4. Intervention in early stages salvages the patients with risk to progress

In our data base there were 94 NBs with at least one of the risk markers mentioned above. The event-free survival of the patients was 43% (40/94). Even with the cytogenetic markers, event-free survival was 86% in the patients with the localized NBs (stage I or II). On the other hand, the survival was 35% in the patients with advanced NBs (stage III, IVs or IV). This finding reveals that intervention in early stages improves clinical outcome of the patients, even if the NBs have risk to progress.

5. Clinical risk of the NBs defined by multivariate markers

Based on the results mentioned above, the '*high risk*' is defined by NBs in any stage with any of following markers, MYCN amplification or INPC unfavorable histology or low Ha-ras/trk A expression. The '*low risk*' is defined by NBs with all another markers, single copy of MYCN and INPC favorable histology and high Ha-ras/trk A expression and localized tumor. The remaining NBs are defined in '*intermediate risk*' category.

6. Estimation of biological profile in NBs detected through infantile mass-screening program

The Japanese mass-screening program was introduced in 1984 for infants at 6 months of age. This program detected 2366 NBs (mass-NBs) until 2001. The event-free survival of patients is 98%. There have been controversial discussion about benefit of the mass-screening. Quebec [13] and German [14] studies reported that the mass-screening provided no effect on mortality caused by NBs. Recent Japanese nation-wide study showed significant reduction of mortality in mass-screening group comparing with children who did not have the mass-screening [15]. The biological analysis of the mass-NBs has not been conclusive. We have been evaluating the biological property of the 248 mass-NBs and following their clinical outcome. They were about 10% of all mass-NBs in Japan. Based on criteria in the

INPC[a] & Ha-ras/trk A[b] Stage	"Favorable"[a] "High" / "Intermediate" / "Low"[b]			"Unfavorable"[a] "High" / "Intermediate" / "Low"[b]			total□ (EFS/UO)[c]
I	⑩○○○ ○○○○○○ ○○▼▼ n n	○○○○○○ ○○○○ n	○○○○ ○▲▼▼ n	○		○○○	99□ (94/5)
II	○○○○ ○○▲▼ n	○○○○ ○○○▲▼	○	○○▼	○○	○	78□ (73/5)
IVs	○○○○○ ○○N	○○○○	○				12□ (12/0)
III	○○ ○▼ n	○ ▲● n	○○○○○ ○○○▼ n	○○		▼	46□ (41/5)
IV	○○○○○ ○	○○			●N	○▲▲● N NN	13□ (9/4)
total□ (EFS/UO)[c]	138□ (133/5)	55□ (51/4)	36□ (32/4)	6□ (5/1)	4□ (3/1)	9□ (5/4)	248□ (229/19)

Fig. 2. Biological profiles of 248 mass-NBs. (a) International neuroblastoma pathological classificaion (INPC), (b) H-ras/trk A expression, c; number of event-free survival (EFS) and patients with unfavorable outcome (UO). *Patients outcome*: small open circle presents a event-free survivor and large one presents 10 event-free survivors. Closed circle is a deceased case. A downward closed triangle is a case with relapse NB and a upward closed triangle is a case with progressive NB. Capital 'N' presents MYCN amplification with more than 10 copies and small 'n' presents the amplification with 3–9 copies.

non-mass NBs mentioned above, the 248 mass-NBs were classified into risk categories. Seventy-one percent of them were detected as localized tumors (stage I and II) and 29% were in stages III, IVs and IV. The MYCN amplification was detected in only 13 mass-NBs (5%). The distribution of mass-NBs with MYCN amplification and the better event-free survival [69% (9/13)] of the patients might suggest the benefit of early detection and intervention through the mass-screening. Total 62 NBs (25%) were evaluated to have high-risk property; 55 had INPC unfavorable histology or low Ha-ras/trk A expression and seven had only the MYCN amplification as a risk marker. Among localized 103 NBs (stages I, II) with INPC favorable histology and high Ha-ras/trk A expression, 100 NBs (40%) were classified as *low-risk property* and the MYCN amplified 3 NBs moved to high risk group. The remaining 86 NBs (35%) in other categories were classified to be *Intermediate risk* (Fig. 2).

7. Conclusion

The multivariate evaluation showed the diversity of non-mass- and mass-NBs with variety risk to progress. The cytogenetic markers, MYCN, INPC histology and Ha-ras/trk A expression could predict the risk with high sensitivity and specificity.

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Significance of Survivin mRNA Expression in Prognosis of Neuroblastoma

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Neuroblastoma (NB) is the most common malignant solid tumor in childhood, and among all childhood malignancies is second in prevalence only to leukemia. In NB we need to both make an accurate diagnosis and rapidly analyze the expression of genetic prognostic factors such as *MYCN*, H-*ras*, and *trk*A. Moreover, it has recently become important to analyze the expression of survivin mRNA, a member of the inhibitor of apoptosis protein family. Expression of the survivin gene is related to tumorigenesis and inhibition of apoptosis in some malignant tumors. We investigated its expression by reverse transcription-polymerase chain reaction (RT-PCR) in NB cell lines (SK-N-SH, NB-39, and IMR-32), two normal blood cell samples, and 13 clinical NB tumor samples. All three NB cell lines had high levels of mRNA expression for this gene, but normal blood cells had no expression. We detected expression of survivin mRNA in 7 of the 13 NB tumor samples (54%). Two NB patients were in stage I disease, 6 in stage II, and 5 in stage IV_A. Quantitative analysis by RT-PCR revealed that the ratio between survivin mRNA and human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) mRNA was very low in stages I and II (0—0.017). In contrast, in advanced NBs (stage IV_A) the ratio was much higher (0—0.050). The prognoses of the three patients in the advanced stage who had high ratios of expression were poor. A high level of expression of survivin mRNA indicates a high grade of malignancy, high likelihood of recurrence, and poor prognosis.

Key words survivin; reverse transcription-polymerase chain reaction; prognostic factor; neuroblastoma

Neuroblastoma (NB) is a very common malignant solid tumor in childhood. Prognosis in NB patients tends to vary greatly, and many studies have demonstrated that both clinical and molecular biological factors are correlated with outcome.[1] For example, patients under the age of 1 year at diagnosis usually have good prognoses, but those diagnosed over the age of 1 year have poor prognoses.[2] Increased expression of the molecular biological factors *MYCN*, H-*ras* and *trk*A is well known in NB.[3—11]

Recently, there has been great interest in apoptosis, or programmed cell death, the mechanism by which cells essentially suicide.[12] Many inhibitors of apoptosis are known to contribute to tumorigenicity and increased spread of tumor cells.[13] Survivin is a recently described member of the inhibitor of apoptosis protein (IAP) family.[14] This gene exists on chromosome 17q and inhibits apoptosis by blocking the effects of caspase-9, which is activated in extrinsic and intrinsic pathways.[14—17] Survivin is expressed in many malignant tumors, including breast, lung, stomach, colon and pancreatic cancers, bladder tumors, malignant lymphoma, and NB.[18] It is not usually present in normal tissues and is rarely found in mature tissues.[17] Thus, survivin expression is likely to be an important prognostic factor in tumor malignancy, and we considered that survivin mRNA expression would be useful in determining tumor malignancy and prognosis in NB.

We therefore used reverse transcription-polymerase chain reaction (RT-PCR) to investigate the expression of survivin mRNA in NB cell lines, normal blood cell samples, and clinical NB tumor samples.

Here, we describe how the degree of expression of survivin mRNA is a very useful prognostic indicator.

MATERIALS AND METHODS

Cell Lines, Clinical NB Tumor Samples, and Normal Blood Cell Samples Three NB cell lines (IMR-32,[19,20] SK-N-SH,[20,21] and NB-39[20]) were examined. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 g/l sodium bicarbonate under 5% CO_2 at 37 °C. Two normal adult blood cell samples and 13 clinical NB tumor samples were also examined. Two of the tumor samples were from recurrent tumors. These tumor tissues had been stored at −80 °C since collection. The clinical diagnoses for these patients had been made by histopathology. Informed consent was obtained from all patients before the study began.

RNA Extraction Total RNA from the three cell lines and 13 NB tumor samples was extracted with TRI_{ZOL} reagent (Gibco BAL) by the acid-guanidium-phenol chloroform extraction method.[22] Total RNA from the two normal blood cell samples was extracted with TRI_{ZOL} LS Reagent (Gibco BAL) by acid-guanidium-phenol chloroform extraction method.[16]

Reverse Transcription-Polymerase Chain Reaction For determination of survivin mRNA expression, total RNA (1 μg) was reverse-transcribed in a 10 μl reaction mixture with a first strand cDNA synthesis kit (Rever Tra-α-^{TM}, Toyobo). RT was performed with Oligo-dT. The mixture was incubated at 48 °C for 30 min, followed by annealing at 95 °C

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for 10 min and then holding at 4 °C. For *MYCN* mRNA expression, the mixture was incubated at 42 °C for 50 min, followed by annealing at 72 °C for 15 min and then holding at 4 °C. For *trk*A, the mixture was incubated at 42 °C for 20 min, followed by annealing at 99 °C for 5 min and then holding at 4 °C. PCR amplification was carried out in 10× reaction mixture containing 1.2 pmol of the respective primer. We used a KOD-Plus PCR kit (Toyobo).[23—25] The PCR cycling conditions were as follows: for survivin, initial denaturation at 48 °C for 30 min, 10 min at 95 °C, followed by 25 cycles at 95 °C for 15 s and 60 °C for 1 min; for *MYCN*, initial denaturation at 95 °C for 10 min, followed by 25 cycles at 94 °C for 15 s, 57 °C for 5 s, and 72 °C for 10 s; for *trk*A, initial denaturation at 94 °C for 2 min, followed by 25 cycles of denaturing at 94 °C for 15 s, annealing at 60 °C for 90 s, extension at 68 °C for 20 s, and then holding at 4 °C. We used human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) as an internal marker and NB cell lines (IMR-32, NB-39, and SK-N-SH) as positive controls. The primer sequences are listed in Table 1.

Analysis and Quantities of PCR Products PCR products were electrophoresed through 2.0% agarose gel, stained with ethidium bromide (Wako), and visualized under a UV lamp. We used a bioanalyzer (Agilent Technologies) to accurately determine band sizes (Fig. 1). For each sample we determined the ratios of survivin/h-GAPDH mRNA, *MYCN*/h-GAPDH mRNA, and *trk*A/h-GAPDH mRNA.

RESULTS AND DISSCUSION

We used RT-PCR to analyze survivin mRNA expression in three cell lines. Fig. 1 shows the result of bioanalyzer. Electrophoresis was used to approximate the bands and the bioanalyzer was used to determine accurate band sizes. The band size for survivin mRNA was 261 bp, and that of h-GAPDH as an internal marker was 209 bp. Survivin mRNA was expressed in all three NB cell lines (IMR-32, NB-39, and SK-N-SH). Expression of *MYCN* and *trk*A mRNA in the cell lines, as determined by bioanalyzer, is also shown in Fig. 1. Fig. 2 shows the expression of mRNA of survivin, *MYCN* and *trk*A relative to that of h-GAPDH mRNA in each cell line. Expression of *MYCN* mRNA was recognized in NB-39 and IMR-32, and that of *trk*A mRNA in NB-39 and IMR-32, but in SK-N-SH there was no expression of *MYCN* or *trk*A mRNA. The two normal adult blood cell samples did not express survivin mRNA (Fig. 3). Therefore, expression of survivin mRNA was apparent only in the NB cell lines. Survivin mRNA was detected in 7 of the 13 tumor samples (54%)

Table 1. PCR Primers

Gene	Sequence	Detected size (bp)
survivin sense	5′-AAG AAC TGG CCC TTC TTG GA-3′	261
survivin anti-sense	5′-GGC TCT TTC TCT GTC CAG T-3′	
MYCN sense	5′-GAC CAC AAG GCC CTC AGT AC-3′	240
MYCN anti-sense	5′-GTG GAT GGG AAG GCA TCG TT-3′	
*trk*A sense	5′-TGG AGA AGA AGG ACG AAA CA-3′	412
*trk*A anti-sense	5′-GCC TTG ACA GCC ACC AGC AT-3′	
h-GAPDH sense	5′-TCC TCT GAC TTC AAC AGC GAC ACC-3′	209
h-GAPDH anti-sense	5′-TCT CTC TTC CTC TTG TGC TCT TGG-3′	

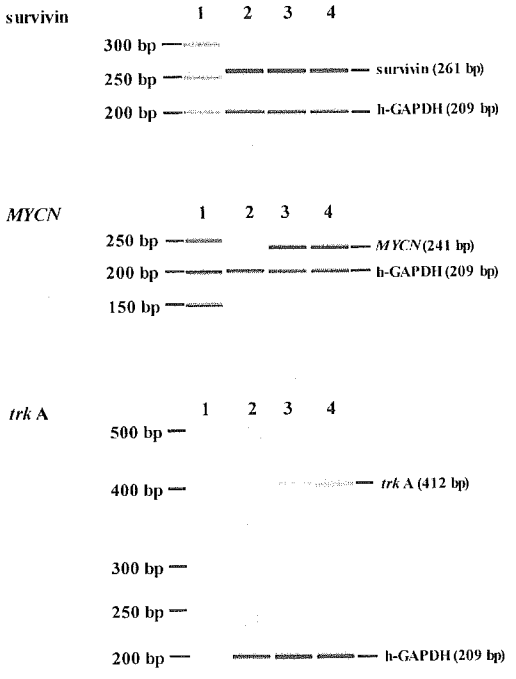

Fig. 1. Expression of Survivin, *MYCN* and *trk*A mRNAs in NB Cell Lines by RT-PCR

Bands detected by bioanalyzer. Lane 1: marker; lane 2: SK-N-SH; lane 3: 1 NB-39, lane 4: IMR-32. The sizes of the PCR products were 261 bp (survivin), 241 bp (*MYCN*), 412 bp (*trk*A), and 209 bp (h-GAPDH).

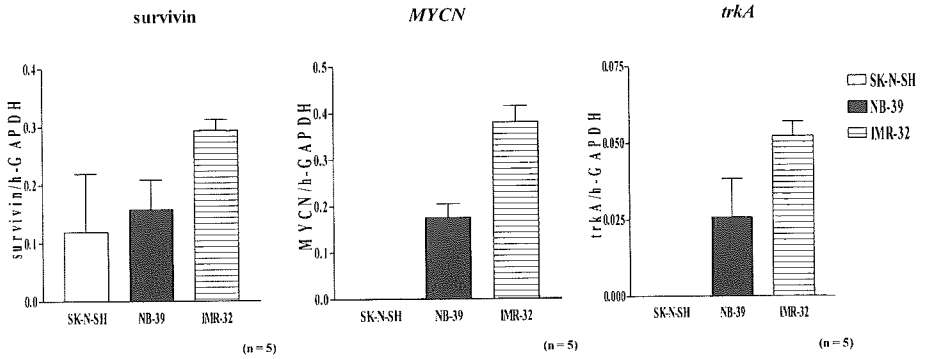

Fig. 2. Expression of Survivin, *MYCN* and *trk*A mRNAs in NB Cell Lines (SK-N-SH, NB-39, and IMR-32)

The relative expression of each gene is given as the ratio of its mRNA expression to that of h-GAPDH.

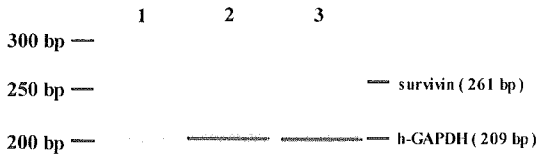

Fig. 3. Expression of Survivin mRNA in Normal Blood Cell Samples by RT-PCR

Bands detected by bioanalyzer. Lane 1: marker; lane 2: male, 24 years; lane 3: female, 23 years. The sizes of RT-PCR products were 261 bp (survivin) and 209 bp (h-GAPDH).

Table 2. Relationship between Expression of Survivin, *MYCN*, and *trk*A and Clinical Prognosis in NB Patients

Tissue	Stage	Age	Survivin	*MYCN*	*trk*A	Recurrence and dead
T1	I	8 m	+	−	+	−
T2	I	9 m	−	−	+	−
T3	II	2 m	−	−	+	−
T4	II	8 m	+	−	+	−
T5	II	7 m	+	−	+	−
T6	II	4 m	+	+	−	−
T7	II	8 m	−	−	+	−
T8	II	7 m	−	−	+	−
T9	IV_A	11 y	++	+	−	+
T10	IV_A	6 y	−	−	+	−
T11	IV_A	2 y	−	−	+	−
T12	IV_A	5 y	++	−	+	+
T13	IV_A	5 y	++	−	+	+

For survivin: +, low mRNA expression; ++, high expression; −, no expression. For *MYCN* and *trk*A: +, mRNA expression; −, no mRNA expression. For recurrence and death: +, occurred; −, did not occur.

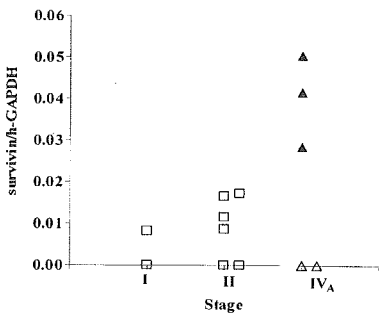

Fig. 4. Expression of Survivin mRNA in NB Tumor Samples

Survivin mRNA abundance is expressed as a ratio to that of h-GAPDH mRNA. □: diagnosis at <1 year; alive, △: diagnosis at ≧1 year; alive, ▲: diagnosis at ≧1 year; tumor recurrence, died.

(Table 2). The ratio between survivin mRNA and h-GAPDH mRNA was analyzed quantitatively by RT-PCR in the 13 tumor samples and the expression levels plotted. The results are shown in Fig. 4. Three of the 5 stage IV_A (advanced stage) patients had higher relative expression of survivin mRNA (0.029—0.050) than did patients in the earlier clinical stages (I, II). These three patients in stage IV_A all had recurrences and died. In contrast, in the other 10 patients the relative expression was low (0—0.017), and all 10 were alive without recurrence. Therefore, a ratio of about 0.02 is the cut-off point between good and poor prognosis (Fig. 4).

Moreover, we also determined the levels of expression of *MYCN* and *trk*A mRNA in the 13 patients (Table 2). Abnormal amplification and expression of *MYCN* and no, or low levels of, expression of *trk*A mRNA are well known to occur in advanced NB patients with poor prognoses.[3—5,7—10] For T1—T8 patients (equivalent to early stage I and II, under the age of 1 year at diagnosis, and disease found by mass screening), 7 of the 8 patients except T6 had no *MYCN* mRNA expression and were positive for *trk*A mRNA expression. T1, T4, T5, and T6 patients expressed survivin mRNA, but the relative level of expression was low. All 8 patients were alive without recurrence. In contrast, the advanced NB patients (stage IV_A: T9, T12, and T13) had relatively high levels of survivin mRNA expression; they developed recurrences and died, even though the T12 and T13 patients expressed *trk*A mRNA. This means that a high level of expression of survivin mRNA indicates a high grade of tumor malignancy, high likelihood of recurrence, and poor prognosis.

Survivin is a novel member of the IAP family and is expressed not only in several apoptosis-regulated fetal tissues, but also in a few adult differentiated tissues.[14—17] Furthermore, survivin is overexpressed in most common human cancers.[14—17] Survivin mRNA was expressed in all NB cell lines and was also expressed in 7 of 13 clinical NB tumor samples. It was not expressed in normal blood cell samples. Three of the 13 NB patients had unfavorable outcomes and had high levels of expression of survivin mRNA. Although we still need to analyze a greater number of advanced NB tumor samples, relative expression of survivin mRNA appears promising as a prognostic indicator in NB patients.

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Usefulness of Tyrosine Hydroxylase mRNA for Diagnosis and Detection of Minimal Residual Disease in Neuroblastoma

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Neuroblastoma (NB) is the most common malignant solid tumor in childhood and, among all childhood malignancies, is second only to leukemia. NB originates before birth in the neural crest, which develops into the adrenal medullae and sympathetic ganglia. In the adrenal medulla, tyrosine hydroxylase (TH) is the first enzyme in the pathway of catecholamine synthesis. We used reverse transcription polymerase chain reaction (RT-PCR) to examine the expression of TH mRNA in NB and Ewing's sarcoma cell lines, small round cell tumors (SRCTs) containing NB, and other clinical tumor samples (osteosarcoma, osteochondroma, and Wilms' tumor). In total, we analyzed 33 clinical tumor samples. TH mRNA was expressed in all three NB cell lines examined, but not in two ES cell lines or in a breast cancer cell line. We detected TH mRNA in 23 of 25 NB tumor samples (92%), but in none of the SRCTs or other clinical tumor samples. This RT-PCR technique showed a sensitivity for TH mRNA of one NB cell per 10^5 negative cells. Based on these results, the detection of TH mRNA is very useful both as a tumor marker for NB and for detecting minimal residual disease. Therefore, we can use this method to detect tumor cell contamination before hematopoietic stem cell transplantation.

Key words neuroblastoma; tyrosine hydroxylase; hematopoietic stem cell transplantation; minimal residual disease; reverse transcription polymerase chain reaction

Neuroblastoma (NB) is a very common malignant solid tumor in childhood. NB belongs to the small round cell tumors (SRCTs), which include other solid tumors such as Ewing's sarcoma (ES), rhabdomyosarcoma, and malignant lymphoma.[1] SRCTs are histologically ambiguous, so it is necessary to analyze adequate tumor markers for an accurate diagnosis. Patients who are over the age of 1 year at diagnosis usually have poor prognoses.[2]

NB is also characterized by elevated levels of catecholamine production. Tyrosine hydroxylase (TH) is very important as the first and rate-limiting step in the synthesis of catecholamines.[3—6] Therefore, we used reverse transcription polymerase chain reaction (RT-PCR) to examine the expression of TH mRNA in some cell lines, SRCTs, and other clinical tumor samples, to assess whether we can use it as a tumor marker and detect cell contamination in hematopoietic stem cells. Specific TH mRNA could be detected in NB cell lines and clinical NB tumor samples, but not in other cell lines and tumor samples. Moreover, the technique had a high sensitivity of $1/10^5$.

We think that this method should be used for detecting minimal residual disease because the prognoses of patients in NBs depend on being positive or negative for TH mRNA in bone marrow (BM) samples within 4 months after chemotherapy.[7] Moreover, the risk of relapse after autologous peripheral blood stem cell (PBSC) or BM transplantation is high if there is NB cell contamination.[8,9]

Here, we describe a very useful method for detecting minimal residual disease. The method can also be used as a tumor marker.

MATERIALS AND METHODS

Cell Lines and Tumor Samples Three NB cell lines (IMR-32,[10,12] SK-N-SH,[11,12] and NB-39,[12] two ES cell lines (NCR-EW2,[13] SCMC-ES1[14]), and one breast cancer cell line (MCF-7[15]) were examined. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 g/l sodium bicarbonate under 5% CO_2 at 37 °C. Thirty-three clinical tumor samples (25 NBs, 1 ES, 1 osteosarcoma, 1 osteochondroma, 1 Wilms' tumor, 1 malignant lymphoma, and 3 rhabdomyosarcoma) were examined. One of the three rhabdomyosarcoma samples was from a recurrent tumor. These tissues had been stored at −80 °C since collection. The clinical diagnoses for these patients were made by histopathology. Informed consent was obtained from all patients before they entered this study.

RNA Extraction Total RNA was extracted from the six cell lines using the acid-guanidium-phenol chloroform method after treatment with Catrimox-14™. Total RNA from the 33 clinical tumor samples was extracted by TRIZOL reagent (GIBCO BAL) based on the acid-guanidium-phenol chloroform extraction method.[16]

Reverse Transcription Polymerase Chain Reaction Total RNA (1 μg) was reverse-transcribed in a 10 μl reaction mixture with a first strand cDNA synthesis kit (Rever Tra-α-TM, TOYOBO). RT was performed with Oligo-dT. The mixture was annealing at 42 °C for 20 min, followed by incubated at 99 °C for 5 min, and then held at 4 °C. PCR amplification was carried out in 10× reaction mixture containing 1.2 pmol of the respective primers. We used a HOT START PCR kit from KOD-Plus- (TOYOBO).[17—19] The PCR conditions were one cycle of template denaturing at 94 °C for

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Table 1. PCR Primers

Gene	Sequence	Location
TH sense	5′-TGT CAG AGC TGG ACA AGT GT-3′	Exon 8
TH anti-sense	5′-GAT ATT GTC TTC CCG GTA GC-3′	Exon 9
GAPDH sense	5′-TCC TCT GAC TTC AAC AGC GAC ACC-3′	Exon 5
GAPDH anti-sense	5′-TCT CTC TTC CTC TTG TGC TCT TGG-3′	Exon 8

2 min, followed by 28 cycles of denaturing at 94 °C for 15 s, annealing at 60 °C for 90 s, extension at 68 °C for 20 s, and then holding at 4 °C. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal marker and IMR-32, NB-39, and SK-N-SH neuroblastoma cell lines as positive controls. NCR-EW2, SCMC-ES1, and MCF-7 were examined as negative controls. The primer sequences are listed in Table 1.[20] The primers for TH are located in exons that are not affected by alternative splicing. To avoid contamination with genomic DNA, the reverse primers for both TH and GAPDH were located in successive exons.

Analyses of PCR Products The PCR products were electrophoresed through 2.0% agarose gel, stained with ethidium bromide (WAKO), and visualized under a UV lamp. We used a Bioanalyzer (Agilent Technologies) to accurately determine band sizes.

Detection for Sensitivity A NB cell line (IMR-32) was examined as a positive control and an ES cell line (NCR-EW2) as a negative control. We counted the number of each cell type and diluted them to make suspensions containing one NB cell per 10 ES cells, one per 10^2, one per 10^3, one per 10^4, one per 10^5, and one per 10^6. We investigated the sensitivity of this RT-PCR technique for detecting TH mRNA.

RESULTS AND DISCUSSION

We analyzed six cell lines for detecting a specific TH mRNA with the RT-PCR technique. The electrophoresis and Bioanalyzer results are presented in Figs. 1A and 1B. The band size for TH was 299 bp, and the internal marker GAPDH was 209 bp. TH mRNA was detected in three NB cell lines (IMR-32, NB-39, and SK-N-SH), but it was not detected in the ES cell lines (NCR-EW2, SCMC-ES1) or the breast cancer cell line (MCF-7).

We investigated 33 clinical tumor samples by the same method as that used for the cell lines. The electrophoresis and Bioanalyzer results are presented in Tables 2 and 3. In 23 of 25 NB tumor samples (92%), TH mRNA could be detected, but it was not detected in the T1 and T2 samples (Table 2). TH mRNA was not detected in the osteosarcoma (T26), osteochondroma (T27), Wilms' tumor (T28), and SRCT (T29—T33) samples (Table 3). The rhabdomyosarcoma from a recurrent tumor (T33) also did not express TH mRNA.

We examined the sensitivity for detecting minimal residual disease by this RT-PCR technique. We used a NB cell line (IMR-32) as a positive control and an ES cell line (NCR-EW2) as a negative control. The electrophoresis and Bioanalyzer results are presented in Figs. 2A and 2B. On the electrophoresis, a TH mRNA band could be seen in samples with concentrations down to one NB cell per 10^5 ES cells, but not at a concentration of one NB cell per 10^6 ES cells (Fig. 2A).

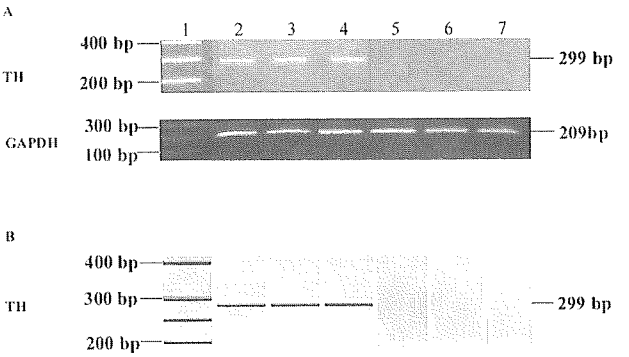

Fig. 1. RT-PCR Products Detected in Cell Lines

(A) Bands detected by electrophoresis. Lane 1, marker; lane 2, IMR-32; lane 3, NB-39; lane 4, SK-N-SH; lane 5, NCR-EW2; lane 6, SCMC-ES1; lane 7, MCF-7. (B) Bands detected by Bioanalyzer. Lanes are as for electrophoresis. A 299 bp RT-PCR product for TH was seen by both electrophoresis and Bioanalyzer, and a 209 bp product for GAPDH was also seen.

Table 2. NB Patients' Clinical Profile and TH mRNA Bands Detected by Electrophoresis and Bioanalyzer

Tissue	Sex	Age	Diagnosis	TH mRNA
T1	F	1 y	NB	−
T2	F	10 m	NB	−
T3	M	10 m	NB	+
T4	M	10 m	NB	+
T5	M	9 m	NB	+
T6	M	3 y	NB	+
T7	M	7 m	NB	+
T8	M	4 m	NB	+
T9	F	10 m	NB	+
T10	F	1 y	NB	+
T11	F	6 y	NB	+
T12	F	1 y	NB	+
T13	F	8 m	NB	+
T14	F	6 m	NB	+
T15	M	4 y	NB	+
T16	F	3 y	NB	+
T17	M	4 y	NB	+
T18	F	10 y	NB	+
T19	M	9 y	NB	+
T20	F	7 m	NB	+
T21	F	9 m	NB	+
T22	F	10 m	NB	+
T23	M	3 y	NB	+
T24	M	5 y	NB	+
T25	F	5 y	NB	+

NB: neuroblastoma.

Similarly, using the Bioanalyzer, TH mRNA could be detected at one NB cell per 10^5 ES cells but not at one NB cell per 10^6 ES cells (Fig. 2B).

Abnormal amplification and expression are well known for *MYCN*,[21—23] *trk-A*,[24—27] and protein gene product 9.5 (PGP9.5)[28] in NB. However, these markers are not very useful for diagnosis. Recently, by using monoclonal antibodies

Table 3. Other Tumor Patients' Clinical Profile and TH mRNA Bands Detected by Electrophoresis and Bioanalyzer

Tissue	Sex	Age	Diagnosis	TH mRNA
T26	M	13 y	OS	–
T27	F	12 y	OC	–
T28	M	8 m	WT	–
T29	F	6 m	ML	–
T30	M	3 y	ES	–
T31	M	11 y	RMS	–
T32	F	3 y	RMS	–
T33	M	4 y	RMS	–

OS: osteosarcoma, OC: osteochondroma, WT: Wilms' tumor, ML: malignant lymphoma, ES: Ewing's sarcoma, RMS: rhabdomyosarcoma.

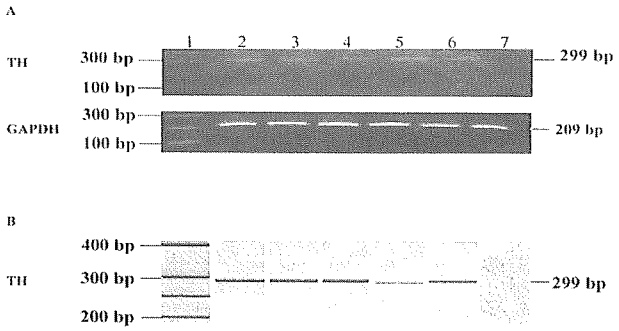

Fig. 2. Sensitivity of RT-PCR Technique for TH mRNA

Lane 1, marker; lane 2, one NB cell per 10 ES cells; lane 3, one NB cell per 10^2 ES cells; lane 4, one NB cell per 10^3 ES cells; lane 5, one NB cell per 10^4 ES cells; lane 6, one NB cell per 10^5 ES cells; lane 7, one NB cell per 10^6 ES cells. (A) Bands detected by electrophoresis. (B) Bands detected by Bioanalyzer. The 299 bp RT-PCR product for TH was detected by both electrophoresis and Bioanalyzer, and a 209 bp product for GAPDH was also seen.

that react selectively to cells of neuroectodermal origin, the diagnostic usefulness has been improved. However, the monoclonal antibodies often produce false positive results,[29,30] and the clinical importance of detecting positive cells as evidence of infiltration is still debated.[31] As NB has a specific catecholamine metabolism, it may be more useful to detect TH, which is the first and rate-limiting enzyme of catecholamine synthesis. We found that TH mRNA was expressed in all NB cell lines (100%) and in 23 of 25 (92%) clinical NB tumor samples. On the other hand, it was not expressed in any of the other cell lines and clinical tumor samples.

Thus, we found that TH mRNA is expressed specifically in NB, and this specific expression can be use to distinguish NB from SRCTs. TH mRNA can also be used as a tumor marker for the accurate diagnosis of NB.

The expression of TH mRNA did not correlate with the patient's age or sex, and it might not be a prognostic factor for NB patients.

Another important problem is determining whether tumor cell contamination exists when a patient's PBSC or BM is used for autologous transplantation. Our method can detect contamination of one cell in 10^5.

In future, our method may be very useful for diagnosing NB patients and detecting minimal residual disease in clinical samples.

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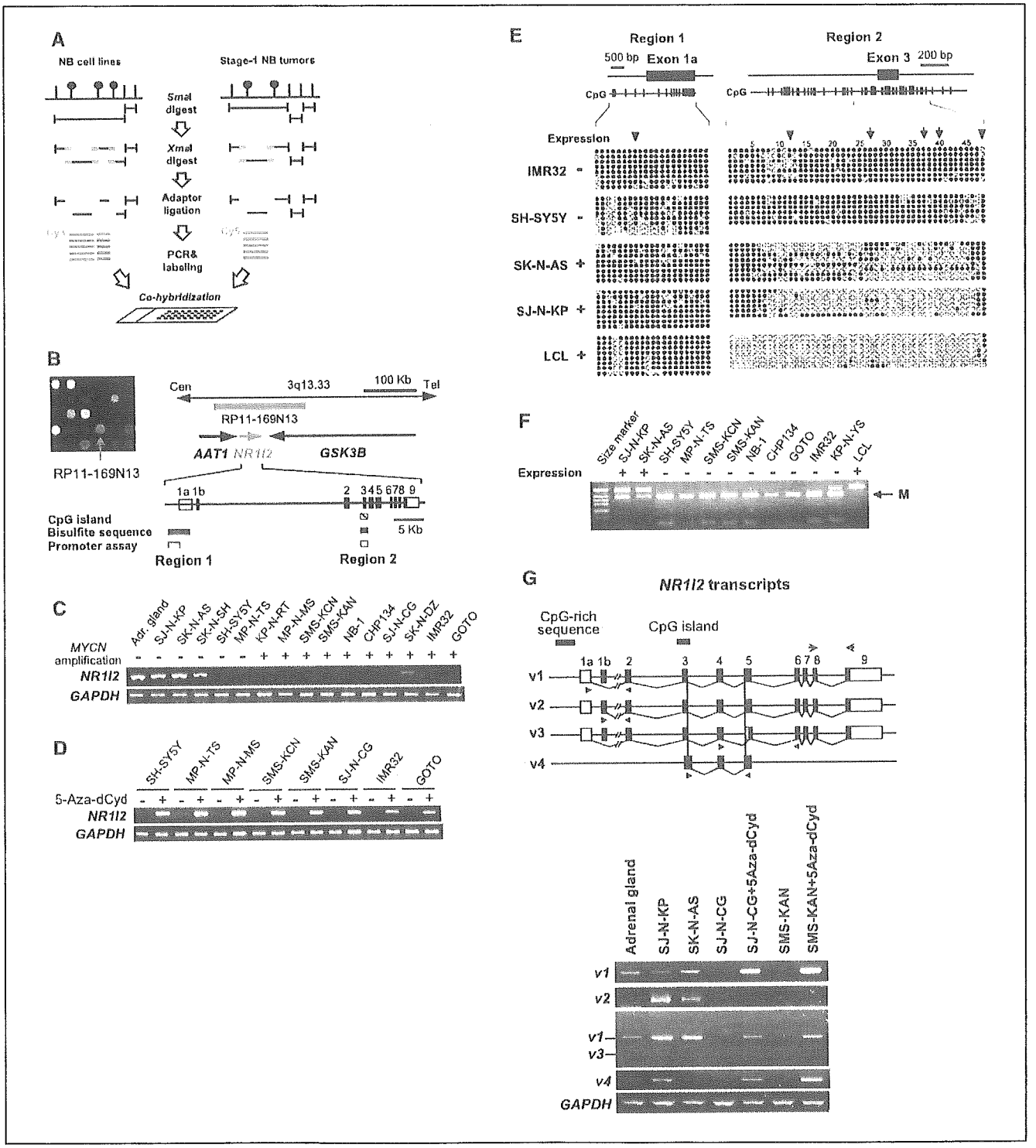
human cell lines and tumors of neuroblastoma through hypermethylation of a CpG island showing promoter activity. *NR1I2* was methylated and silenced mainly in late-stage neuroblastoma tumors with *MYCN* amplification and in older children. Exogenous restoration of NR1I2 expression suppressed growth of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

Table 1. List of positive BACs in BAMCA analysis and summary of screening of candidate methylated genes (Cont'd)

BAMCA ratio[†]		mRNA expression				Methylation[‡]	
GOTO	IMR32	Stage I	IMR32	GOTO	GOTO (+5-aza-dCyd)[§]	Stage I	Stage IVa
3.46	4.57	−	+	+			
1.91	10.78						
1.68	2.10	+	−	−	+	−	+
		+	+	+			
		+	+	+			
3.01	5.19	+	+	+			
2.10	2.05	+	+	+			
1.72	2.33	+	+	+			
3.07	5.54	+	+	+			
2.54	2.32	+	+				
		+	+	+			
3.52	2.17	+	−	+			
3.31	2.48	+	−	−	−		
3.89	5.45	+	+	+			
3.72	12.90	+	−	+			
2.06	1.45	+	+	+			
2.51	3.51	−	−	−			
		+	+	+			
		+	+	−			
1.81	1.85	+	+	+			
		+	+	+			
1.75	16.90	+	−	−	−		
		+	−	−	±	−	±
2.93	3.24	+	−	−	+	−	±
		+	−	−	+	−	±
1.54	3.39	+	+	+			
		−	+	+			

Methylation of *NR1I2* CpG island in neuroblastoma cell lines. We next examined the methylation status of the slightly CpG-rich 5′ region (region 1) and the CpG island including exon 3 (region 2) of the *NR1I2* gene, which had been detected by the National Center for Biotechnology Information (NCBI) human genome database[6] as shown in Fig. 1*E*. Bisulfite sequencing analysis of region 2 revealed aberrant DNA hypermethylation in IMR32 and SH-SY5Y cell lines lacking expression of *NR1I2*, but hypomethylation in two lines expressing the gene (SK-N-AS and SK-N-KP) and in a normal lymphoblast cell line (LCL). On the other hand, no significant difference in methylation pattern within region 1 was observed among those four neuroblastoma cell lines and LCL, regardless of expression status. We did COBRA to confirm the relationship between expression and methylation status within region 2 in a larger set of neuroblastoma cell lines. Predominant methylated alleles were detected in all lines lacking *NR1I2* expression (Fig. 1*F* and data not shown).

Because several splicing variants of *NR1I2*, including the variant starting transcription from exon 3 (variant 4, v4), have been reported in various human tissues (19),[7] we did RT-PCR using specific primers for each variant to examine which transcripts might be silenced through a DNA methylation within region 2 in neuroblastoma cell lines (Fig. 1*G*). Variant 1, the most major variant in various human tissues, and variant 2 were expressed in unmethylated neuroblastoma cell lines, but not in methylated lines, whereas variant 4 without open reading frame was not expressed in one of the unmethylated cell lines (SK-N-AS). The expression of variant 1 and variant 4 was restored after the treatment with 5-aza-dCyd in methylated neuroblastoma cell lines, whereas the expression of variant 2 was not. Variant 3, lacking a part of exon 5, was not expressed in neuroblastoma cell lines regardless of methylation status within CpG island and 5-aza-dCyd treatment. Those results suggested that the methylation of CpG residues in region 2 might be mainly responsible for the silencing of variant 1 of the *NR1I2* gene starting transcription from exon 1a in neuroblastoma, although region 2 does not contain its transcriptional start site.

Promoter activity of the CpG island located around exon 3 of *NR1I2*. Because the CpG island (region 2) of *NR1I2* was located around exon 3, we first determined whether region 2 had promoter activity by means of a luciferase reporter assay. This fragment alone (Fig. 2*A*, *2/L*) revealed clear promoter activity, whereas region 1 fragment upstream of exon 1 (Fig. 2*A*, *1/L*) showed almost none (Fig. 2*B*). In addition, we next determined whether region 2 acts as an enhancer to stimulate transcription from exon 1 by testing the luciferase activity in construct

[6] http://www.ncbi.nlm.nih.gov/.
[7] http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html.

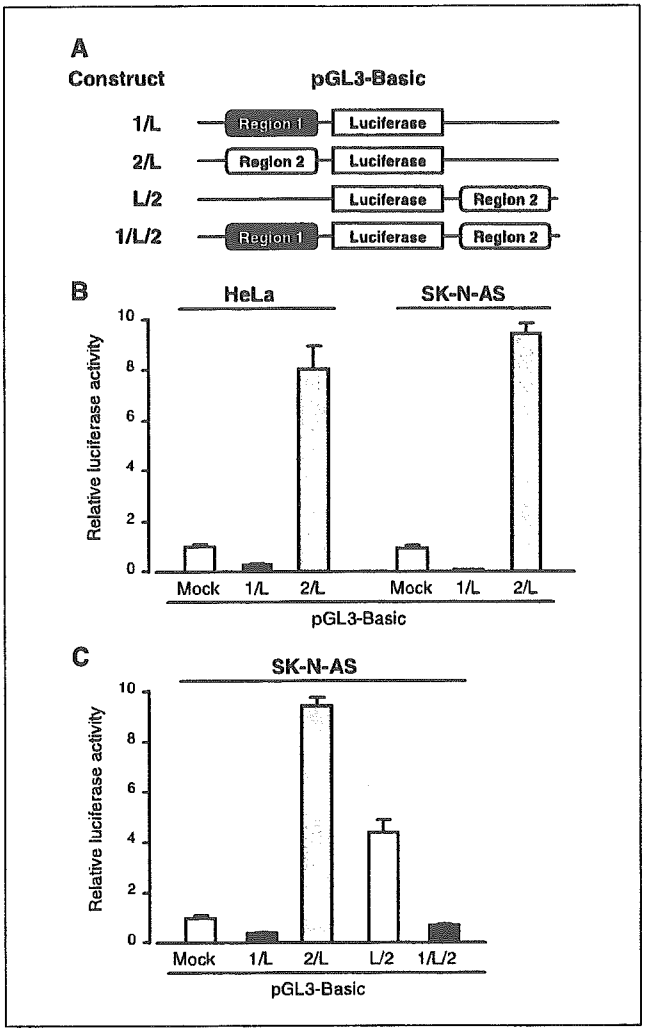


Figure 2. Promoter activity of the CpG island of *NR1I2*. pGL3-Basic vector, each containing a 1,060 bp 5′ fragment (region 1) or a 480 bp CpG island (region 2) of *NR1I2* in front of and/or downstream of the luciferase gene (*1/L*, *2/L*, *L/2*, and *1/L/2*; *A*), or pGL3-Basic empty vector (mock), was transfected into HeLa or SK-N-AS cells to evaluate promoter activity of region 1 and region 2 (*B*) and enhancer activity of region 2 (*C*). Luciferase activities were normalized versus an internal control. *Columns*, means of three separate experiments, each done in triplicate; *bars*, SE.

containing region 1 in front of the luciferase reporter gene and region 2 downstream of the luciferase gene (Fig. 2*A*, *1/L/2*). Although region 2 downstream of the luciferase gene (Fig. 2*A*, *L/2*) showed some promoter activity, region 2 revealed no enhancer activity for region 1 (Fig. 2*C*).

Analysis of *NR1I2* methylation and expression in primary neuroblastoma tumors. We next examined methylation status of the *NR1I2* CpG island in 51 surgically resected primary neuroblastomas using COBRA (Table 2). Clearly methylated alleles were detected in nine of the tumors (17%; Fig. 3*A*). The appearance of partial methylation observed in those nine tumors can be explained by the unavoidable contamination of nontumorous cells in the specimens. Five of the nine tumors (55%) had undergone *MYCN* amplification, whereas only 3 of 42 (7%) unmethylated tumors showed amplification of *MYCN* (Table 2). Moreover, methylation of region 2 of the *NR1I2* gene was more frequently detected in advanced tumors (stages III and IVa; P = 0.0234, Fisher's exact test), tumors from patients with poor outcome (dead from disease; P = 0.0135, Fisher's exact test), and tumors from patients >1 year old (P = 0.052, Fisher's exact test), although the difference did not quite reach statistical significance in terms of patient age. In the 47 neuroblastoma cases where high-quality RNAs were available for expression analysis, a clear correlation between the methylation status of the CpG island and expression level of *NR1I2* mRNA was observed (Fig. 3*B* and *C*). By means of real-time quantitative RT-PCR experiments, we saw a statistically significant inverse correlation between expression of *NR1I2* mRNA and tumor stage (P = 0.0137, Mann-Whitney U test) or *MYCN* amplification (P = 0.0003, Mann-Whitney U test; Fig. 3*C*).

Suppression of cell growth after restoration of NR1I2 expression. To gain further insight into the potential role of NR1I2 in neuroblastoma carcinogenesis, we investigated whether restoration of NR1I2 expression would suppress growth of neuroblastoma cells lacking endogenous *NR1I2* expression using two *NR1I2* expression constructs, a Myc-tagged full coding sequence of *NR1I2* alone (pCMV-Tag3-*NR1I2*) and one fused to the constitutively active herpes virus VP-16 transactivation domain (pCMV-Tag3-*VP-NR1I2*). The VP-16-NR1I2 chimeric protein showed stronger transactivating activity than NR1I2 alone in a reporter assay using a reporter construct containing *NR1I2* response elements from the *CYP3A4* promoter (data not shown). After selecting drug-resistant colonies in transient transfection experiments, we found that colonies of *NR1I2*-transfected cells were remarkably fewer than in cultures of control transfectants and the effect of VP-16-NR1I2 was much greater than that of NR1I2 (Fig. 4*A*). Furthermore, VP-16-NR1I2 stable transfectants

Table 2. Correlation between patient profiles and *NR1I2* methylation status in 51 cases with neuroblastoma

Characteristics	Cases *n*	COBRA on *NR1I2* region 2*		
		Negative *n*	Positive *n*	*P*†
Total	51	42	9	
Age (y)				
<1	33	30	3	0.052
≥1	18	12	6	
Stage‡				
I, II, IVS	30	23	2	**0.0234**
III, IVa	21	16	7	
MYCN				
Nonamplified	43	39	4	**0.024**
Amplified	8	3	5	
Outcome				
Alive	44	39	5	**0.0135**
Dead	7	3	4	

NOTE: Statistically significant values are in boldface.
*COBRA was done as described in Materials and Methods.
†*P* values are from Fisher's exact test and were statistically significant when <0.05.
‡Tumor stage was classified according to the International Neuroblastoma Staging System.

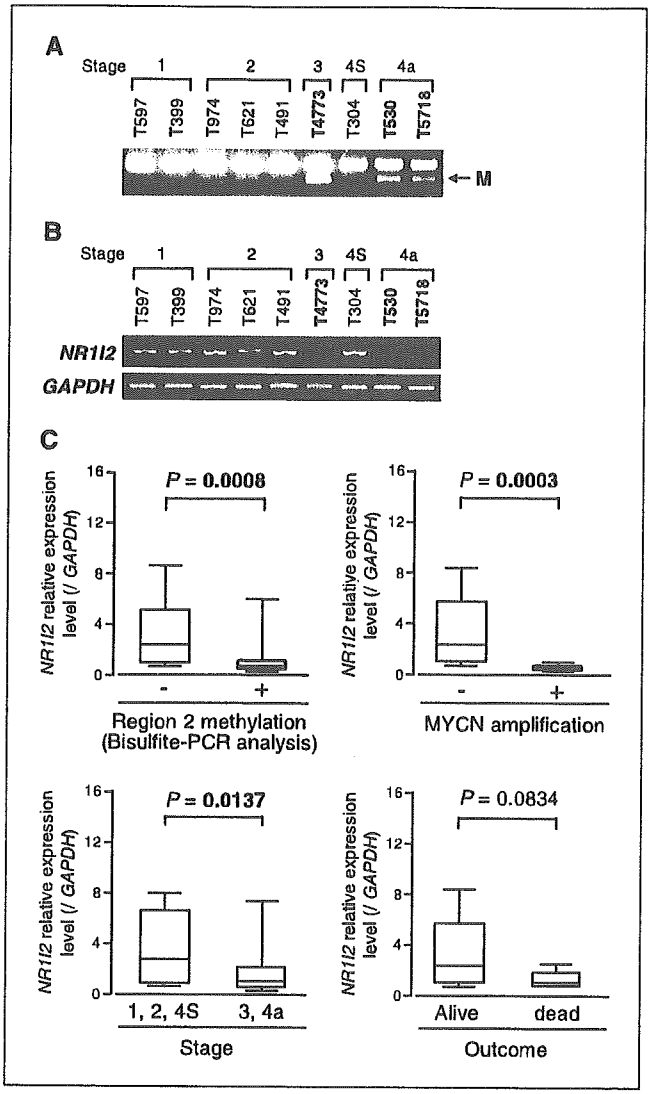


Figure 3. Methylation status and expression levels of *NR1I2* in primary neuroblastoma tumors. *A*, representative results of COBRA of the *NR1I2* CpG island (region 2). *M*, methylated alleles restricted by *Hha*I. *B*, representative results of RT-PCR analysis of *NR1I2* mRNA expression. *GAPDH* was used as an internal control. Note that tumors showing methylation in Fig. 2*A* (T4773, T530, and T5718) showed decreased expression of *NR1I2*. *C*, expression of *NR1I2* mRNA in primary neuroblastoma tumors, compared with methylation status of the CpG island of *NR1I2* region 2, *MYCN* amplification status, tumor stage, and patient outcomes. The levels of *NR1I2* mRNA expression were determined by real-time quantitative RT-PCR experiments. Significantly higher expression of *NR1I2* was observed in tumors without methylation of the CpG island ($P = 0.0008$; Mann-Whitney *U* test), in stage I, II, and IVS tumors ($P = 0.0137$, Mann-Whitney *U* test), and in *MYCN*-nonamplified tumors ($P = 0.0003$, Mann-Whitney *U* test) compared with tumors with methylation of the CpG island, stage III or IVa tumors, and *MYCN*-amplified tumors. Higher expression of *NR1I2* was also observed in living, disease-free patients compared with those who had died of their tumors, although the difference did not reach statistical significance ($P = 0.0834$, Mann-Whitney *U* test).

established from a cell line (SMS-KAN) without endogenous expression of this gene showed a lower growth rate than control vector-transfected cells regardless of VP-16-NR1I2 expression level (Fig. 4*B* and *C*). The same result was obtained in other cell line (data not shown).

Screening of possible target genes for NR1I2. To identify possible transcriptional targets for NR1I2, we did expression array analysis in an independent VP-16-NR1I2 stable transfectant established from SMS-KAN, in a comparison with vector-transfected cells. We twice obtained independent experimental data (first, Cy3-B1/Cy5-vector; second, Cy5-B1/Cy3-vector) and compiled a list of 105 genes that showed ratios >1.5 in both experiments (Supplementary Table S1). Semiquantitative RT-PCR analysis revealed up-regulation of *CYP3A4*, a known transcriptional target of *NR1I2*, in VP-16-NR1I2 stable transfectant, indicating the reliability of our system for detecting target genes (Supplementary Fig. S1). To validate the expression array data, we did semiquantitative RT-PCR analyses of 10 other genes (Fig. 4*D*), which have been previously reported as tumor-associated genes, using two independent VP-16-NR1I2 stable transfectants established from SMS-KAN cells (KAN-B1 and KAN-B2) and one stable transfectant from GOTO cells (GOTO-A1). GOTO-A1 also showed a lower growth rate than control vector-transfected cells (data not shown). The RT-PCR results for KAN-B1 confirmed up-regulation of seven genes except for *BARD1*, *EXT1*, and *MCM5* (Fig. 4*D*). Notably, only *PLA2G2A* showed increased expression in all stable transfectants examined compared with their control cells (Fig. 4*D*; Supplementary Fig. S1).

Discussion

In the study presented here, we identified a novel target for CpG island methylation, *NR1I2*, observed mainly in advanced neuroblastomas, through a genome-wide exploration of highly methylated DNA fragments using BAMCA. A clear inverse correlation emerged between CpG island methylation and the expression status of *NR1I2* both in cell lines and primary tumors of neuroblastoma (i.e., hypermethylation and silencing of *NR1I2* were more frequent in advanced neuroblastoma tumors). Together with a shown growth suppressive effect of exogenous NR1I2, the data suggested that *NR1I2* was likely to be a tumor suppressor gene associated with clinical and/or biological aggressiveness of neuroblastoma. Our results further underscored the promise of BAMCA as a high-throughput screening method for methylated sites in cancer genomes on an array platform.

BAMCA, however, has some disadvantages: (*a*) It examines only a limited number of CpG sites within a CpG island because only *Sma*I/*Xma*I sites are used to search differentially methylated CpGs. (*b*) It identifies BACs, which contain differentially methylated sequences between test and reference DNAs, although sequences are not always associated with promoter regions of genes. (*c*) It misses differentially methylated sequences/genes without BACs spotted on the BAC array used. To improve the sensitivity of array-based methylation screening, it is possible to use other methylation-sensitive restriction sites as indicators, including *Not*I site (20). However, BAMCA may quickly provide the list of possible target genes for methylation within identified BAC clones using information from human genome database without cloning and sequencing of enriched fragments, suggesting that it may have important applications in population-based studies of CpG island methylation.

NR1I2 locates at 3q13.33, a chromosomal region that is not often involved in loss of heterozygosity or copy number losses in neuroblastoma (21, 22). Indeed, most of the cell lines we used in this study showed normal copy number in 3q (13), suggesting that a homozygous inactivation of *NR1I2* might occur by biallelic

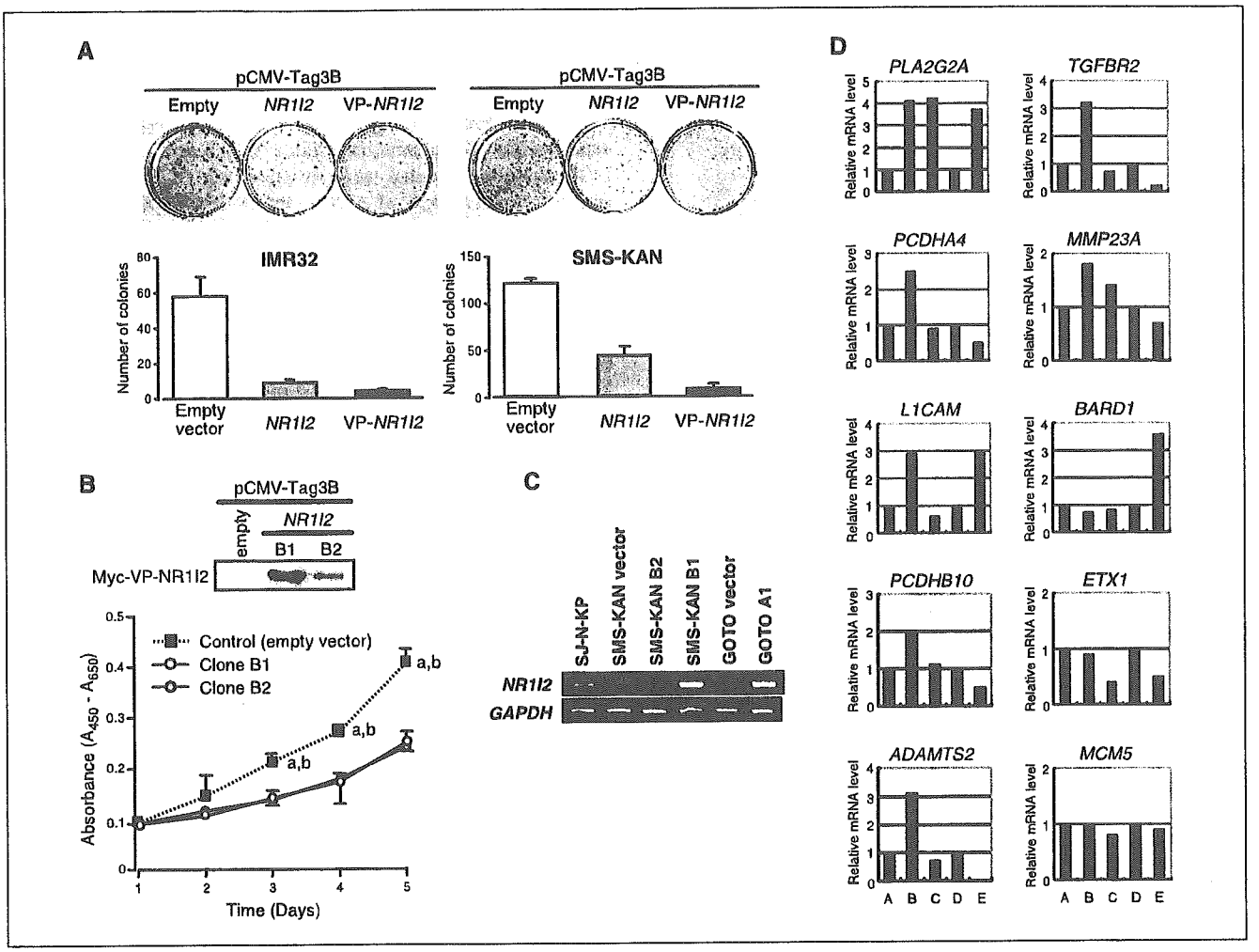


Figure 4. Effect of restoration of NR1I2 expression on growth of neuroblastoma cells. *A, top,* colony formation assays using neuroblastoma cell lines. Cells without *NR1I2* expression (IMR32 and SMS-KAN) were transfected with Myc-tagged construct containing *NR1I2* (pCMV-Tag3B-*NR1I2*), *VP-NR1I2* (pCMV-Tag3B-*VP-NR1I2*), or empty vector (pCMV-Tag3B), and selected for 3 weeks with G418. *Bottom,* quantitative analysis. *Columns,* mean of three separate experiments, each done in triplicate; *bars,* SE. *B,* inhibitory effect of stably transfected *NR1I2* on the growth of SMS-KAN cells transfected with pCMV-Tag3B-*VP-NR1I2* or empty vector and selected with G418 to establish clones stably expressing NR1I2. *Top,* two clones transfected with pCMV-Tag3B-*VP-NR1I2* (B1 and B2) were subjected to Western blot analysis using 10 μg of protein extract and anti-Myc-Tag antibody. Both expressed Myc-tagged VP-NR1I2 protein. *Bottom,* effect of stable NR1I2 expression on the growth of SMS-KAN cells. Cell viability was determined by water-soluble tetrazolium salt assay at the indicated times. *Points,* means of three separate experiments; *bars,* SE. Statistical analysis used the Mann-Whitney *U* test: *a,* control versus clone B1; *b,* control versus clone B2. All $P < 0.05$. *C,* the mRNA expression level of *NR1I2* in endogenously NR1I2-positive cells (SK-N-KP) as well as stably transfected cells (SMS-KAN-B1, SMS-KAN-B2, and GOTO-A1) and their mock-transfected control (KAN-vector and GOTO-vector). *D,* confirmation of microarray results by semiquantitative RT-PCR of possible target genes using two stable transfectants established from SMS-KAN (KAN-B1 and KAN-B2) and one transfectant from GOTO (GOTO-A1) with their mock-transfected control cells *A,* SMS-KAN mock-control; *B,* KAN-B1; *C,* KAN-B2; *D,* GOTO mock-control; *E,* GOTO-A1. PCR products were electrophoresed in 3% agarose gel and band quantification was done with LAS-3000 (Fujifilm, Tokyo, Japan). After normalization with *GAPDH*, expression level of each gene in each transfectant relative to its corresponding mock-transfected control was recorded as a fold increase in relative expression level. Primer sequences and cycling numbers for PCR of each gene are available on request.

methylation. Similar findings have been reported for several genes, such as *RASSF1* (3p21.3), *DAPK* (9q34.1), and *THBS1* (15q15), which are located in regions not frequently deleted, although some methylated genes, such as *ARF* and *INK4A* (9p21), and *CASP8* (2q23), are in fact on regions frequently deleted in neuroblastoma (23). Therefore, both biallelic methylation and monoallelic methylation with allele loss may be important mechanisms for inactivating tumor-associated genes in this disease.

Our promoter assays showed that CpG island around exon 3 (region 2) shows promoter activity, but CpG-rich 5′ region containing exon 1 and its 5′ upstream sequences (region 1) does not. Region 2 shows no enhancer activity for region 1 (Fig. 2). The methylation status of region 2, but not region 1, was highly and inversely correlated with the expression of *NR1I2*, especially the most major variant of *NR1I2*, variant 1, starting from exon 1a. Those results suggest that the methylation status of CpG residues in region 2 might be responsible for the silencing of this gene and contributed to loss of function of NR1I2 protein in neuroblastoma. A few studies, including ours, have shown that promoter activity can occur in fragments, especially CpG islands, not containing transcriptional starting sites (17, 24, 25). It is possible that methylation that occurred in those CpG islands with promoter activity may silence gene expression from specific starting sites.

Among numerous hypermethylated genes reported in neuroblastoma (3–8), *CASP8* seems to be inactivated through promoter methylation in advanced neuroblastoma tumors where *MYCN* is amplified (4). Those findings, along with ours, suggest that (*a*) unknown mechanisms contribute to progression of neuroblastoma by causing genetic alterations, including *MYCN* amplification, as well as methylation-mediated inactivation of a subset of tumor suppressor genes; (*b*) methylation-mediated inactivation of a subset of tumor suppressor genes may cause genetic changes that lead to progression of neuroblastoma; or (*c*) *MYCN* amplification and/or other alterations in advanced neuroblastomas may bring about a CpG island methylator phenotype (3). Gonzalez-Gomez et al. (23) reported that higher aggressiveness, represented at the molecular level by concurrent *MYCN* amplification and 1p loss in neuroblastoma, was not paralleled by an accumulation of methylation events among various genes they examined, suggesting that CpG island methylation in advanced neuroblastoma could be specific to a subset of genes.

The *NR1I2* gene encodes an orphan nuclear receptor that plays a key role in the regulation of xenobiotic response by controlling expression of drug metabolizing and clearance molecules (26–28). NR1I2 protein activates expression of genes encoding proteins such as CYP3A4 and ABCB1, which reduce the concentrations of xenochemicals and toxic bile acids (29). However, effects of NR1I2 on cell growth or expression of growth-regulating genes have never been clarified, although we have shown here that the induction of ectopic NR1I2 inhibited growth of neuroblastoma cells. Other nonsteroidal nuclear receptors, such as all-*trans* retinoic acid receptor and vitamin D_3 receptor, which form heterodimers with the 9-*cis* retinoic acid receptor in the same way as NR1I2, mediate antiproliferative and differentiation-promoting activities toward several malignant cell types (30, 31). Therefore, growth-suppressive activity might be one of the normal functions of NR1I2 although its mechanisms remain unknown.

To achieve some clarity with respect to the growth inhibitory activity of NR1I2, we tried to determine its putative transcriptional targets. Among 105 genes through an expression array analysis, we selected 10 genes for validation by semiquantitative PCR based on their possible cancer-associated function (Online Mendelian Inheritance in Man),[8] and identified one candidate, *PLA2G2A*, encodes secretory phospholipase A2, as a possible target of NR1I2, although it will be needed to determine whether *PLA2G2A* is a direct or indirect target. This product qualifies as a tumor suppressor because mice lacking *PLA2G2A* expression show increased colonic polyposis (32). Interestingly, *PLA2G2A* was mapped to chromosome 1p36, a region frequently implicated in the pathogenesis of neuroblastoma (33). Further screening of possible targets of NR1I2 will be necessary to clarify how NR1I2 regulates neuroblastoma cell growth.

Because only 7 of 51 neuroblastoma patients in our study died during follow-up periods, we did not perform a survival analysis. However, the high prevalence of *NR1I2* silencing through DNA methylation that we observed in aggressive neuroblastomas, along with the shown growth suppression activity of NR1I2, indicate that this molecule might serve as a diagnostic marker to predict prognosis.

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[8] http://www.ncbi.nlm.nih.gov/Omim/omimhelp.html.

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