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Original Paper

Loss of Heterozygosity for Chromosome 1p in Familial Neuroblastoma

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Loss of heterozygosity (LOH) and deletion of chromosome 1p are very often found in sporadic neuroblastoma. Nevertheless, very few data are available concerning 1p LOH in familial neuroblastoma. Families with recurrent neuroblastoma are rare and analysis of chromosome 1p in these families might give useful information for identifying the putative neuroblastoma suppressor gene. We used combined cytogenetic and molecular techniques to study 1p LOH in two neuroblastoma families. Family M has 2 out of 3 children with neuroblastoma and family C has 2 children, 1 of whom has neuroblastoma and type 1 neurofibromatosis (NF1). All patients of both families showed tumour cells with chromosome 1p deletion (1pdel), but only the patient from family C also had *MYCN* gene amplification. In all cases the deleted chromosome 1 was of maternal origin. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

FAMILIES WITH recurrent neuroblastoma occur with low frequency. Mendelian analysis of large pedigrees of families with more than two generations in which at least one subject has neuroblastoma indicates an autosomal dominant mode of inheritance [1–3]. Knudson and Strong [4] postulated that hereditary neuroblastoma is the result of a first mutation occurring in the germ cells while the second mutation occurs in the somatic cells. The same authors calculated that familial neuroblastoma accounts for approximately 25% of all cases. Conversely, the percentage of observed familial recurrent neuroblastoma ranges between 1% and 2%. Why such a great difference exists between the expected and observed cases is not clear. One simple explanation is the paucity of data on hereditary neuroblastoma. Alternatively, the peculiar behaviour of this tumour, which very often shows regression, might explain why many tumours spontaneously mature and why they are not observed in the paediatric population. This could be supported by the mass screening study indicating more tumours in neonates than in older children [5]. Furthermore, several neuroblastomas are associated with other pathologies i.e. neurofibromatosis.

Although familial neuroblastoma has been studied for modality of neuroblastoma trait transmission, very few data are available concerning the molecular defects. Conversely, sporadic neuroblastoma has been widely studied for loss of heterozygosity (LOH) of chromosome 1p, 11p and 14q, for *MYCN* oncogene amplification and other molecular defects [6]. In this paper we report the molecular study of chromosome 1p in two families: family M which shows an F1 with two out of three children having neuroblastoma and family C in which 1 of the 2 children has neuroblastoma associated with type 1 neurofibromatosis.

PATIENTS AND METHODS

Family M (Figure 1)

A 5-year-old male (II-1) was admitted to G. Gaslini Children's Hospital (GGCH) in March 1994 with a 1 month history of fever, abdominal pain and anaemia. A CT scan revealed a large solid mass measuring $9 \times 9 \times 7$ cm with internal calcifications displacing the right kidney. Bone

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marrow evaluation was normal. According to INSS (International Neuroblastoma Staging System), this patient was considered stage 4 due to the presence of distant metastasis in the bone and was enrolled in the ICGNB (Italian Cooperative Group for Neuroblastoma) protocol. After the first course of high-dose chemotherapy with the combination of cyclophosphamide, VP-16, carboplatin and thiotepa, the patient suffered a gram-positive sepsis and died of toxicity a month after diagnosis.

Three months later his sister (II-2), a 3-year-old female, was admitted to the Division of Paediatric Oncology after radical surgery of a pelvic mass performed in another hospital. Postsurgical evaluation was negative for metastasis and the patient was staged as 2B neuroblastoma. The child received six courses of standard-dose chemotherapy and is currently in complete remission 22 months after diagnosis. During the daughter's disease, the mother had a third pregnancy. At birth and every 3 months the child (II-3) has been observed, and at 1 year of age she is alive and well without signs of neoplastic disease.

Family C (Figure 2)

A $3^1/_2$-year-old male (III-2) affected by NF1, diagnosed in another hospital (presence of many cutaneous café-au-lait spots and small hypodense, poorly contrast-enhancing areas in the brain), was admitted to GGCH in June 1993 for anaemia, loss of weight and the presence of an abdominal mass. A CT scan revealed a large mass with multiple internal calcifications. The urinary cathecholamine level was elevated and the bone marrow aspirate contained neuroblasts. MIBG scintigraphy was positive in the mass and in bone. According to INSS, the patient was considered as stage 4 neuroblastoma due to the presence of liver, bone marrow and bone metastasis. The child was enrolled in the Italian therapeutic protocol NB-92. In January 1994, he was in complete remission. After 13 months, MIBG and $^{99}T_c$ scintigraphy showed a bone relapse and bone marrow completely replaced by neuroblasts. After chemotherapy, the child died of disease in November 1995. During the disease of this son, the mother had a second pregnancy. The newborn was a female; she is alive without any sign of tumour disease.

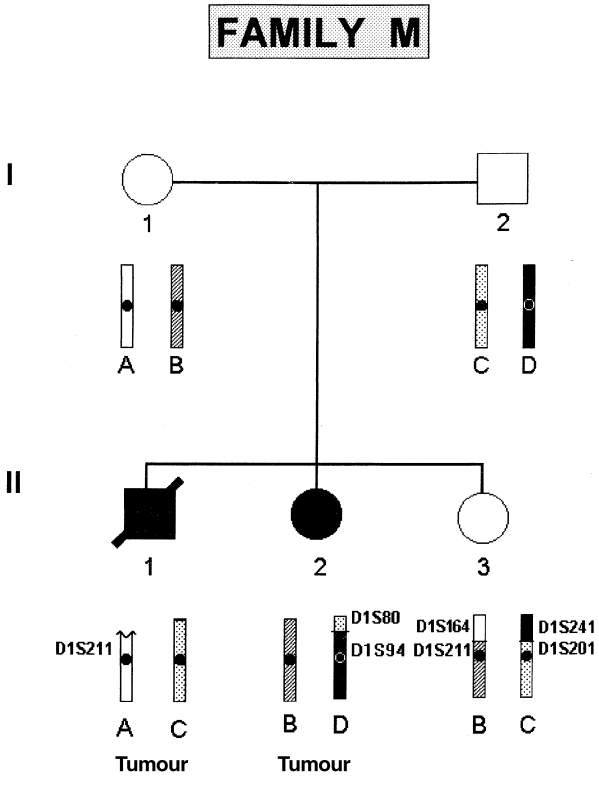

Figure 1. Pedigree of the family M. Chromosomes are shown as vertical rectangles, shading used to distinguish the 4 parental chromosome's.

Tumour and blood samples

Samples were obtained from the primary tumours surgically removed from both patients at the G. Gaslini Children's Hospital. A blood sample was also taken from each patient and from the parents.

Nucleic interphase fluorescence in situ *hybridisation*

The method for fluorescence *in situ* hybridisation (FISH) has been described in detail elsewhere [13]. The assay was performed using tumour touch preparation. Probe concentrations were 20 ng/μl for p1-79 (locus *D1Z2*) and 10 ng/μl for the chromosome 1 α-satellite probe QC. The biotin labelled probe was detected by avidin conjugated with fluorescent isothiocyanate (FITC), and the digoxygenin labelled probe was for anti-digoxygenin antibody conjugated with rhodamin. Nuclei were counterstained with 20 ng/ml DAPI and embedded in antifade solution (20 mM DABCO, 1M Tris-HCl, 90% glycerol). Fluorescent signals were observed using an Olympus BH-2 microscope and photographed with a Kodak 400 ASA film.

DNA purification and analysis

DNA was extracted from tumour tissue and blood leucocytes for each patient, according to the standard method [7]. 8 μg of DNA from each sample was digested with appropriate restriction endonuclease enzyme. The resulting fragments were separated by electrophoresis in agarose gel (0.8%) and blotted on to a nylon membrane filter, according to the standard Southern blot technique [8].

Restriction length polymorphism analysis

The filter was hybridised with random-primer [α^{32}P]dCTP labelled probes, washed and autoradiographed at −80°C as described elsewhere [9, 10]. The following combinations of restriction enzymes and probes were used for the different

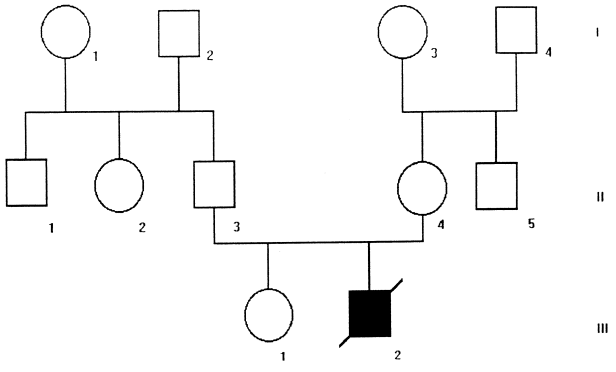

Figure 2. Pedigree of the family C and the diverse pathologies of the parents of the propositus (III-2). I-1: intestinal polyposis; I-4: dermatomyositis; II-4: café-au-lait spots; III-2 neuroblastoma and NF1.

loci (in brackets) of the distal part of the chromosome 1p: CEB15 (*D1S172*)/Taq I, 1p-24 (*D1S94*)/Sph I, 1p-31 (*D1S112*)/Ssp I, HS1 (*HSPG2*)/Bam HI.

PCR analysis of the D1S80 *locus*

Oligonucleotides (5′TGCGTGTGAATGACCCAGGAGCGTAT3′ and 5′TCTGCTTGTTTATTTTGTCTTGTTGGAG3′) derived from the sequence described by Kasai and associates [11] were used for amplification of the *D1S80* locus. PCR amplification was performed as reported elsewhere [12].

Microsatellite analysis

LOH in tumour DNAs and constitutional allelotype for chromosome 1 were investigated with polymorphic (CA)n repeat dinucleotide sequences by the PCR technique for each available DNA sample. Matched tumour and constitutional DNA were used as a template for PCR amplification with a specific set of 19 microsatellite loci. The following microsatellites were studied: *D1S243*, *D1S468*, *D1S214*, *D1S160*, *D1S244*, *D1S170*, *D1S241*, *D1S201*, *D1S164*, *D1S211*, *D1S197*, *D1S200*, *D1S220*, *CRP*, *APO-A2*, *D1S104*, *F13B*, *D1S306*, *D1S249*. The PCR conditions were according to the Genome database.

RESULTS

Combined cytogenetic and molecular analysis of family M show that chromosome 1p was deleted in the tumours of both patients, II-1 and II-2 (Figure 1). Tumour II-1 was composed of a homogeneous population of diploid cells which in FISH displayed two red signals (centromeric) and one green signal (telomeric), showing deletion of the locus *D1Z2*. Tumour II-2 displayed a mixed-cell population diploid and triploid for chromosome 1 and only the triploid cells missed one of the green signals. In fact, in these cells we observed three red signals and two green signals. LOH analysis performed with RFLP, PCR and microsatellites produced informative results only for a few loci of tumour II-1. Interpretation of the results was possible only by allelotype analysis of the mother (II-1) and father (II-2). In fact, the constitutional DNA of II-1 was not available and LOH for 1p was deduced observing the allelic distribution of I-1 and I-2.

A complete study of chromosome 1 including loci for the short and long arm allowed us to identify the allelotype of all the members of the family and understand the distribution of the alleles among the offsprings. We have assigned the chromosome A and B to the mother and C and D to the father (Figure 1). The II-1 patient inherited chromosome A and chromosome C, the sister II-2 had chromosome B from the mother and chromosome D from the father. This chromosome shows a homologous recombination with telomeric part of chromosome C at the region 1p36. The youngest sister II-3 shows a more complex chromosome homologous recombination. The *MYCN* oncogene was found present in a single copy in neuroblastoma cells of both patients.

FISH analysis performed with the tumour cells of patient (III-2) belonging to family C (Figure 2) shows deletion of locus *D1Z2* both of the primary tumour and neuroblasts infiltrating the bone marrow. Molecular study of 1p deletion confirmed extensive damage encompassing the region from 1p35 to 1pter. We had the opportunity of performing a study of 1p deletion also at the time of relapse of the patient. The child relapsed with metastasis in the bone marrow which was completely infiltrated by neuroblasts. Neuroblastoma cells again presented the 1p deletion from 1p35 to 1pter. Study of allelotype showed that deletion of chromosome 1p was of maternal origin. Finally, tumour cells displayed *MYCN* gene amplification.

DISCUSSION

Combined cytogenetic and molecular study of familial neuroblastoma might produce additional information for a better understanding of the genetic defects of this paediatric tumour. Families with recurrent neuroblastoma are rare, and sometimes it is possible to observe a neuroblastoma patient in families with other kinds of tumours present in the pedigree.

Study of chromosome 1p deletion, the most common damage occurring in sporadic neuroblastoma [5, 6], shows

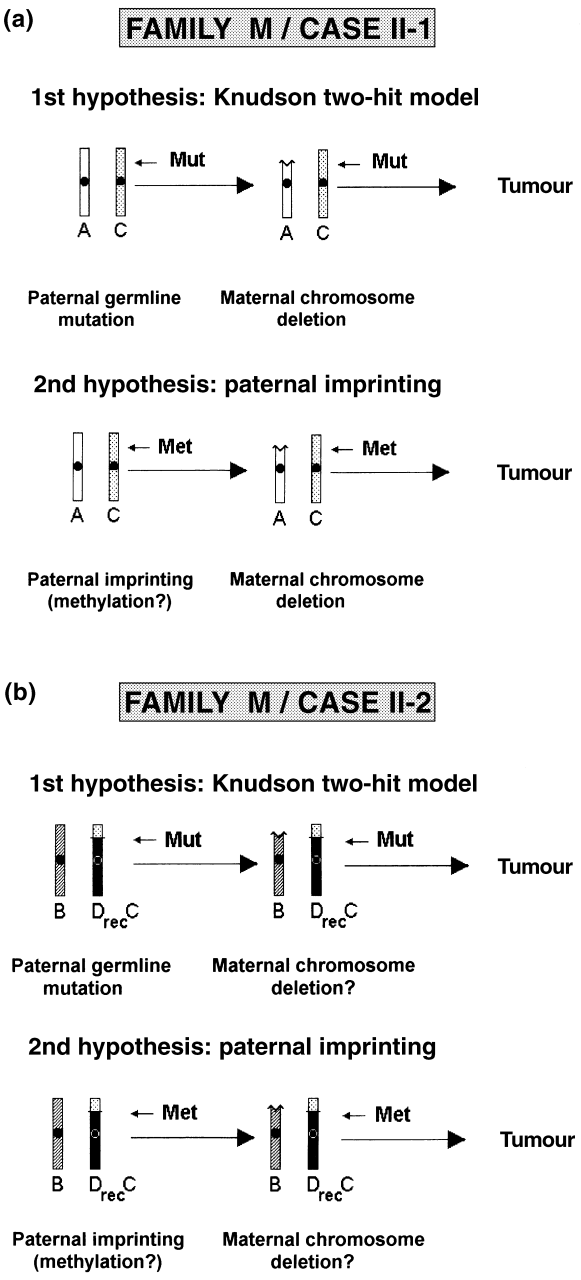

Figure 3. Alternative hypotheses proposed for tumour development of (a) patient II-1 and (b) patient II-2 of family M.

evidence of LOH for chromosome 1p in all members with neuroblastoma observed in the 2 families. In the study of the 2 families, one of the suitable tools was FISH analysis. We were able to detect 1p deletion by double-colour FISH as in tumour-touch preparation as in bone marrow cytospins. FISH analysis was essential for detecting the 1p defect of II-1 patient of family M where constitutional DNA was not available and in II-2 of the same family where a diploid and triploid cell population was present. The previous studies of familial neuroblastoma lack this information. Kushner and associates [2] reviewed more than 40 families with neuroblastoma and no data about karyotype analysis were available, making difficult any association between 1p deletion and the mode of transmission of the neuroblastoma trait. Although we observed only 2 families, all patients with neuroblastoma showed 1p deletion and the defect occurs in both families in the chromosome inherited from the mother. We did not detect any abnormality of chromosome 1p in the parents of the 2 families, suggesting that no mutation was present at 1p36 in the inherited chromosome. However, we cannot exclude the presence of a small deletion that was not detected by our molecular analysis. Very few data are available concerning the constitutional 1p defect in patients. Biegel and associates [14] described a case with 1p36 deletion observed in peripheral lymphocytes.

Taking account of the results obtained by the study of family M, we can postulate the two-hit model proposed by Knudson and Strong [4]. Figure 3(a) and (b) show that the tumour might arise in both II-1 and II-2 patients by deletion of 1p maternal chromosome associated with a mutation of the 1p paternal chromosome (first hypothesis). Alternatively, the maternal 1p deletion might be associated with inactivation (methylation?) of paternal chromosome 1p. Also in this second hypothesis the two alleles of the putative neuroblastoma suppressor gene should be inactivated.

Tumour cells of the patient belonging to family C showed a large 1p deletion as found in neuroblastoma cells of the II-1 of family M. Both children had stage 4 neuroblastoma but only the patient from family C also had *MYC*N gene amplification. It is worth noting that the chromosome 1p deletion detected in the primary tumour of the patient from family C was also observed in the metastasis and in the neuroblasts infiltrating the bone marrow at the time of relapse. This should account for the monoclonality of the tumour and might also indicate that the therapeutic treatment was unable to eliminate completely the malignant cells.

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