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# Neuroblastoma in Two Siblings Supports the Role of 1p36 Deletion in Tumor Development

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ABSTRACT: *Familial neuroblastoma occurs rarely. We studied a family with three children; one of them has a disseminated (stage 4) and another has a localized (stage 2) neuroblastoma. We observed subtelomeric locus D1Z2 (1p36) deletion in both tumors by using double-color fluorescence in situ hybridization. The* **MYNC** *gene was found in single copy in both tumors. Loss of heterozygosity (LOH) and restriction fragment length polymorphism analyses were performed by using* **DNA** *from frozen tumor cells and from microdissected tumor areas excised from paraffin-embedded sections. We detected somatic LOH at locus D1S468 (1p36) in a tumor-cell population with a trisomy 1 of the stage-2 patient. Neuroblastoma cells of the stage-4 patient were diploid and showed allelic loss at the following loci: D1S172, D1S80, D1S94, D1S243, D1S468, D1S214, D1S241, and D1S164. Haplotype study showed that the siblings inherited the same paternal 1p36→pter chromosome region by homologous recombination and that. in the two tumors, arm 1p of different chromosomes of maternal origin was damaged. Our results suggest that the siblings inherited the predisposition to neuroblastoma associated with paternal 1p36 region and that tumors developed as a consequence of somatic loss of the maternal 1p36 allele.* © *Elsevier Science Inc.,* ***1999.*** *All rights reserved.*

## INTRODUCTION

Neuroblastoma (NB), a neural crest-derived neoplasia, usually occurs as a sporadic tumor, and families with recurrent neuroblastoma are rare. In 1945, Dodge and Benner [1] reported a neuroblastoma in siblings and, after that, other observations on familial neuroblastoma were described (for review, see [2–4]).

Tumor cells *from* patients with disseminated sporadic neuroblastoma very often show a nonrandom deletion of the chromosome arm 1p [5]. Data from loss of heterozygosity (LOH) studies indicate that a putative NB suppressor gene should be located at the 1p36 region [6–8]. More recently, Kaghad et al. [9] identified the p73 gene as a **strong** candidate for the NB suppressor gene. On the other **hand,** Maris et al. [10] excluded an NB predisposition gene **being** located at 1p36 by linkage analysis in familial neuroblastoma. Although several cytogenetic and molecular studies on sporadic neuroblastoma have been carried out, **there** are very few data on familial cases. The study of 1p deletion in familial neuroblastoma can help to better understand the role of this chromosomal abnormality and to identify the gene(s) involved in the tumor development.

In the present paper, we report the cytogenetic and **molecular** studies of chromosome arm 1p in a family with **recurrent** neuroblastoma.

## PATIENTS AND METHODS

A 5-year-old boy (II-1) was admitted to the G. Gaslini Children's Hospital with a month-long history of fever, abdominal pain, and anemia. A large solid abdominal mass **was** histologically diagnosed as an unfavorable neuroblastoma [11]. The patient was staged as **4** [12] **and.** after a high-dose chemotherapy cycle, died from toxicity. Three months later. his 3-year-old sister (II-2) was admitted to **the** Children's Hospital with a diagnosis of histologically favorable neu-

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roblastoma. The postsurgical evaluation of metastasis was found to be negative. and the patient was staged as 2B. So far, after standard-dose chemotherapy. she is in complete remission. In the course of the daughter's disease, the mother became pregnant. Ultrasonographies were performed every 2 months throughout the pregnancy and 3 months after birth: the results were negative. Two vears after birth. the girl (II-3) is living without any sign of neoplastic disease. The pedegree of the familv is shown in Figure 3.

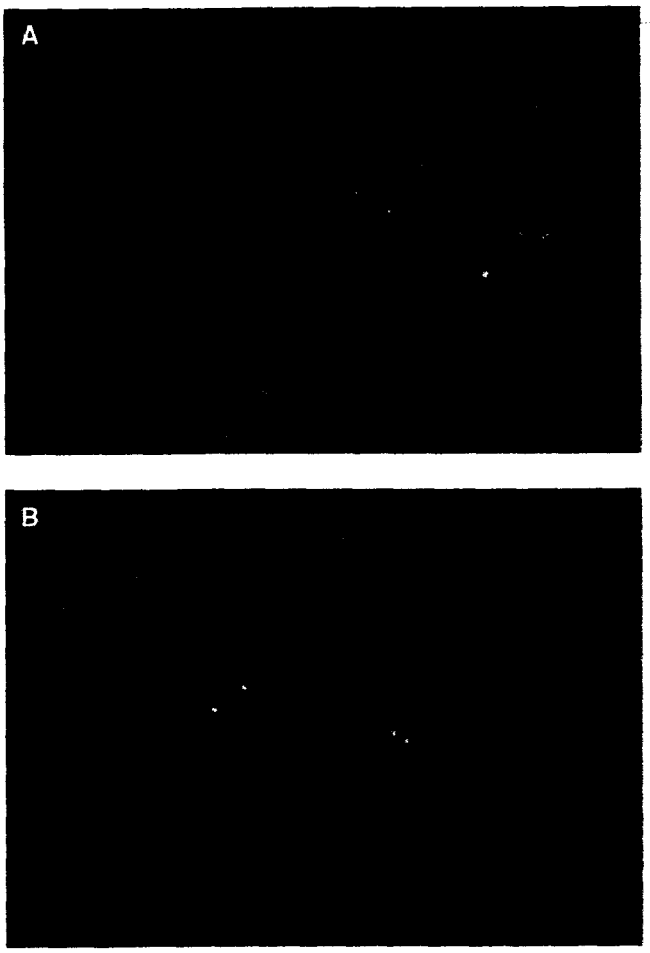
To detect the 1p36 deletion in tumors. we performed double-color fluorescence in situ hybridization (FISH) on interphase nuclei on tumor touch preparations from patients II-1 and II-2 as described elsewhere [13]. The probes that we used were the p1-79 for subtelomeric locus *D1Z2* and the chromosome 1 a-satellite for the centromeric region. Fluorescent signals were observed by an Olympus BH-2 microscope.

We also carried out an LOH study by using restriction fragment length polymorphisms (RFLPs) and mini- and microsatellite analyses for chromosome arm 1p loci. We extracted DNA from tumor tissues of both patients and from peripheral blood leukocytes of the mother. the father. and the sisters (II-2. II-3) according to the standard method [14]. A blood sample from patient II-1 was not available. High-molecular-weight DNAs (10 μg) purified from the tumors of patients II-1 and II-2 and from the leukocytes of patients II-2, II-3, the mother, and the father were digested with appropriate restriction enzymes. The resulting fragments were separated by agarose gel (0.8%) electrophoresis and blotted onto a nylon membrane filter, according to Southern blot techniques [15]. The filter was hvbridized With randomly primer [$\alpha^{32}$P]dCTP labeled probes, washed. and exposed to x-ray film at −80°C as described elsewhere [14]. RFLP analysis was done with the following restriction probes (locus) and enzymes: CEB15 (*D1S172*)/*Taq*I, p1−24 (*D1S94*)/*Sph*I, pl−31 (*D1S112*)/*Ssp*I. and perlecan (*HSPG2*)/*Bam*HI. A polymerase chain reaction (PCR) study for locus *D1S80* was carried out according to Martine et al. [16]. The resulting amplified fragments were separated by 1.2% agarose gel electrophoresis stained with ethidium bromide. To further study the extension of the chromosome arm 1p deletion. we performed PCR by using a series of microsatellites: tumor DNA of patient II-1 and tumor and constitutional DNA of patient II-2 were used as templates for PCR amplification for the following loci: *D1S243*, *D1S468*. *D1S214*. *D1S160*. *D1S244*. *D1S170*, *D1S241*, *D1S201*. *D1S164*. *D1S211*. *D1S197*. *D1S200*. and *D1S220*. The PCRs were carried out according to the conditions described by Genome Data Base (Johns Hopkins University. Baltimore. MD. USA. 1990). Finally, to stud!; constitutional DNA from patient II-1 and to determine which parental chromosome 1 of patient II-2 had been deleted, we obtained tumor and normal cells from 5-w paraffin-embedded tissue sections. Briefly. tumoral cells were ——dissected in such a way that DN.4 was prepared only from areas containing more than 90% of neoplastic cells. T[redacted]ells from the uninvolved area of the tissue provided' aormal somatic DNA. which was compared with the tu- **DNA.** Thr sections were slight!! counterstained with Harry's Haematoxlin ior 15 se: and then microdissected under light microscopy control as previously described [17]. The microdissected areas were digested overnight at 37°C in a TE (Tris-HCI 100 mM. 1 mM EDTA, pH 8.0) buffer containing 200 mg/mL of proteinase K (Boehringer. Germany). DNA (1/10 of the final volume) was used as template for the PCR reaction.

## RESULTS AND DISCUSSION

Double-target FISH showed that the tumor from patient II-1 was composed of disomic cells for chromosome 1 that displayed loss of a green telomeric signal and retained two centromeric red signals (Fig. 1A). The neuroblastoma of the sister (II-2) shows two cell populations—one disomic and another trisomic for chromosome 1: the latter accounted for about 30%. *Loss* of a green signal was observed only in the nuclei with trisomy 1 (Fig. 1B).

**Figure 1** The figure is representative of double-color FISH analysis performed on interphase nuclei of tumor II-1 (A) and tumor II-2 (B) by using ar α-satellite probe (red' specific for the centromere of chromosome 1 and a probe for subtelomeric 1p36 locus *D1Z2* (green). In (A) two red signals for locus QC and only one green signal for locus *D1Z2* at each nucleus are visible In (B) the nuclei show three red and two green signals



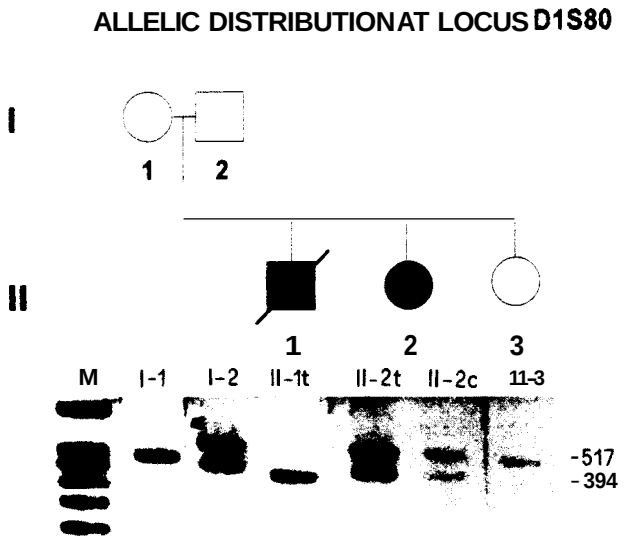



Figure 2 PCR for locus *D1S80*. Two PCR products are generated: one at 394 bp and the other at 517 bp. The mother (I-1) is homozygous for the 517-bp allele, whereas the father (I-2) is heterozygous. The tumor of patient (II-1t) shows only the paternal allele at 394 bp; the constitutional DNA of the patient was not available. The tumor (II-2t) and the constitutional (II-2c) DNA of patient II-2 retains the two alleles, and no LOH was observed. The constitutional DNA (II-3) of the youngest sister was homozygous for the 517-bp allele.

Nonrandom chromosome 1p36 deletion has often been detected in metastatic sporadic neuroblastoma (stage 4), as it was in the tumor of the patient II-1, rarely in localized tumors (stages 1 and 2), and it has been associated with a poor outcome of patients [18]. In our experience, at stage 2, whose tumor shows chromosome arm 1p damage, have a high chance of relapsing (of 28 stage-2 NBs, two out of three have 1p36 deletion and relapsed, personal data). This fact suggests that the 1p36 deletion characterizes aggressive behavior of neuroblastoma cells in localized tumors, too. In this regard, it is notable that patient II-2 had a stage-2 NB and showed a tumor-cell clone with a chromosome 1p36 deletion. PCR for locus *D1S80* shows two products, a 394-bp and a 517-bp band (Fig. 2). The mother was homozygous for the 517-bp allele, and the father was heterozygous (394 and 517). The presence of the 394-bp allele in the tumor cells of patient II-1 indicates that the allele deleted was the 517-bp allele of maternal origin. In the tumor of II-2, minisatellite analysis failed to show LOH. LOH for locus *D1S172* was observed in the tumor of patient II-1, whereas the loci *D1S94*, *HSPG2*, and *D1S112* results were uninformative. Heterozygosity for *D1S94*, *HSPG2* and *D1S112* was retained in the neuroblastoma cells of patient II-2 (Table 1). Because the peripheral blood of patient II-1 was not available, loci *D1S160*, D1S244, *D1S170*, *D1S197*, and *D1S220* were uninformative. In contrast, allele segregation in the kindred allowed us to easily assign LOH for *D1S468*, *D1S214*, *D1S241*, and *D1S164* at the maternal Chromosome 1 [Table 1]. The microsatellite study of tumor paraffin-embedded tissue confirmed that the maternal chromosome A was damaged in patient II-1. A comparison be-

Table 1 LOH for chromosome arm 1p in the siblings

| Locus[a] | Map location | Patient II-1 A | Patient II-1 C | Patient II-2 B | Patient II-2 $D_{rec}C$ |
|---|---|---|---|---|---|
| D1S172 | 1p36.3 | ■ | 1 | 3 | 1 |
| D1S80 | 1p36.3 | ■ | 2 | 1 | 2 |
| D1S94 | 1p36.33 | □ | 1 | 1 | 2 |
| D1S243[b] | 1p36.33 | ■ | 1 | □ | 2 |
| D1S468[b] | 1p36.3 | ■ | 3 | ■ | 3 |
| D1S214[b] | 1p36.22–21 | ■ | 4 | 2 | 3 |
| D1S160 | 1p36.2 | □ | 1 | 2 | 3 |
| D1S244 | 1p36.11–12 | □ | 1 | 1 | 2 |
| D1S170 | 1p36.11–12 | □ | 1 | 1 | 1 |
| HSPG2 | 1p36.1p35 | □ | 1 | 1 | 2 |
| D1S112 | 1p36.11 | □ | 1 | 1 | 1 |
| D1S241 | 1p35 | ■ | 3 | 1 | 2 |
| D1S201 | 1p35 | — | — | 3 | 1 |
| D1S164 | 1p35 | ■ | 2 | 3 | 2 |
| D1S211 | 1p32.3 | 3 | 1 | 2 | 1 |
| D1S197 | 1p32.2 | □ | 1 | 1 | 1 |
| D1S200 | 1p32.2–3 | 1 | 2 | 1 | 2 |
| D1S220 | 1p31–p32 | □ | 1 | 1 | 1 |

[a] Alignment of loci for chromosome arm 1p according to Genome Data Base (Johns Hopkins University, Baltimore, MD, USA. 1990).
Symbols: ■, deleted; □, not informative; —, not done.
[b] LOH was studied by using DNA from microssected paraffin-embedded tissue.

Table 2 Chromosome 1 genotype of mother, father, and daughters II-2 and II-3

| Locus[a] | Map location | I-1 A | I-1 B | I-2 C | I-2 D | II-2 B | II-2 $D_{rec}C$ | II-3 $B_{rec}A$ | II-3 $C_{rec}D$ |
|---|---|---|---|---|---|---|---|---|---|
| D1S172 | 1p36.3 | 2 | 3 | 1 | 1 | 3 | 1 | 2 | 1 |
| D1S80 | 1p36.3 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 1 |
| D1S94 | 1p36.33 | 2 | 1 | 1 | 2 | 1 | 2 | ND | ND |
| D1S243 | 1p36.33 | 1 | 2 | 1 | 2 | 2 | 2 | 1 | 2 |
| D1S468 | 1p36.3 | 2 | 1 | 3 | 3 | 1 | 3 | 2 | 3 |
| D1S214 | 1p36.22–21 | 1 | 2 | 4 | 3 | 2 | 3 | 1 | 3 |
| D1S160 | 1p36.2 | 1 | 2 | 1 | 3 | 2 | 3 | 1 | 3 |
| D1S244 | 1p36.11–12 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 2 |
| D1S170 | 1p36.11–12 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| HSPG2 | 1p36.1p35 | ND | ND | 1 | 2 | 1 | 2 | ND | ND |
| D1S112 | 1p36.11 | ND | ND | 1 | 1 | 1 | 1 | ND | ND |
| D1S241 | 1p35 | 2 | 1 | 3 | 2 | 1 | 2 | ND | ND |
| D1S201 | 1p35 | 1 | 3 | 2 | 1 | 3 | 1 | 1 | 2 |
| D1S164 | 1p35 | 1 | 3 | 2 | 2 | 3 | 2 | 1 | 2 |
| D1S211 | 1p32.3 | 3 | 2 | 1 | 1 | 2 | 1 | 2 | 1 |
| D1S197 | 1p32.2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| D1S200 | 1p32.2–3 | 1 | 1 | 2 | 2 | 1 | 2 | 1 | 2 |
| D1S220 | 1p31–p32 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| CRP | 1q21–q23 | 2 | 2 | 1 | 1 | 2 | 1 | ND | ND |
| APO-A2 | 1q21–q23 | 3 | 2 | 3 | 1 | 2 | 1 | 2 | 3 |
| D1S104 | 1q21–q23 | 1 | 2 | 1 | 2 | 2 | 2 | 2 | 1 |
| F13B | 1q31–q32.1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 |
| D1S306 | 1q32.1 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 1 |
| D1S249 | 1q32.1 | 1 | 1 | 3 | 2 | 1 | 2 | 1 | 3 |

Abbreviation: ND, not done.
[a] Alignment of loci for chromosome 1 according to Genome Data Base (Johns Hopkins University, Baltimore, MD, USA, 1990)

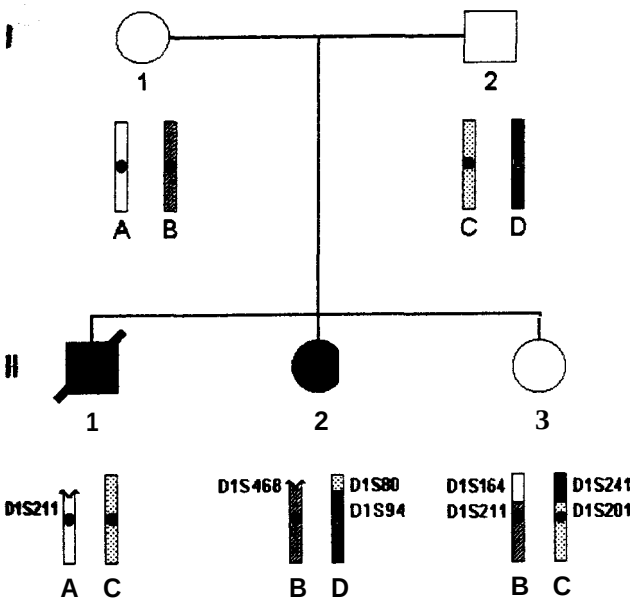



**Figure 3** Schematic representation of genotype distribution. chromosomal recombination in the family members. and chromosome 1p deletion in tumor-cell patients. The genotype of patient II-1 was studied by using tumor DNA. For chromosomes C. B, and D, the loci upstream and downstream of the crossing-over are reported. Loci *D1S211* and *D1S468*, downstream at the deletion breakpoints of chromosomes A and B. are respectively reported.

tween normal and tumor **DNA** of patient 11-2 showed LOH for locus *D1S468* of maternal chromosome B (Table 1).

*NMYC* gene amplification was evaluated by the dot blot technique [14] and extra copies of the *NMYC* gene were not detected either in the tumor of patient II-1 or that of II-2 (data not shown).

The genotypes of individuals I-1, I-2, II-2, and 11-3 were studied by using the microsatellites already reported and the following ones for the 1q region: *CRP. APO-AZ. D1S104*, *F13B*, *D1S306*, and *D1S249*. Chromosome segregation and recombination observed in **the** family are shown in Table 2 and represented in Figure 3. The genotype of patient II-1 was studied by using the tumor **DNA;** it showed that the patient inherited the maternal chromosome **A** and the paternal C (not shown in Table 2). The maternal chromosome **B** was transmitted to patient II-2. and the paternal chromosome presents a crossing-over between loci *D1*S80 and *D1S94*. As a result, patients II-1 and II-2 inherited the same paternal distal region of chromosome C The healthy sister (II-3) has the recombinant maternal chromosome A/B and the recombinant paternal chromosome C/D (Fig. 3).

Because no *NMYC* amplification was detected in either II-1 or II-2 tumors, extra copies of the gene appeared not to be required for the tumor development that should depend, bona fida. on chromosome arm 1p deletion. However, Weiss et al. [19] provided evidence that the *NMYC* gene contributes to the genesis of neuroblastoma in a mouse model. In sporadic tumors. we found that *NMYC* amplification and 1p LOH are significantly associated [20]. A majority of tumors with *NMYC* gene amplification have chromosome arm 1p deletion, too, but not the contrary. Data from sporadic tumors again suggest that 1p36 deletion is probably involved in neuroblastoma development than is *NMYC* gene amplification [20].

Knudson and Meadows [21] proposed the two-hit model for neuroblastoma, and the 1p36 damage detectable in the two patients is consistent with this hypothesis. The frequent 1p36 deletion observed in neuroblastoma, as in this family, indeed suggests the presence of a tumor-suppressor gene. We could assume that both siblings, II-1 and II-2, inherited the predisposition to neuroblastoma associated with paternal telomeric chromosome C and that tumors developed as a consequence of the somatic loss of maternal 1p36 allele(s). This appears in contrast with data reported by Maris et al. [10] and the possibility that other genes [22–24], located at different chromosomes. participate in neuroblastoma development cannot be excluded. The discrepancy between our data and that of Maris could be due to diverse reasons. One is that Maris et al. [10] included in their study neuroblastoma, ganglioneuroblastoma, and ganglioneuroma, the last two rarely showing chromosomal abnormalities, whereas our study included neuroblastoma only.

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