Deletion Map of Chromosome 9 and p16 (CDKN2A) Gene Alterations in Neuroblastoma

Junko Takita, Yasuhide Hayashi, Takashi Kohno, Naohito Yamaguchi, Ryoji Hanada, Keiko Yamamoto, and Jun Yokota

ABSTRACT

We reported previously that loss of heterozygosity (LOH) on chromosomes 2q, 9p, and 18q frequently occurs in neuroblastoma and that patients with 9p LOH in the tumors showed statistically significant association with an advanced stage of the disease and poor prognosis. To determine the role of chromosome 9 loss in neuroblastoma, we performed deletion mapping of chromosome 9 in 80 cases of neuroblastoma using 11 polymorphic microsatellite markers and a restriction fragment length polymorphism marker. LOH at one or more loci on chromosome 9 was detected in 33 of 80 cases (41%). Chromosome 9p was lost in 24 of 80 cases (30%), whereas chromosome 9q was lost in 18 of 80 cases (23%). There were two commonly deleted regions mapped to 9p21 between the D9S171 marker and the IFNB1 marker and 9q34–qter distal to the D9S176 marker. In addition, patients with LOH at 9p21 but not at 9q34–qter in the tumors showed statistically significant association with poor prognosis (P = 0.023). Because the commonly deleted regions at 9p21 include the p16 (CDKN2A) gene, the status of the p16 gene was further examined in 80 fresh tumors and 19 cell lines of neuroblastoma. A missense mutation was detected at codon 52 in a fresh tumor. The p16 gene was not expressed in 13 of 19 cell lines (72%), and 5 of the 13 cell lines displayed methylation of the CpG island surrounding the first exon of the p16 gene. These results suggest that the p16 gene is a candidate tumor suppressor gene for neuroblastoma, and its inactivation may contribute to the progression of neuroblastoma.

INTRODUCTION

Recent molecular studies have revealed that the genesis and progression of human cancer is largely attributed to accumulation of a series of genetic events that culminate in the transformation of a cell into a malignant clone (1). Central to this theory are the roles of oncogenes and tumor suppressor genes, the activation and inactivation of which, respectively, cause disruption of critical events in cell division and differentiation (1). The paradigm of alterations in the tumor suppressor gene is a mutation of one allele and a loss of the other allele. Reduction to homozygosity of the tumor suppressor gene can be detected as LOH2 of informative polymorphic markers in the region of the tumor suppressor gene. Thus, allelic losses are hallmarks of chromosomal regions harboring tumor suppressor genes (2).

Although NB is one of the most common childhood tumors, little is known about the genetic changes that contribute to the development of tumor. It has been reported that LOH occurs frequently on at least three chromosome arms, 1p, 11q, and 14q, in NB (3–10). In addition, we demonstrated recently that three additional loci on chromosomes 2q, 9p, and 18q were deleted with high frequency in NB. Moreover, several studies have shown the correlation of genetic changes with prognosis of the patients with NB (11). N-myc (NMYC) oncogene amplification has been known to be an appreciable prognostic factor in an advanced stage of the disease. It is also indicated that chromosome 1p deletion frequently occurs in an advanced stage of the disease, and there may be two tumor suppressor genes on chromosome 1p associated with progression of the disease (9, 10). In addition, we also reported that 9p LOH was significantly associated with advanced stages of the disease and with poor prognosis (12).

To determine the locus on chromosome 9 that may harbor putative tumor suppressor genes involved in the progression of NB, we performed deletion mapping of chromosome 9 in 80 cases of NB using 11 microsatellite markers and a RFLP marker. The result indicated that there were two commonly deleted regions on chromosome 9, 9p21 and 9q34–qter, in NB, and that LOH at 9p21 was significantly associated with poor prognosis. The p16 (CDKN2A) tumor suppressor gene has been mapped to 9p21 and is inactivated in a variety of malignancies by various mechanisms, including deletion, point mutation, and methylation of the CpG island in the 5′ end of the p16 gene (13–17). Therefore, we further examined the alterations of the p16 gene in NB. Although deletions and mutations of the p16 gene are infrequent, transcriptional silencing and DNA methylation were frequently detected in NB cell lines. Thus, it was indicated that the p16 gene is a candidate tumor suppressor gene involved in the progression of NB.

MATERIALS AND METHODS

Primary Tumors and Cell Lines. Tumor samples were randomly obtained from 80 patients admitted to various institutions between May 1987 and July 1993 at surgery or at autopsy. Corresponding normal tissues were available in all cases. The patients were staged according to the classification of staging in NB (18). Of the 80 cases, 21 were classified as being stage I, 27 as stage II, 8 as stage III, 16 as stage IV, and 8 as stage IVS. In our 80 NB patients, 74 patients were infants under 1 year of age at diagnosis, and 6 patients were over 1 year. Patients with stage I, II, or IVS were treated with either surgery alone or surgery plus chemotherapy consisting of vincristine and cyclophosphamide with or without radiotherapy. Patients with stage III or IV were treated with multidrug chemotherapy consisting of cyclophosphamide, Adriamycin, cisplatin, and etoposide with or without surgery and radiotherapy. In 53 cases, histological data was available; thus, tumors were histologically classified as described by Ota and Shimizu (19). There were 4 cases of GNB classified as well differentiated, 8 cases of GNB classified as composite, 9 cases of GNB classified as poorly differentiated, 27 cases of NB classified as rosette-fibrillary, and 5 cases of NB classified as round cell. We also used 19 NB cell lines, NB1, NB9, NB16, NB19, NB39, NB69, LAN1, LAN2, LAN5, KP-N-NS, GOTO, CHP-134, IMR-32, TNO-1, TGW, SCMCN2, SCMCN3, SCMCN4, and SCMCN5, for the analysis of the p16 gene alterations in NB.

DNA, RNA, and Protein Extraction. DNA was isolated from tumors, normal tissues, and cell lines by proteinase K digestion and phenol/chloroform isomyl alcohol (24:1) extraction as described previously (20). mRNA was extracted from cells growing in culture using the FastTrack 2.0 mRNA isolation kit according to the manufacturer’s instructions (Invitrogen). Cellular protein was extracted by lysing 1×106 cells with 40 μl of lysis buffer [50 mM

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2 To whom requests for reprints should be addressed. Phone: 81-3-3542-2511, Ext. 4650; Fax: 81-3-3542-0807.

3 The abbreviations used are: LOH, loss of heterozygosity; GNB, ganglioneuroblastoma; NB, neuroblastoma; SSCP, single-strand conformational polymorphism.
HEPES-NaOH (pH 7.0), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin.

**PCR-LOH Analysis.** DNA from tumors and corresponding normal tissues were analyzed for LOH by PCR amplification of microsatellite sequences. Microsatellite markers for PCR-LOH analysis were listed in Fig. 1. Total reaction volumes were 10 μl containing 50–100 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 μM of each deoxynucleotide triphosphate, 0.01% gelatin, 125 ng of each primer, 1.14 μCi of [α-32P]dCTP, and 1 unit of Taq DNA polymerase (Pharmacia Biotech, Inc.). Because unequal amplification of alleles occurs within 35 cycles of PCR (21), PCR amplifications were performed for 35 cycles consisting of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 90 s in a Gene Amp PCR System 9600 (Perkin-Elmer) as described (12).

**Southern and Northern Blot Analyses.** Approximately 10 μg of purified DNA were digested with appropriate restriction enzymes and separated by electrophoresis on 0.8% agarose gel. DNA was transferred from the gel to nylon membranes (12). The membranes were hybridized with a PCR-generated fragment corresponding to exon 1 of the p16 gene (p16-1), a full-length p16 cDNA fragment, INFb1, and pNB-1 labeled with [α-32P]dCTP. LOH at the INFb1 locus was examined by Southern blot analysis (12). Approximately 3 μg of mRNA was denatured with 40% formamide/32% formaldehyde and was electrophoresed on a 1.2% agarose gel containing 25% formaldehyde. Then mRNA was transferred to nylon filters. The filters were hybridized with p16-1 and full-length cDNA probes labeled with [α-32P]dCTP and were exposed to Kodak XAR-5 film at –80°C. Prehybridization, hybridization, and posthybridization washes were performed basically as described (20).

**PCR-SSCP Analysis.** All samples were screened for mutations in exons 1 and 2 of the p16 gene by PCR-SSCP analysis (22). exon 1 was amplified as one fragment, whereas exon 2 was split into two fragments for PCR-SSCP analysis. The primer sets for the p16 gene were: exon 1, PQ1S, 5’-TCTGCGGAGGAGGAAGCAGCA (sense) and PQ1A, 5’-TCTGCGGAGGAGGAGGAAGCAGCA (antisense); first exon 2, PQ2AS, 5’-ACACGTTCTTTTCTCGCTATCCCG (sense) and PQ2AA, 5’-CCAGCAGACGTGGCGGACTTCTGAC (antisense); and second exon 2, PQ2BS, 5’-TCTGAGCTGCTGGGT (sense) and PQ2BA, 5’-TCTGAGCTTCTGGA-GCTCTCAG (antisense).

**PCR conditions for exon 1 of the p16 gene were 35 cycles of 50 s at 94°C, 50 s at 65°C, and 50 s at 72°C. PCR conditions for exon 2 of the p16 gene were 35 cycles of 50 s at 94°C, 50 s at 64°C, and 50 s at 72°C. The PCR products were separated by electrophoresis on 6% polyacrylamide gel with 5% glycerol and without glycerol. Moreover, to better assure detection of mutations by PCR-SSCP, we used two cases of lung carcinomas with p16 mutations as positive controls.

**Sequencing.** PCR products selected for sequencing were ligated with the pCRIIA-cloning vector (Inviogen) and transformed into competent DH5α cells (Life Technologies, Inc.). Both strands were sequenced for each PCR product from at least three independent clones.

**Definition of LOH.** The signal intensity of the polymorphic alleles was quantified and calculated by the scanning densitometer and data analysis system (The Discovery Series; Quantity One, pdi, NY). LOH was considered to be present if reduction rates of signal intensities in tumors were more than 40%, as described previously (23). We could score allelic imbalance, such as trisomy, besides LOH by this definition. Therefore, it is possible that certain chromosome loci scoring LOH are trisomic rather than monosomic.

**Statistical Analyses.** Significance of the differences in various biological and clinical features of the disease among the patient group was examined by Fisher’s exact test. The vital status of the patients was observed until December 31, 1995. The survival curves for each group of the patients were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test for univariate analysis. Multivariate analysis was performed using the Cox proportional hazards model.

**Western Blot Analysis.** Fifty μg of protein were separated in a 4–20% gradient SDS/polyacrylamide gel and electrophobotted to Hybond-Enhanced Chemiluminescence (ECL) nitrocellulose membrane (Amersham Corp., Arlington Heights, IL). After being blocked with 5% nonfat dry milk and 0.1% Tween 20 in Tris-buffered saline, membranes were incubated at 37°C for 2 h with the 1:400 dilution of a rabbit polyclonal anti-p16 antibody (PharMingen), the epitope of which is unknown, and a rabbit polyclonal anti-p16 antibody for amino acids 137–156 at the COOH terminus of the p16 protein (Santa Cruz Biotechnology). The blot was subsequently probed by the ECL Western blotting detection system (Amersham Corp.). Equal loading of protein was confirmed by staining the membrane after detection.

**RESULTS**

**Frequency and Common Regions of LOH on Chromosome 9 in NB.** Eighty cases of NB were examined for LOH on chromosome 9 using 11 microsatellite polymorphic markers and a RFLP DNA marker. The incidence of LOH at each locus is summarized in Fig. 1. All cases showed heterozygous genotypes in their normal tissue at one or more loci on chromosome 9, and LOH at one or more loci was detected in 33 of 80 cases (41%). LOH on chromosome 9p was detected in 24 of 80 cases (30%), whereas LOH on chromosome 9q was detected in 18 of 80 cases (23%). Five of 33 cases (15%) showed LOH at all informative loci, whereas the other 28 tumors showed partial deletions of chromosome 9 (Fig. 1). Case 23 showed LOH at
CHROMOSOME 9 DELETIONS AND p16 IN NEUROBLASTOMA

the D9S162, IFNB1, and IFNA loci, but heterozygosity was retained at the D9S171 locus (Fig. 2). In case 60, LOH was detected at the D9S171 locus, but heterozygosity was retained at all loci distal to the IFNB1 locus (Fig. 2). Cases 33, 75, and 76 showed LOH at D9S158, but heterozygosity was retained at all informative loci proximal to the D9S176 locus (Fig. 2). The result from these five patients implicates the presence of two commonly deleted regions that are mapped between the IFNB1 and D9S171 loci at chromosome 9p21 and distal to the D9S176 locus at chromosome 9q34–qter (Fig. 1). The size of genetic distance of a commonly deleted region on chromosome 9p21 is 5 cM and that on 9q34–qter is more than 34 cM.

Relationship between LOH on Chromosome 9 and Clinicopathological Findings of NB. Because age, stage, and N-myc amplification are known to be associated with prognosis of patients with NB, the relationship between LOH on chromosome 9 and these clinicopathological findings was examined. There were 56 patients with stage I+II+IVS, 8 patients with stage III, and 16 patients with stage IV. N-myc amplification was detected in 10 of 80 cases. Because 62 of 80 (78%) were found by a mass screening program, the ratio of infantile and early-stage patients in this study was higher than that in previous studies (5, 10). However, age, stage, and genotype of N-myc were also significantly associated with short survival of the patients in our 80 cases (P < 0.001). Therefore, clinical outcome of patients in this study was similar to that of patients in previous studies (5, 10). All patients were followed-up for at least 36 months and up to 82 months. The mean follow-up periods of patients with LOH on chromosomes 9, 9p, and 9q were 44, 46, and 47 months, respectively. The medium ranges of survival of the patients with LOH on chromosomes 9, 9p, and 9q were 50, 42, and 57 months, respectively. LOH on chromosome 9p was significantly associated with the stage of the disease (P = 0.029) and the survival of the patients (P = 0.023). However, neither LOH on chromosome 9q nor LOH on chromosome 9 showed association with any of these parameters (Table 1). Ten of 24 patients with 9p LOH died within 24 months, whereas only 9 of 56 patients without 9p LOH died within this period (Fig. 3). However, significant association was not observed between 9q LOH and survival of the patients (P = 0.551; Table 2). Accordingly, LOH on chromosome 9 was not significantly associated with survival (P = 0.387; Table 2).

There was no correlation between 9p LOH and other clinicopathological findings, such as age, course of diagnosis (found by mass screening or clinical symptoms), histological types, and genotype of N-myc and 1p LOH. When multivariate analysis of survival was performed by age (under 1 year versus over 1 year) and genotype of N-myc amplification (present or absent) as covariates, no significant

Table 1 Correlation of LOH on chromosome 9 with biological and clinical variables in neuroblastoma

<table>
<thead>
<tr>
<th>LOH</th>
<th>Age &lt;1 yr</th>
<th>Age ≥1 yr</th>
<th>Stage I, II, IVS</th>
<th>Stage III, IV</th>
<th>Result of screening</th>
<th>N-myc amplification</th>
<th>Histological type</th>
<th>Survival</th>
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<tbody>
<tr>
<td></td>
<td>29/74</td>
<td>4/6 (0.293)*</td>
<td>20/55</td>
<td>10/16 (0.185)</td>
<td>5/10</td>
<td>28/70 (0.349)</td>
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<td>2/3</td>
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<td></td>
<td>22/74</td>
<td>6/2 (0.339)</td>
<td>15/55</td>
<td>9/16 (0.029)</td>
<td>6/18 (0.807)</td>
<td>20/70 (0.580)</td>
<td>Composite</td>
<td>3/4</td>
</tr>
<tr>
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<td>1/35</td>
<td>1/35</td>
<td>4/10</td>
<td>1/10</td>
<td>Poorly differentiated</td>
<td>3/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/35</td>
<td>1/35</td>
<td>2/10</td>
<td>2/27</td>
<td>NB Rosette fibrillary</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/35</td>
<td>1/35</td>
<td>1/10</td>
<td>2/27</td>
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<td>2/27</td>
<td>0.387</td>
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<td></td>
<td></td>
<td></td>
<td>1/35</td>
<td>1/35</td>
<td>1/10</td>
<td>2/27</td>
<td>0.05</td>
<td>0.551</td>
</tr>
</tbody>
</table>

* Fisher’s exact test.
*P-value, log-rank test.

![Diagram](image-url)
Table 2 Status of the p16 gene in 19 cases of NB cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DNA</th>
<th>mRNA</th>
<th>Protein</th>
<th>Methylation</th>
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</thead>
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<td>TGW</td>
<td>WT</td>
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<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IMR-32</td>
<td>WT</td>
<td>−</td>
<td>+</td>
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<tr>
<td>LAN1</td>
<td>WT</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
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<td>LAN5</td>
<td>WT</td>
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</tr>
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<td>NB9</td>
<td>WT</td>
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<td>+</td>
</tr>
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<tr>
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<td>+</td>
</tr>
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<td>CHP-134</td>
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<td>TNB1</td>
<td>WT</td>
<td>+</td>
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<td>−</td>
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<tr>
<td>GOTO</td>
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<td>−</td>
<td>−</td>
</tr>
<tr>
<td>KP-N-NSW</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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</tbody>
</table>

*p WT, wild type.

Methylation of the p16 Gene in NB Cell Lines. We next examined whether the p16 gene was transcriptionally silenced in NB cell lines by methylation of a CpG island in the p16 gene. DNA was digested with EcoRI and SmaI and analyzed by Southern blot hybridization. Unmethylated fragments of 650 and 350 bp were not detected in five cell lines that did not express p16 mRNA, indicating that the critical SmaI site within exon 1 of all alleles was methylated in these cell lines. However, this site was not methylated in eight other cell lines in which p16 mRNA was not detected. The remaining six cell lines that express p16 mRNA showed unmethylated 650- and 350-bp bands. There were no cell lines in which p16 mRNA was expressed in spite of having the methylated SmaI site in exon 1.

DISCUSSION

We present here the deletion map of chromosome 9 and alterations of the p16 gene in NB. In this study, we detected two commonly deleted regions: one was between the IFNB1 and D9S171 loci at chromosome 9p21 and the other was distal to the D9S176 locus at chromosome 9q34–qter. Recently, it has been demonstrated that both short and long arms of chromosome 9 are frequently deleted in many types of human cancers. Loss of the short arm of chromosome 9, in

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particular 9p21–22, occurs in a variety of human cancers, including melanoma (24), renal cell carcinoma (25), lung cancer (26), bladder cancer (27), head and neck cancer (28), and ovarian cancer (29). Chromosome 9q, in particular 9q34–qter, is also frequently deleted in several human cancers (27, 29–31). Interestingly, both of the short and long arms are deleted in some human cancers, such as bladder cancer, ovarian cancer, esophageal carcinoma, and renal cell carcinoma (27, 29–31). Therefore, as in the case of several other types of cancers, at least two tumor suppressor genes on both short and long arms of chromosome 9 may contribute to genesis and progression of NB. Furthermore, we found that 9p LOH significantly correlates with an advanced stage of the disease and poor prognosis of the patient, and 9q LOH did not correlate with these clinical parameters. In the present study, 9p LOH was significantly associated with poor prognosis independently of N-myc amplification. Thus, it is possible that a tumor suppressor gene located on chromosome 9p21 plays an important role in the progression of NB through a different pathway from N-myc amplification. Although the age of children at diagnosis is also a factor to predict the outcome of patients, 9p LOH was not correlated with the age of patients. This might be due to the small number of patients over 1 year in this study. Therefore, further studies with a large population of children over 1 year may lead to conclusive data for the correlation between age of patients and 9p LOH. Although 1p LOH is also considered to correlate with poor survival, we found no association between 1p LOH and 9p LOH. Because we used only two markers at 1p32 for detection of 1p LOH (12), it could influence the statistical analysis.

The p16 gene, a candidate tumor suppressor gene involved in many types of human cancers (13–15), have been mapped to chromosome 9p21 between the IFNBI and D9S171 loci, which is one of the commonly deleted regions on chromosome 9 in NB. However, no homozygous deletions have been reported in NB (32). It is also reported that there were no p16 gene mutations and no LOH at the IFNBI locus close to the p16 gene locus (32). In this study, we found no homozygous deletions in both primary tumors and cell lines, and a missense mutation was detected only in a primary tumor. Because this type of mutation has not been reported previously, we do not know if it is functionally significant. These data suggest that the p16 gene is not a target tumor suppressor gene inactivated in NB. However, it is possible that the p16 gene is inactivated by alternative mechanisms in most tumors, such as intronic deletions and mutations not detected by sequence analysis of exons or Southern blot analysis. Moreover, recent evidence indicated that transcriptional repression by DNA methylation of promoter and 5′ regulatory sequences may be a pathway for inactivation of the p16 gene in several types of human cancers (16–17). To clarify whether the p16 gene is inactivated in NB, we examined the status of the p16 gene using Southern, Northern, and Western blot analyses. The p16 gene was not expressed in most NB cell lines. Absence of the p16 mRNA in the samples lacking the p16 protein suggests that p16 expression is likely to be regulated at the transcriptional level. Moreover, we found that hypermethylation of the 5′ CpG island in the p16 gene is frequent in cell lines lacking p16 expression. Thus, it is likely that the p16 gene is inactivated mostly by 5′ CpG island methylation rather than DNA alterations in NB. Similar results were also reported in several other types of human cancers (16–17). However, the mechanisms for the absence of the p16 mRNA in the remaining cell lines is not clear. We cannot rule out the possibility that mutation harbors in the promoter region of the p16 gene with the consequence of gene inactivation. Recent studies indicated that expression of not only the p16 gene but also the p15 gene is suppressed by homozygous deletion, point mutation, and hypermethylation of the 5′ CpG island of this gene in several human cancers (33, 34). Particularly, in leukemia, the methylation of the 5′ CpG island in the first exon of the p15 gene is more frequent than that in the p16 gene (17). Therefore, to determine the pathways to inactivate these genes and whether the p15 and/or p16 genes are involved in the progression of NB, more detailed analysis of these genes will be necessary.

In conclusion, it was demonstrated here that at least two tumor suppressor genes on chromosome 9 are involved in the genesis and/or progression of NB. Particularly, the gene on chromosome 9p is likely to be associated with progression of NB, and the p16 gene is a candidate target tumor suppressor gene involved in the progression of NB. However, because we have not examined p16 methylation in primary tumors, the biological significance of p16 inactivation in the genesis and progression of NB is still unclear. For this reason, we are currently investigating the association of p16 expression and methylation in tumors with prognosis of patients with NB.
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