic factors or the clinical outcome. The serum NM23-H1 protein level was higher in neuroblastoma patients than in control children ($P < 0.0001$). Patients with *MYCN* amplification had higher serum NM23-H1 levels than those with a single copy of *MYCN*. Overall survival was assessed in the 86 patients found clinically, and was found to be worse in patients with higher serum MN23-H1 levels (≥ 250 ng/mL) than in those with lower levels (< 250 ng/mL; $P = 0.034$). The higher level of NM23-H1 was correlated with a worse outcome in patients with a single *MYCN* copy, or in those younger than 12 months of age. Serum NM23-H1 protein levels may contribute to predictions of clinical outcome in patients with neuroblastoma. (*Cancer Sci* 2005; 96: 653–660)

The *NM23* gene was identified by differential hybridization of a cDNA library with total RNA extracted from slightly and highly metastatic melanoma cell lines.[1] The *NM23* gene has been identified as a family of genes encoding different isoforms of nucleoside diphosphate kinase (NDPK).[2] *NM23* genes play critical roles in cellular proliferation, differentiation, oncogenesis, and tumor metastasis.[1,3] The mechanisms for these pleiotropic effects are not well understood. Eight isotypes of the human *NM23* gene (*NM23-H1*, *NM23-H2*, *NM23-H3/DR-NM23*, *NM23-H4*, *NM23-H5*, *NM23-H6*, *NM23-H7*, and *NM23-H8*) have been identified.[2] Among these, only *NM23-H1* and *NM23-H2* have been studied extensively in human cancers.

The level of *NM23-H1* expression is inversely correlated with the tumor's metastatic potential in experimental rodent cells and in human tumors such as breast, ovarian, cervical and gastric cancer, hepatocellular carcinoma, and melanomas.[4] Therefore, *NM23-H1* is implicated in the regulation of metastasis in a variety of human cancers. However, overexpression of the *NM23-H1* gene has been reported in various neoplasms including neuroblastoma, hematological malignancies, and pancreatic, lung, ovarian and gastric cancers.[5–8] Overexpression of *NM23-H1* is indicative of a poor patient prognosis for patients with neuroblastoma, acute myelogenous leukemia (AML), or non-Hodgkin's lymphoma (NHL).[8–10]

In neuroblastoma, a gain of 17q is the most frequent genetic abnormality, followed by 1p deletion and *MYCN* amplification, both of which correlate closely with 17q gain. The three genetic events are strong predictors of unfavorable prognosis.[11,12] The *NM23* genes are located at the edge of the common chromosomal region of 17q gain. Godfrid *et al.* identified genes that are activated in the *MYCN* downstream pathway using SAGE libraries of *MYCN*-transfected and control neuroblastoma cell lines.[13] The *NM23-H1* and *NM23-H2* genes are strongly induced in *MYCN*-expressing cells. Neuroblastoma tumor and cell line panels reveal a striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes. These findings suggest that *NM23-H1* and *NM23-H2* expression may be increased by 17q gain in neuroblastoma, and can be further upregulated by *MYCN* overexpression. These observations suggest a role of *NM23-H1* and *NM23-H2* in the tumorigenesis of an unfavorable type of neuroblastoma.

We previously established an enzyme-linked immunosorbent assay (ELISA) technique for determining the serum level of NM23-H1 protein.[14] Serum levels of NM23-H1 in patients with NHL and AML are significantly higher than those in controls, and elevated NM23-H1 levels correlate with poor prognosis in these patients.[10,15] It has been strongly suggested that serum NM23-H1 protein is produced directly by tumor cells and its level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14,16] These results indicate that the serum level of NM23-H1 protein may be clinically useful as a prognostic factor in NHL and AML. The present study assessed the clinical implications of serum NM23-H1 protein levels in patients with neuroblastoma, in whom tumor samples were used to determine the biological prognostic factors.

Materials and Methods

Patients and controls

Serum NM23-H1 protein was measured in 217 untreated neuroblastoma patients who were admitted to various institutions in Japan and underwent biopsy or surgery between 2000 and 2002. The 217 patients included 131 who were found by

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a mass-screening (MS) program for infants at 6 months of age by measuring urinary catecholamine metabolites and 86 who were found clinically.[17] Of the 86 patients, 29 who were younger than 12 months old were mostly found before MS, and 57 who were 12 months old or older underwent MS with a negative result, or did not undergo MS. Patients were staged according to the International Neuroblastoma Staging System (INSS).[18] Patients of any age with stage 1 or 2 disease, and those younger than 12 months of age with stage 3 disease were treated by surgery or surgery and chemotherapy consisting of cyclophosphamide and vincristine; patients 12 months or older with stage 3 or stage 4 disease and those younger than 12 months of age with stage 4 disease were treated according to the protocol published by the Japanese Neuroblastoma Study Group.[19] Serum samples from 23 children consisting of 22 with inguinal hernias and one with an edematous scrotum before surgery were analyzed for comparison. The median age of the children was 23 months (range: 3–49 months). Informed consent was obtained from patients and/or their parents, and the ethics committee of Saitama Cancer Center approved the study design.

Venous blood samples

Peripheral venous blood samples were collected in sterile test tubes with heparin and placed on ice. The samples were centrifuged at 2000*g* for 15 min at 4°C, and stored at −20°C. As a marker of hemolysis, free serum hemoglobin (Hb) was determined according to the method of Testa *et al.*[20]

ELISA for human NM23-H1 protein

NM23-H1 protein levels in serum were determined using a sandwich ELISA assay, as described previously.[14,15] Recombinant NM23-H1-GST protein was used as a standard.

Examination of *MYCN* copy number, TRKA expression and ploidy

DNA preparation, digestion, and Southern blot analysis using the *MYCN* probe were carried out as described previously.[12] The presence of more than three copies of the *MYCN* gene per haploid genome was considered to indicate amplification.[21] *TRKA* expression was examined by northern blotting as reported previously.[22] DNA index was analyzed on a Becton-Dickinson FACScan flow cytometer by DNA cell-cycle analysis software (version C).

Statistical analysis

The significance of differences in various clinical and biological aspects of the disease among the patient groups was examined by using the Mann–Whitney *U* or Kruskal–Wallis test (non-parametric analysis). Spearman's correlation coefficient (rs) by ranks was used to evaluate the correlation between paired values. Survival analysis was performed according to the Kaplan–Meier method, and the significance of differences in survival was determined by using the generalized Wilcoxon's and log-rank tests. A multivariate analysis of prognostic factors was performed using Cox's proportional-hazards regression model. All statistical analyses were performed with Excell Statcel and Stat Flex software (version 5.0, Artech Co. Ltd, Osaka, Japan), and $P < 0.05$ was taken to indicate significance.

Results

Examination of serum NM23-H1 protein levels in neuroblastoma patients and control children

The serum level of NM23-H1 was examined in 217 neuroblastoma patients and 23 control children. The serum levels of NM23-H1 were significantly higher in patients with neuroblastoma ($n = 217$, mean ± SD 176 ± 280 ng/mL) than in the control children ($n = 23$, 27 ± 41 ng/mL, $P < 0.0001$; Fig. 1a). The serum NM23-H1 levels of the control children were higher than those of the healthy adults (data not shown). The serum NM23-H1 levels in patients with neuroblastoma were significantly higher than those in patients with various hematological malignancies (data not shown). Next, the relationship between serum levels of NM23-H1 and Hb was examined in 217 neuroblastoma patients and 23 control children, because the NM23-H1 protein leaked from red blood cells by hemolysis may have elevated the serum NM23-H1 levels.[23] The results showed a weak correlation (rs = 0.3958, $P = 7.5356 \times 10^{-10}$, Spearman's correlation coefficient by ranks), although some patients had a higher Hb level but a lower NM23-H1 level, or a lower Hb level but a higher NM23-H1 level (Fig. 1b). When we chose samples from 156 patients

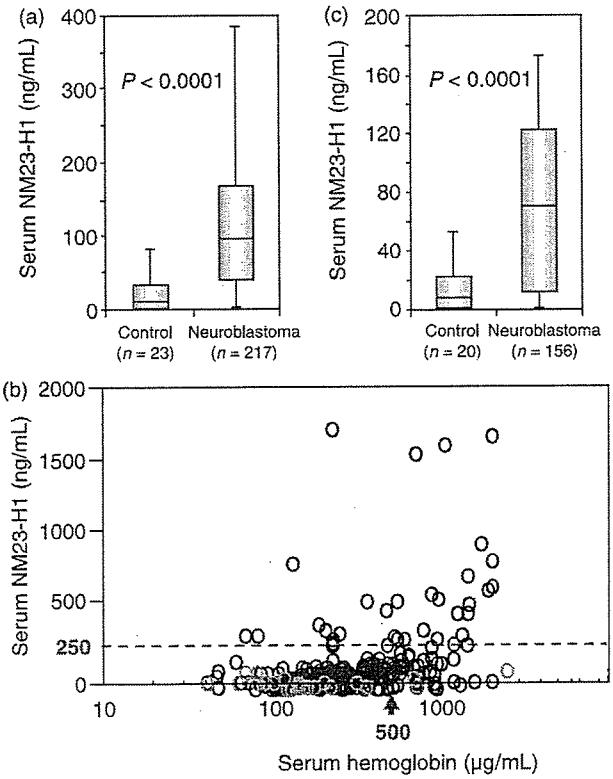

Fig. 1. Serum NM23-H1 levels in patients with neuroblastoma and in control children. (a) Box plots of NM23-H1 serum levels for 217 patients with neuroblastoma and 23 control children with any serum hemoglobin levels. (b) Relationship between the serum levels of NM23-H1 and hemoglobin in all samples examined (black circles, neuroblastoma patients [$n = 217$]; red circles, control children [$n = 23$]). (c) Box plots of NM23-H1 serum levels for 156 patients with neuroblastoma and 20 control children with serum hemoglobin levels less than 500 μg/mL.

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Table 1. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 217 patients with neuroblastoma and 23 control children

Clinicopathological findings	Number of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
Control children	23	27 ± 41	
All patients	217	176 ± 280	< 0.0001 (MW)
Method of detection			
Mass-screening	131	135 ± 206	
Found clinically	86†	239 ± 357	0.0595 (MW)
Age of patients			
< 12 months	134	168 ± 292	
≥ 12 months	83	190 ± 260	0.2427 (MW)
Stage of the disease			
1 + 2 + 4s	122	136 ± 159	
3 + 4	95	227 ± 378	0.8088 (MW)
Primary site			
Mediastinum	31	145 ± 212	
Adrenal	101	187 ± 290	
Abdomen	78	184 ± 302	0.3393 (KW)
Others	7	74 ± 82	
MYCN copy number			
1	186	143 ± 204	
> 3	31	378 ± 519	0.0006 (MW)
TRKA expression	173		
Medium or high	125	150 ± 209	
None or low	48	238 ± 373	0.4629 (MW)
Ploidy	168		
Diploid	69	188 ± 273	
Hyperdiploid	99	185 ± 284	0.9012 (MW)
Others	7	112 ± 126	

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test. †Table 2.

and 20 control children with serum Hb less than 500 μg/mL, the correlation between serum NM23-H1 and Hb levels was negligible (rs = 0.2351, $P = 0.0035$). Even in these patients, the serum levels of NM23-H1 were significantly higher (n = 156, 113 ± 184 ng/mL) than in the control children ($n = 20$, 20 ± 35 ng/mL, $P < 0.0001$; Fig. 1c).

Relationship between serum NM23-H1 protein levels and clinicopathological features in neuroblastoma

The relationship between serum NM23-H1 levels and various clinical and biological features in the 217 patients is shown in Table 1. The serum NM23-H1 levels tended to be higher in patients found clinically than in those found by MS ($P = 0.0595$), and were significantly higher in patients with amplified *MYCN* copies than in those with a single *MYCN* copy ($P = 0.0006$; Table 1). There was a correlation between *MYCN* amplification and the elevated serum NM23-H1 level (≥ 250 ng/mL) in all 217 patients (rs = 0.6970, $P = 0.0005$). However, serum Hb concentrations did not correlate with *MYCN* amplification ($P = 0.6320$), or other factors (data not shown). There was no significant difference in the serum NM23-H1 levels between two groups of patients classified by age of the patients, stage of the disease, expression levels of *TRKA*, or tumor cell ploidy (Table 1).

Serum NM23-H1 levels and overall survival

Of the 217 patients, the 86 patients who were found clinically were included and the 131 patients found by MS were excluded from survival analysis, because all the 131 patients were alive at the last follow-up (18–51 months), and the clinical and biological features are different for the patients found by MS and those found clinically.[12] The relationship between serum NM23-H1 levels and various clinical and biological features in the 86 patients was similar to that found for all 217 patients (Tables 1, 2). The 86 patients were divided into two groups according to various cut-off points over 100 ng/mL, which was the upper limit in control serum (mean + 2 × SD = 20 + 2 × 35 = 90). The cut-off points used here were 100 ng/mL (< 100, $n = 39$, *vs* ≥ 100, $n = 47$), 150 ng/mL (< 150, $n = 54$, *vs* ≥ 150, $n = 32$), 200 ng/mL (< 200, $n = 60$, *vs* ≥ 200, $n = 26$) and 250 ng/mL (< 250, $n = 64$, *vs* ≥ 250, $n = 22$). The cut-off value of greater than 250 ng/mL showed the most significant prognostic effects with generalized Wilcoxon's and log-rank test analysis (data not shown). Therefore, we used 250 ng/mL of serum NM23-H1 as a cut-off value. As shown in Figure 2a, patients with the higher serum NM23-H1 levels had worse overall survival than those with the lower levels ($P = 0.0219$ according to the generalized Wilcoxon test, $P = 0.0340$ according to the log-rank test). Overall survival was significantly worse for patients who were 12 months or older than for those younger than 12 months of age ($P = 0.0364$ and $P = 0.0158$), for patients at stages 3 and 4 than for those at stages 1, 2 and 4S ($P = 0.0157$ and $P = 0.0082$), and for patients with *MYCN* amplification than for those with a single copy of *MYCN* ($P = 0.0195$ and $P = 0.0054$; Fig. 2b,c,d). These results

Table 2. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 86 patients with neuroblastoma found clinically

Characteristics	No. of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
All patients	86	239 ± 357	
Age			
< 12 months	27	282 ± 471	
≥ 12 months	59	219 ± 294	0.7694 (MW)
Stage			
1 + 2 + 4s	21	154 ± 187	
3 + 4	65	266 ± 394	0.3900 (MW)
Primary site			
Mediastinal	11	124 ± 207	
Adrenal	46	285 ± 383	
Abdominal	26	220 ± 375	0.0982 (KW)
Others	3		
MYCN copy number			
1	59	157 ± 193	
> 3	27	418 ± 534	0.0028 (MW)
TrkA expression	63		
Medium + high	28	154 ± 189	
0 + low	35	296 ± 422	0.1865 (MW)
Ploidy	66		
Diploid	37	255 ± 436	
Hyperdiploid	27	234 ± 352	0.4304 (MW)

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test.

indicate that the serum NM23-H1 level serves as a useful prognostic factor for neuroblastoma, as well as the other well-known prognostic factors.

Subsequently, we classified the 86 patients into two groups according to the age of the patients, stage of the disease, or copy numbers of *MYCN*, and evaluated the influence of the serum NM23-H1 levels on the overall survival in each one of the six groups (Fig. 3). Of the 29 patients younger than 12 months of age, the seven patients with higher levels of NM23-H1 had a worse outcome than the 22 patients with the lower levels ($P = 0.0401$ according to the generalized Wilcoxon test and $P = 0.0273$ according to the log-rank test; Fig. 3a). The seven patients with higher levels of NM23-H1 had the following attributes: stage 1 + 2 + 4S ($n = 3$); stage 3 + 4 ($n = 4$); with non-amplified *MYCN* ($n = 4$); with more than three *MYCN* ($n = 3$). Likewise, of the 19 patients with a stage 3 tumor, four patients with higher levels had a worse outcome than the 15 patients with lower levels ($P = 0.0005$ and $P < 0.0001$; Fig. 3c). The four patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 0$); > 12 months of age ($n = 4$); with non-amplified *MYCN* ($n = 1$); with more than three *MYCN* ($n = 3$). Of the 59 patients with a single copy of *MYCN*, the 11 patients with higher levels had a worse outcome than the 48 patients with lower levels of serum NM23-H1 ($P = 0.0301$ and $P < 0.0366$; Fig. 3e). The 11 patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 4$); > 12 months of age ($n = 7$); stage 1 + 2 + 4S ($n = 2$); stage 3 + 4 ($n = 9$). In contrast, a higher serum NM23-H1 level did not influence overall survival in the 57 patients 12 months old or older, in the 46 patients with stage 4 disease, or in the 27 patients with *MYCN* amplification (Fig. 3b,d,f).

Four prognostic factors, including the age of the patients, stage of the disease, *MYCN* copy number, and the serum NM23-H1 level, were available for multivariate analysis in the 217 patients (Table 3a) and 86 patients (Table 3b). According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients (Table 3).

Discussion

The *NM23-H1* gene is overexpressed in various hematological malignancies and other neoplasms including neuroblastoma. Overexpression of *NM23-H1* mRNA is indicative of a poor prognosis in patients with neuroblastoma, and mutations and increased copy numbers of *NM23-H1* have been reported in advanced neuroblastoma.[6,24] In the present study, we found that the serum NM23-H1 level was significantly higher in patients with neuroblastoma than in the control children (Fig. 1), and that the serum NM23-H1 level predicted a poor outcome for patients with tumors (Fig. 2a). Furthermore, the higher level of NM23-H1 was correlated with a worse outcome in patients younger than 12 months of age, in those with stage 3 disease, or in those with a single *MYCN* copy (Fig. 3). In contrast, a higher serum NM23-H1 level did not influence overall survival in patients who were 12 months old or older, in those with stage 4 disease, or in those with *MYCN*

Table 3. Univariate and multivariate analysis for predictors of survival in neuroblastoma

Prognostic factors	Univariate (χ^2, log-rank)	*P*-value	Multivariate (relative risk & 95% CI)	*P*-value
Patients found by mass-screening or clinically ($n = 217$)				
Serum NM23-H1(< 250/> 250 ng/mL)	11.211	0.0008	1.7294 (0.7997–3.7398)	0.1639
Age (< 12/≥ 12 months)	32.353	< 0.00001	3.8979 (1.3818–10.996)	0.0101
Stage (1, 2, 4s/3, 4)	33.142	< 0.00001	8.2514 (1.8173–37.466)	0.0063
NMYC amplification (–/+)	43.997	< 0.00001	2.3253 (1.0541–5.1297)	0.0366
Patients found clinically ($n = 86$)				
Serum NM23-H1(< 250/> 250 ng/mL)	4.493	0.0340	1.6143 (0.7386–3.5282)	0.2299
Age (< 12/≥ 12 months)	5.825	0.0158	1.4742 (0.4877–4.4563)	0.4916
Stage (1, 2, 4s/3, 4)	6.994	0.0082	3.5721 (0.7158–17.826)	0.1206
NMYC amplification (–/+)	7.749	0.0054	1.9682 (0.9016–4.2967)	0.0892

CI, confidence interval.

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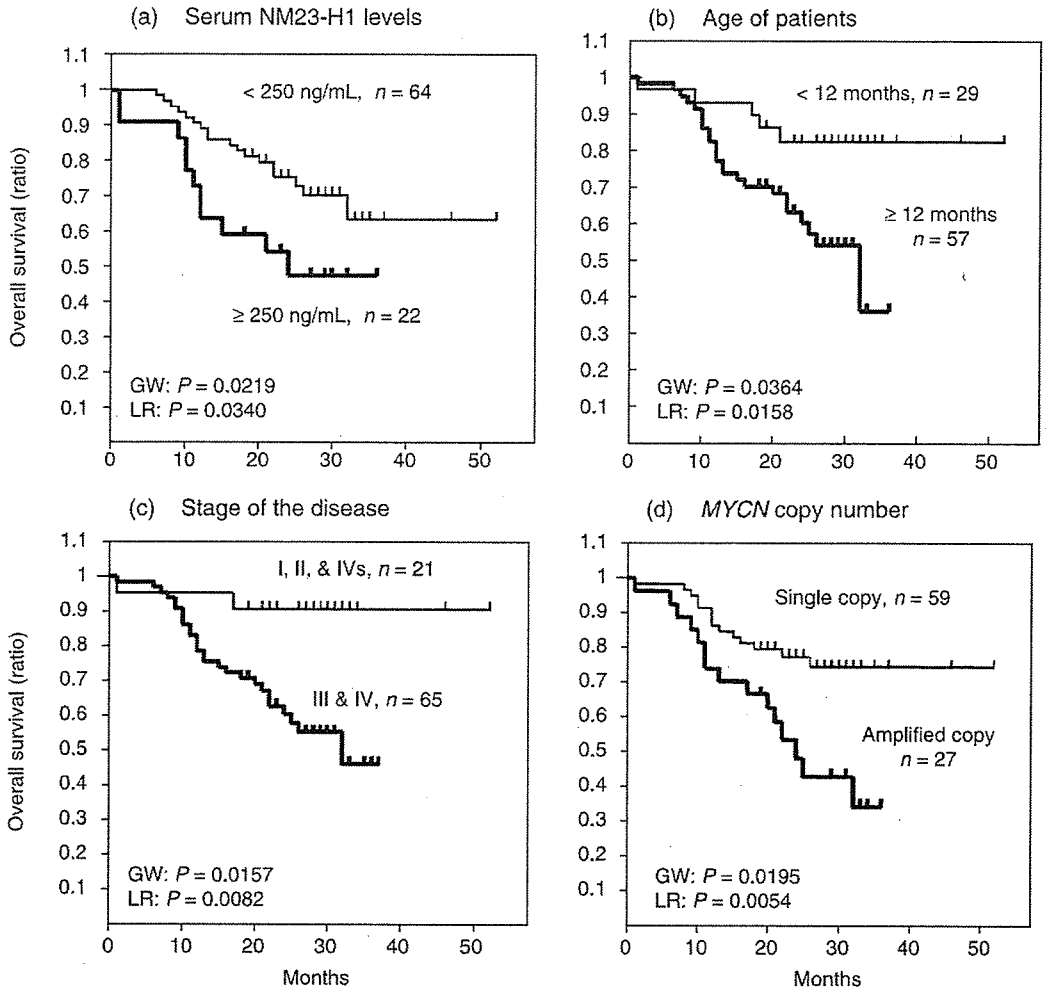

Fig. 2. Overall survival curves for 86 patients with neuroblastoma who were found clinically. (a) Overall survival curves for 22 patients with a serum NM23-H1 level ≥ 250 ng/mL, and for 64 patients with a level < 250 ng/mL. (b) Overall survival curves for 57 patients 12 months of age or older, and for 29 patients younger than 12 months. (c) Overall survival curves for 65 patients at stages 3 and 4 of the disease, and for 21 patients at stages 1, 2 and 4s. (d) Overall survival curves for 27 patients with *MYCN* amplification, and for 59 patients with a single copy of *MYCN*. GW, generalized Wilcoxon's test; LR, log-rank test.

amplification (Fig. 3). These findings suggest that the NM23-H1 level may be an important factor for predicting the outcome of patients in these low or intermediate risk groups (i.e. patients younger than 12 months of age, with stage 3 disease, or with a single copy of *MYCN*). In addition, the serum NM23-H1 level may be a clinically useful prognostic factor, because the measurement of serum NM23-H1 protein is easily and quickly carried out prior to treatment.

According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients shown in Table 3. These results might be due to the short observation time, the small number of cases, or the strong correlation between *MYCN* amplification and the elevated serum NM23-H1 level.

Although all the 131 patients found by MS were alive at the last follow-up (18–51 months) and were excluded from survival analysis, they contained 15 patients (the last follow-up: 19–37 months) with higher levels than 250 ng/mL of serum NM23-H1. It might be interesting to follow up these patients to clarify the clinical significance of serum NM23-H1 in the MS group.

Prognostic factors in neuroblastoma have been thoroughly investigated and include *MYCN* copy number, *TRKA* expression level, chromosomal ploidy, 1p loss, and 17q gain in tumor cells. Laborious and time-consuming work is required to examine these biological factors in tumor tissues. Therefore, serum markers that are easily measurable and can predict a clinical outcome are desired. Serum levels of lactate dehydrogenase (LDH) and ferritin are high in advanced stage neuroblastomas, but both may reflect a rapid cellular turnover or a large tumor burden.[25,26] Neuron-specific enolase (NSE) is a cytoplasmic protein that is associated with neural cells,

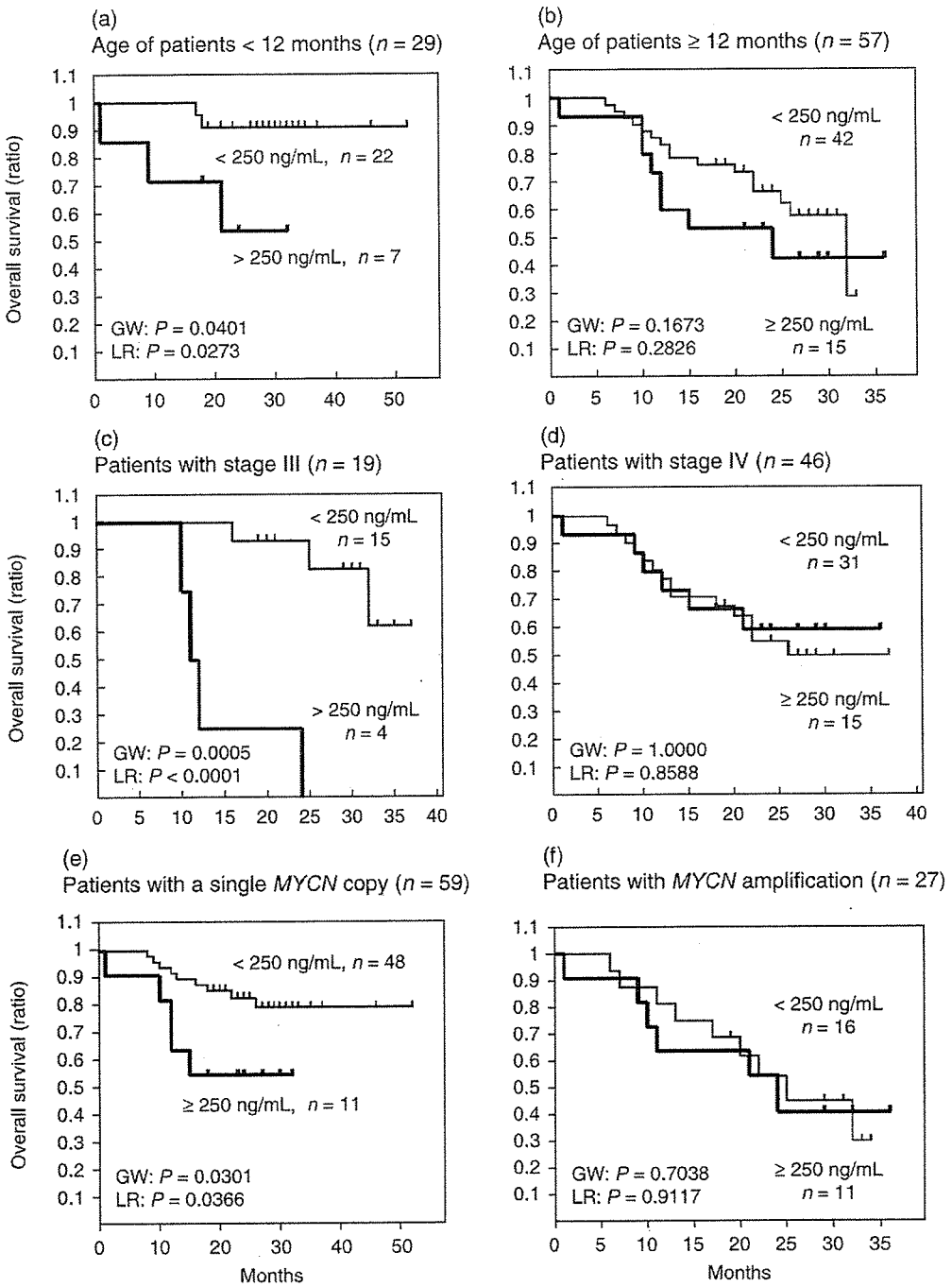

Fig. 3. Clinical significance of the serum NM23-H1 levels in the groups classified according to the age of the patients, or stage of the disease, or copy number of *MYCN*. (a) Survival curves for seven patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 22 patients with a level < 250 ng/mL. Both groups of patients were younger than 12 months of age. (b) Survival curves for 15 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 42 patients with a level < 250 ng/mL. Both groups of patients were 12 months old or older. (c) Survival curves for four patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 15 patients with a level < 250 ng/mL. Both groups of patients were at stage 3 of the disease. (d) Survival curves for 15 patients with the serum NM23-H1 level ≥ 250 ng/mL, and for 31 patients with the level < 250 ng/mL. Both groups of patients were at stage 4 of the disease. (e) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 48 patients with a level < 250 ng/mL. Both groups of patients had a single copy of *MYCN*. (f) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 16 patients with a level < 250 ng/mL. Both groups of patients had *MYCN* amplification in the tumor. GW, generalized Wilcoxon's test; LR, log-rank test.

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and serum NSE is a useful marker for patients with advanced neuroblastoma in whom the elevated levels are associated with a poor outcome.[27] The disialoganglioside GD2 is found on the surface of most neuroblastoma cells, and elevated plasma levels have been found in patients.[28] Nevertheless, none of these markers is used at present to predict clinical outcomes or to choose treatment protocols. Therefore, serum NM23-H1 levels might be useful for clinical purposes.

The elevated serum level of NM23-H1 was correlated with a poor prognostic feature, namely, *MYCN* amplification (Table 1). Godfrid *et al.* identified genes that are part of the *MYCN* downstream pathway using SAGE libraries of *MYCN* transfected and control neuroblastoma cell lines.[13] The chromosome 17q genes *NM23-H1* and *NM23-H2* were strongly induced in *MYCN*-expressing cells. A striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes was found in the cell lines. The present multivariate analysis showed no influence of serum NM23-H1 level on overall survival, and this finding might be caused by the overlap of patients with *MYCN* amplification with those with a high serum level of NM23-H1. However, within the group of patients with a single copy of *MYCN*, patients with a higher level of NM23-H1 had a worse outcome (Fig. 3e). The findings suggest that *NYCN* amplification may influence serum NM23-H1 levels as well as clinical outcome, and that neuroblastomas with a single copy of *MYCN* and a higher serum NM23-H1 level may have had a mutation or an increased copy number of the *NM23-H1* gene.[6,24,29] *MYCN* overexpression in some neuroblastomas with a single copy of *MYCN* may have resulted in higher serum NM23-H1 levels and a poor outcome; however, a recent study showed that *MYCN* overexpression did not affect the prognosis of advanced-stage neuroblastomas with a single *MYCN* copy.[30]

In patients with NHL and AML, it is thought that serum NM23-H1 protein is produced directly by the tumor cells, and its serum level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14] High concentrations of NM23 protein were found in the serum and body fluid of patients with lung cancer overexpressing the *NM23* genes.[31] Tumor cells may secrete this protein through some unknown mechanism, because there is no signal peptide sequence for secretion in the NM23 molecule. Serum NM23-H1 in patients with neuroblastoma might be derived from tumor cells and might be induced by *MYCN* amplification/overexpression or by *NM23-H1* overexpression independent of *MYCN*.

The serum level of NM23-H1 protein is clinically useful as an important prognostic factor in NHL or AML, and the present study showed that the protein could be a factor predicting an outcome of patients with neuroblastoma. It would be interesting to examine whether the serum NM23-H1 level generally predicts a poor outcome for patients with other tumors. The mechanisms by which the NM23-H1 protein is secreted into the serum and how it affects patient outcome are unclear. We are now studying the possibility that a high concentration of serum NM23-H1 may positively affect tumor cell growth or negatively affect normal cells.

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Methylation-Associated Silencing of the *Nuclear Receptor 1I2* Gene in Advanced-Type Neuroblastomas, Identified by Bacterial Artificial Chromosome Array-Based Methylated CpG Island Amplification

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Abstract

To identify genes whose expression patterns are altered by methylation of DNA, we established a method for scanning human genomes for methylated DNA sequences, namely bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA). In the course of a program using BAMCA to screen neuroblastoma cell lines for aberrant DNA methylation compared with stage I primary neuroblastoma tumors, we identified CpG methylation-dependent silencing of the *nuclear receptor 1I2* (*NR1I2*) gene. *NR1I2* was methylated in a subset of neuroblastoma cell lines and also in advanced-stage primary tumors with amplification of *MYCN*. Its methylation status was inversely associated with gene expression. Treatment with the demethylating agent 5-aza-2′-deoxycytidine restored *NR1I2* transcription in neuroblastoma cell lines lacking endogenous expression of this gene. A CpG island located around exon 3 of *NR1I2* showed promoter activity, and its methylation status was clearly and inversely correlated with *NR1I2* expression status. The gene product, NR1I2, has a known function in regulating response to xenobiotic agents but it also suppressed growth of neuroblastoma cells in our experiments. We identified some possible transcriptional targets of NR1I2 by expression array analysis. The high prevalence of *NR1I2* silencing by methylation in aggressive neuroblastomas, together with the growth-suppressive activity of NR1I2, suggests that this molecule could serve as a diagnostic marker to predict prognosis for neuroblastomas. (Cancer Res 2005; 65(22): 10233-42)

Introduction

Neuroblastoma, the most common extracranial solid tumor of childhood, has distinct biological characteristics in different prognostic subgroups. Children (>12 months at diagnosis) with stage IV or *MYCN*-amplified stage III tumors are at high risk of mortality (>60%), children with non-*MYCN*-amplified local-regional tumors (i.e., stages I, II, and III) and infants (<12 months at diagnosis) with stage IVS disease are generally at low risk of mortality (<10%), and infants with stage IV disease and children with stage III disease without *MYCN* amplification are at intermediate risk (1, 2), although the biological basis for that clinical diversity remains unclear. In addition to genetic changes including the *MYCN* amplification, epigenetic alterations often play important roles in the pathogenesis of human cancers, including neuroblastoma (3). For example, hypermethylation of promoter sequences of *CASP8, RASSF1A, CD44, TSP-1*, and *HSP47* genes has been observed in neuroblastoma tumors (4–8), and silencing of *CASP8* through methylation of its promoter tends to be associated with *MYCN* amplification (4). A reported positive correlation between promoter hypermethylation of *CASP8* and *RASSF1A* (5) suggests that hypermethylation of multiple genes may influence the phenotype of neuroblastoma.

Because hypermethylation in CpG-rich promoter or exonic regions seems to be a critical contributor to inactivation of tumor suppressor genes in many human cancers through transcriptional silencing (9), identification of hypermethylated CpG-rich sequence in cancer cell genomes could accelerate identification of unknown tumor suppressors. Although several techniques, including a method known as methylated CpG island amplification (MCA), have been developed (10, 11), we still have limited number of effective and practical high-throughput methods for genome-wide screening of aberrantly methylated CpG-rich sequences. To accomplish high-throughput screening for methylated sites in the entire genome, we developed a bacterial artificial chromosome (BAC) array-based MCA (BAMCA), incorporating our custom-made, BAC-based genomic DNA array combined with MCA (12).

In an effort to identify genes that are silenced by methylation mechanisms and associated with progression of neuroblastoma, we applied BAMCA to human neuroblastoma in the study reported here. Because the pattern of genomic changes observed in most neuroblastoma-derived cell lines is similar to that of advanced primary neuroblastomas (13), we used DNAs from neuroblastoma cell lines and from stage I primary tumors as test and reference samples, respectively. Using this approach, we successfully identified one gene, *nuclear receptor 1I2* (*NR1I2*), also known as *PXR*, whose expression was decreased in a subset of

Note: A. Misawa and J. Inoue contributed equally to this work.

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

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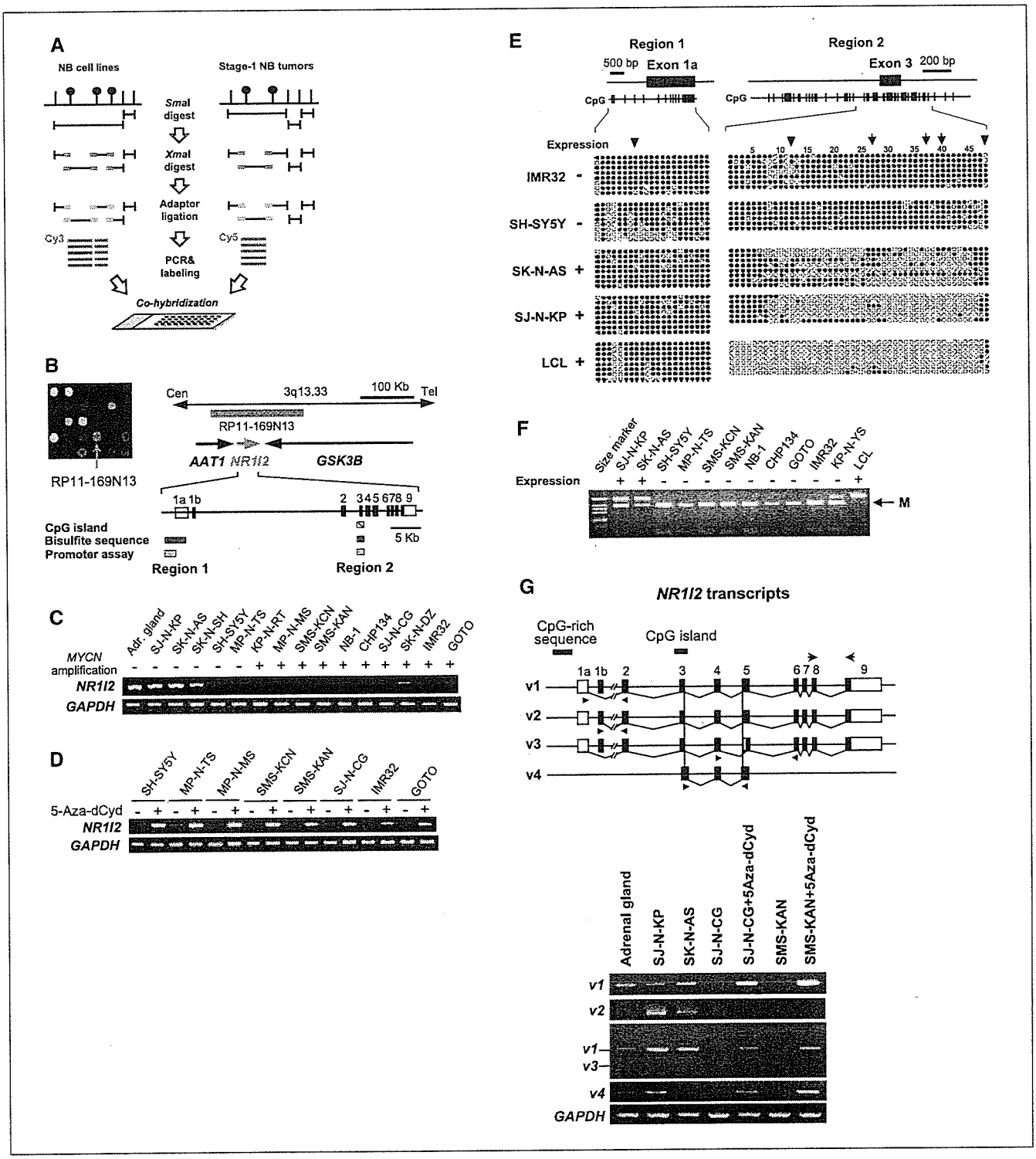
human cell lines and tumors of neuroblastoma through hypermethylation of a CpG island showing promoter activity. *NR1I2* was methylated and silenced mainly in late-stage neuroblastoma tumors with *MYCN* amplification and in older children. Exogenous restoration of NR1I2 expression suppressed growth of neuroblastoma cells lacking endogenous expression of the gene.

Materials and Methods

Cell culture, drug treatment, and primary tissue samples. All 19 human neuroblastoma cell lines we used (SK-N-KS, SK-N-AS, SK-N-SH, SK-N-DZ, SH-SY5Y, MP-N-TS, MP-N-MS, KP-N-RT, KP-N-SIFA, KP-N-SILA, KP-N-TK, KP-N-YS, SMS-KCN, SMS-KAN, SJ-N-CG, NB-1, CHP134, IMR32, and GOTO) had been established from surgically resected tumors and maintained as described previously (13). These cultures



were treated with or without 1 μmol/L 5-aza 2′-deoxycytidine (5-aza-dCyd) for 5 days.

Primary tumor samples were obtained at surgery from 51 neuroblastoma patients who underwent tumor resection at University Hospital, Kyoto Prefectural University of Medicine from 1986 to 2003, with written consent from the parents of each patient in the formal style and after approval by the local ethics committees. Staging was evaluated according to the criteria of the International Neuroblastoma Staging System (14). Of the 51 cases, 12 were classified as stage I, 11 as stage II, eight as stage III, 13 as stage IV, and four as stage IVS. Thirty-seven of the patients were infants <1 year of age at diagnosis. *MYCN* amplification was detected in 8 of 51 cases (15%). In 39 cases (76%), neuroblastoma had been detected by a mass screening program. Patients were treated according to previously described protocols (15, 16). Tumor samples were frozen immediately and stored at −80°C until required.

Bacterial artificial chromosome array-based methylated CpG island amplification. The preparation of DNA probes for screening of methylated regions was carried out by the MCA method described by Toyota et al. (11). Five-microgram aliquots of test DNA were first digested with 100 units of a methylation-sensitive restriction enzyme *Sma*I and subsequently with 20 units of methylation-insensitive *Xma*I. Adaptors were ligated to *Xma*I-digested sticky ends and PCRs were done with an adaptor primer and Cy3-dCTP for labeling. Control DNA was treated in the same manner except that labeling was with Cy5-dCTP (Fig. 1*A*).

Labeled test and control PCR products were cohybridized to our in-house array (MCG Whole Genome Array-4500; ref. 12). Hybridizations were carried out as described elsewhere (17). Arrays were scanned with a GenePix 4000B (Axon Instruments, Foster City, CA) and analyzed using GenePix Pro 4.1 software (Axon Instruments).

Reverse transcription-PCR and real-time quantitative reverse transcription-PCR. Single-stranded cDNAs were generated from total RNAs (17) and amplified with specific primers for each gene. Primer sequences are available on request. Real-time quantitative PCR was done using LightCycler (Roche Diagnostics, Tokyo, Japan) with SYBR green as described previously (18). The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene served as an endogenous control. Each sample was normalized on the basis of its *GAPDH* content. PCR amplification was done in duplicate for each sample.

Methylation analysis. To investigate methylation of DNA, the method of combined bisulfite restriction analysis (COBRA) was done as described earlier (11). Genomic DNAs were treated with sodium bisulfite and subjected to PCR using primer sets designed to amplify the regions of interest. PCR products were digested with *Hha*I, which recognizes sequences unique to the methylated alleles but cannot recognize unmethylated alleles, and electrophoresed. For bisulfite sequencing, PCR products were subcloned and sequenced.

Reporter assay. A 1,060 bp fragment upstream of exon 1 of *NR1I2* (region 1; Fig. 1*A*) and a 480 bp fragment of a CpG island that includes exon 3 (region 2; Fig. 1*A*) were ligated into the pGL3-Basic vector (Promega, Madison, WI) in front of and/or downstream of the luciferase gene. An equal amount of each construct was introduced into cells with an internal control vector (pRL-hTK, Promega), using FuGENE 6 (Roche Diagnostics). A pGL3-Basic vector without insert served as a negative control. Firefly luciferase and *Renilla* luciferase activities were each measured 36 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega); relative luciferase activities were calculated and normalized versus *Renilla* luciferase activity.

Transfection, Western blotting, and colony formation assays. A full-length *NR1I2* cDNA was cloned into the pCMV-Tag3 eukaryotic expression vector (Stratagene, La Jolla, CA) with or without etoposide (VP-16) in-frame along with the Myc epitope. A plasmid expressing a Myc-tagged NR1I2 with or without VP-16 (pCMV-Tag3-VP-*NR1I2* or pCMV-Tag3-*NR1I2*), or the empty vector (pCMV-Tag3-mock), were transfected into cells using FuGENE6 (Roche Diagnostics). Expression of NR1I2 protein in transfected cells was confirmed by Western blotting using anti-Myc-Tag antibody (9B11; Cell Signaling Technology, Beverly, MA). For colony formation assays, transfected cells were selected with 500 μg/mL G418; 3 weeks after transfection, the neomycin-resistant colonies were stained with crystal violet and counted (17).

Cell growth assay. Stable NR1I2 transfectants and controls were obtained by transfecting pCMV-Tag3-VP-*NR1I2* or pCMV-Tag3-mock, respectively, into cells lacking *NR1I2* expression. For measurements of cell growth, 2×10^3 cells were seeded in 96-well plates. The numbers of viable cells were assessed by a colorimetric water-soluble tetrazolium salt assay (cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan).

Oligonucleotide array analysis. mRNA expression profiling was done using the AceGene Human oligo chip 30K (DNA Chip Research, Inc., Kanagawa, Japan), containing 30,000 genes, as described elsewhere (18). The test and reference cDNA probes labeled with aminoallyl-dUTP (Ambion, Inc., Austin, TX) were synthesized using oligo(dT)12-18 primer and coupled with Cy3- or Cy5-monoreactive dye (Amersham Biosciences, Tokyo, Japan), respectively. The hybridized chips were scanned using GenePix 4000B (Axon Instruments) and analyzed using GenePix Pro 4.1 software (Axon Instruments). Signal intensities between the two fluorescent images were normalized by the averaged values for blank spots; this procedure effectively defined the signal intensity-weighted spot for the internal controls of housekeeping genes on each array to have a Cy3/Cy5 ratio of 1.0.

Results

Methylation analysis of neuroblastoma cell lines by bacterial artificial chromosome array-based methylated CpG island amplification. To assess DNA methylation in the more advanced type of neuroblastoma tumors, we did BAMCA

Figure 1. Methylation status and expression levels of *NR1I2* in neuroblastoma (*NB*) cell lines. *A*, BAMCA procedure. The DNAs from neuroblastoma cell lines (test) or stage I neuroblastoma tumors (control) were first digested with *Sma*I in the blunt end and subsequently with *Xma*I in the sticky end (*blue boxes*). Adaptors were ligated to *Xma*I-digested sticky ends (*pink boxes*) and PCR was done with an adaptor primer and Cy3-dCTP (test) or Cy5-dCTP (control) for labeling. Labeled PCR products were cohybridized to BAC array. *B, left*, representative image of BAMCA analysis applied to the IMR32 cell line. *Green*, BAC containing highly methylated fragments in IMR32 compared with stage I tumors; *red*, BAC containing highly methylated fragments in stage I tumors compared with IMR32; *yellow*, unchanged methylation status; *black*, no detectable methylated fragments. The RP11-169N13 BAC (*arrow*) harboring *NR1I2* was detected as spot with a high Cy3 (test)/Cy5 (control) ratio. *Right*, genomic structure of the *NR1I2* gene consisting of nine exons. A 239 bp CpG island exists around exon 3 (Genbank accession nos. NM_003889 for cDNA sequence and NT_005612 for genomic sequence). *Horizontal bars*, regions examined in a promoter assay and bisulfite sequencing analysis (regions 1 and 2). *C*, representative results of RT-PCR analysis of *NR1I2* mRNA expression in normal adrenal gland and neuroblastoma cell lines with (+) or without (−) amplification of *MYCN*. *GAPDH* was used as an internal control. *D*, representative results of RT-PCR analysis to reveal *NR1I2* expression in neuroblastoma cell lines with (+) and without (−) treatment with 5-aza-dCyd. *GAPDH* was used as an internal control. *E, top*, map of the 5′ region (exon 1 and upstream sequence, region 1) and the CpG island around exon 3 (region 2) in *NR1I2*. *Vertical tick marks*, CpG sites. *Bottom*, results of bisulfite sequencing analysis done in *NR1I2*-nonexpressing cell lines (IMR32 and SH-SY5Y) and *NR1I2*-expressing cell lines (SK-N-AS, SJ-N-KP, and LCL). ○, unmethylated CpG sites; ●, methylated CpG sites, respectively; each row represents a single clone. *Arrows*, *Hha*I restriction site. *Arrowheads*, *Sma*I restriction site. *F*, representative results of COBRA of region 2 in neuroblastoma cell lines with (+) or without (−) *NR1I2* expression. A 492 bp PCR product, including exon 3, was restricted by *Hha*I. *M*, methylated alleles. *G, top*, map of four variants (*v1-v4*) and location of each primer set used for RT-PCR analysis. *Black boxes*, coding exons; *gray box*, deleted region in variant 3. *Arrows*, primers used for RT-PCR shown in (*B* and *C*), and in Fig. 2*B* and *C*; *arrowheads*, primer sets specific for each variant. Nucleotide sequences for primers used are available on request. *Bottom*, representative results of RT-PCR analysis. A primer set for variant 3 amplified two products with 441 and 330 bp sizes from variants 1 and 3, respectively.

Table 1. List of positive BACs in BAMCA analysis and summary of screening of candidate methylated genes

	BAC (RP11)	Locus	Gene		CpG island*
			Symbol	Name	
1	73D7	*1q32.1*	*LHX9*	*LIM homeobox 9*	+
2	451A14	*2p24*	No gene		
3	169N13	*3q13.3*	*NR1I2*	*Nuclear receptor subfamily 1, 2group I, member 2*	+
			GSK3B	*Glycogen synthase kinase 3β*	−
			AAT1	*AAT1-α*	−
4	205N12	*4p15.1*	*PCDH7*	*Protocadherin 7*	+
5	17P19	*4q21.2*	*MRPL1*	*Mitochondrial ribosomal protein L1*	−
6	611D20	*9q34*	*NOTCH1*	*Notch homologue 1, translocation-associated (Drosophila)*	+
7	248C1	*10q23.33*	*MPHOSPH1*	*M-phase phosphoprotein 1*	+
8	37L21	*10q24*	*SEMA4G*	*Sema domain, immunoglobulin domain, transmembrane domain and short cytoplasmic domain (semaphorin) 4G*	+
			MRPL43	*Mitochondrial ribosomal protein L43*	+
9	23E5	*11p15.1*	*DELGEF*	*Deafness locus associated putative guanine nucleotide exchange factor*	+
10	56E13	*11p11.2*	*PTPRJ*	*Protein tyrosine phosphatase, receptor type, J*	+
11	79L5	*18q21.2*	*ONECUT2*	*One cut domain, family member 2*	+
12	7F10	*20p11.22*	*PAX1*	*Paired box gene 1*	+
13	124D1	*20q13*	*PREX1*	*Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1*	+
14	93B14	*20q13.33*	*FLJ32154*	*unknown*	+
			SLCO4A1	*Solute carrier organic anion transporter family, member 4A1*	+
			NTSR1	*Neurotensin receptor 1 (high affinity)*	+
15	58O1	*10q22.1*	*SLC29A3*	*Solute carrier family 29 (nucleoside transporters), member 3*	+
			UNC5B	*Unc-5 homologue B (Caenorhabditis elegans)*	+
16	88B12	*10q26.2*	*MGC32871*	*Hypothetical protein*	−
			PTPRE	*Protein tyrosine phosphatase, receptor type, E*	+
17	262M8	*14q21.3*	*PTGDR*	*Prostaglandin D2 receptor*	+
			PTGER2	*Prostaglandin E receptor 2 (subtype EP2), 53 kDa*	+
18	79J21	*15q24*	*ETFA*	*Electron-transfer-flavoprotein, αpolypeptide (glutaric aciduria II)*	−
			ISL2	*ISL2 transcription factor, LIM/homeodomain, (islet-2)*	+

*CpG islands were searched using NCBI human genome database (http://www.ncbi.nlm.nih.gov/).
†Each Cy3-labeled neuroblastoma cell line sample/Cy5-labeled mixed stage I neuroblastoma tumor samples (see Fig. 1*A*).
‡Methylation status in primary tumors was determined by using bisulfite-PCR analysis (see Fig. 2*A*). −, ≤5%; ±, >5% and ≤50%; +, >50%.
§GOTO cells were treated with 1 μmol/L of 5-aza-dCyd for 5 days (see Fig. 1*C*).

(Fig. 1*A*) with our MCG Whole Genome Array-4500 (12) using DNA from each of two neuroblastoma cell lines (IMR32 and GOTO) and mixed DNA from five stage I primary neuroblastoma tumors as test and control DNAs, respectively. As shown in Table 1, 18 BACs, which contain 24 known genes and two uncharacterized transcripts, showed high Cy3 (test)/Cy5 (control) ratios (>1.5) by BAMCA in both cell lines, and were selected as sequences whose CpG sites were frequently methylated in advanced types of neuroblastoma tumors. The same result was obtained in the repeated experiments using the same samples (data not shown), suggesting that BAMCA is a reproducible method. We then selected possible candidates by sequentially analyzing the following: (*a*) the expression status of each gene in stage I primary neuroblastoma tumors and in IMR32 and GOTO cells; (*b*) restoration of gene expression after treatment with 5-aza-dCyd; and (*c*) methylation status of CpG islands around each candidate gene in stage I and stage IVa primary neuroblastoma tumors (data not shown). As shown in Table 1, *NR1I2* located within RP11-169N13 (Fig. 1*B*) emerged as a gene that was (*a*) expressed in stage I tumors but not in the two neuroblastoma cell lines, (*b*) restored after treatment with 5-aza-dCyd, and (*c*) frequently methylated in stage IVa tumors but infrequently in stage I tumors. Those results prompted us to perform detailed analysis of the *NR1I2* gene as a putative tumor suppressor whose silencing by a DNA methylation mechanism might be associated with progression of neuroblastoma.

Analysis of *NR1I2* expression in neuroblastoma cell lines. When we examined *NR1I2* expression in our panel of 19 neuroblastoma cell lines by reverse transcription-PCR (RT-PCR; Fig. 1*C*), no *NR1I2* mRNA was detected in 14 of the lines (73%): in 11 of 12 *MYCN* amplified lines (91%) or in 3 of 7 *MYCN* nonamplified lines (43%). One line, MP-N-TS, lacking expression of *NR1I2* and without *MYCN* amplification, does show c-*MYC* amplification (13). Normal adrenal gland, which is considered the tissue of origin for neuroblastoma tumors, expressed *NR1I2* mRNA.

To investigate whether demethylation could restore *NR1I2* mRNA in neuroblastoma cells lacking endogenous expression, we treated cells with 1 μmol/L of 5-aza-dCyd, a methyltransferase inhibitor, for 5 days. Expression of *NR1I2* mRNA was remarkably increased after the treatment (Fig. 1*D*).