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Original Paper

Molecular Genetic Analysis of Familial Neuroblastoma

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Neuroblastoma has several clinical and molecular genetic parallels with the other paediatric embryonal tumours, such as retinoblastoma, including a hereditary form of the disease. We hypothesised that neuroblastoma susceptibility is due to germline mutations in a tumour suppressor gene and that this predisposition gene may be involved in sporadic neuroblastoma tumorigenesis as well. We therefore aimed to localise the familial neuroblastoma predisposition gene by linkage analysis in neuroblastoma kindreds. Eighteen families segregating for neuroblastoma were ascertained for candidate locus linkage analysis. Although many of the 49 affected individuals in these families were diagnosed as infants with multifocal primary tumours, there was marked clinical heterogeneity. We originally hypothesised that familial neuroblastoma predisposition would map to the telomeric portion of chromosome band 1p36, a genomic region likely to contain a sporadic neuroblastoma suppressor gene. However, neuroblastoma predisposition did not map to any of eight polymorphic markers spanning 1p36.2–.3 in three large kindreds. In addition, there was strong evidence against linkage to two Hirschsprung disease susceptibility genes (*RET* and *EDNRB*), a condition that can cosegregate with neuroblastoma as in one of the kindreds tested here. We conclude that the neuroblastoma susceptibility gene is distinct from the 1p36 neuroblastoma suppressor and two of the currently identified Hirschsprung disease susceptibility genes. © **1997 Elsevier Science Ltd.**

Key words: chromosomes, human, pair 1; chromosomes, human, pair 10; chromosomes, human, pair 13; chromosome mapping; genes, suppressor, tumour; Hirschsprung disease (genetics); linkage (genetics); lod score; neuroblastoma (diagnosis); neuroblastoma (genetics)
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INTRODUCTION

HEREDITARY NEUROBLASTOMA is relatively rare, with less than 5% of patients having a first-degree relative with the condition [1]. When neuroblastoma does occur within families, patients are often diagnosed at an earlier mean age and with multiple primary tumours. In addition, familial neuroblastoma segregates as an autosomal dominant trait with high penetrance. These clinical observations are consistent with the two-mutation hypothesis for cancer initiation first proposed for retinoblastoma [2]. Therefore, we predict that neuroblastoma predisposition is due to the inheritance of a mutation in one allele of a tumour suppressor gene. Furthermore, by analogy with retinoblastoma, germinal mutations in this suppressor gene may be involved in sporadic neuroblastoma initiation as well [3].

There are several regions of the genome that are plausible candidates for the location of a neuroblastoma predisposition gene (Table 1). These regions may be defined by the molecular genetics of sporadic neuroblastomas or by conditions that occasionally cosegregate with this neoplasm in families. The distal short arm of chromosome 1 is the region of the genome most likely to contain a suppressor gene critical in sporadic neuroblastoma [4, 5]. Furthermore, the identification of 2 neuroblastoma patients with constitutional re-arrangements of this region [6, 7] has implicated this suppressor locus as also being involved in susceptibility to neuroblastoma initiation. Other candidate loci, based upon

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Table 1. *Candidate neuroblastoma predisposition loci*

Loci defined by sporadic neuroblastoma genetics
1p36
11q13–23
14q32
17q
Genes defined by genetics of conditions that may cosegregate with neuroblastoma
Hirschsprung disease
RET
EDNRB
EDN3
Neurofibromatosis
NF1

cytogenetic and molecular genetic studies in sporadic neuroblastomas, include the long arms of chromosomes 11 [8], 14 [8–10] and 17 [11, 12]. Additional regions of the genome may also be involved, as suggested by genome-wide allelotyping experiments [13]. Molecular genetic analyses of familial neuroblastomas have not been performed.

Neuroblastoma is rarely associated with congenital malformations or comorbid conditions [5]. However, other disorders of neural crest-derived cells have been occasionally reported coincident with neuroblastoma. Hirschsprung disease occurring with or without congenital central hypoventilation has been described in infants with sporadic, multifocal neuroblastoma [14–17] and has twice been reported to cosegregate with neuroblastoma in families [18, 19]. Hirschsprung disease is a polygenic trait for which three susceptibility genes have been identified so far: the *RET* protoncogene at 10q11 [20], the endothelin-B receptor gene (*EDNRB*) at 13q22 [21] and more recently the ligand for *EDNRB* endothelin-3 (*EDN3*) at 20q13.2–.3 [22, 23]. Interestingly, both *RET* and *EDNRB* have also been implicated in human oncogenesis. The association of neurofibromatosis with neuroblastoma is controversial [24, 25]. However, there is one well-documented kindred segregating for both disorders along with Hirschsprung disease [18]. In addition, there is direct evidence that the *NF1* gene (17q11.2) may function as a tumour suppressor gene in myeloid [26] and neural crest [27] cells. Therefore, the observations of frequent 17q alterations in primary neuroblastomas [11] and *NF1* mutations in some neuroblastoma cell lines [28] make this gene a candidate neuroblastoma predisposition gene.

We originally hypothesised that the familial predisposition to neuroblastoma would be genetically homogeneous and map to chromosomal band 1p36. We now report on our initial experience in neuroblastoma pedigree ascertainment, characterisation and linkage analysis. Our findings that the neuroblastoma predisposition locus is distinct from the 1p36 suppressor locus and two of the Hirschsprung disease predisposition genes (even in a family segregating for both conditions) are discussed in the context of our ongoing studies aimed at isolating a familial neuroblastoma predisposition gene.

MATERIALS AND METHODS

Families and specimens

A protocol for specimen collection and molecular genetic analysis of inherited neuroblastoma has been approved by the Children's Hospital of Philadelphia Institutional Review Board and is active in both the Children's Cancer and Pediatric Oncology Groups. Families in which more than one first-degree relative had a definitive diagnosis of neuroblastoma, ganglioneuroblastoma or ganglioneuroma were ascertained and histopathologically confirmed. Blood was obtained from all available first-degree relatives consenting to participate for the establishment of Epstein–Barr virus transformed lymphoblastoid cell lines. Tumour (and/or bone marrow) specimens were obtained, when available, from all affected individuals through the referring institution or cooperative group reference laboratory. Archival paraffin-embedded tissues were used when frozen specimens were not available. DNA was extracted from all specimens by standard methods [29] and the sample made anonymous prior to genotyping.

Three kindreds informative for linkage were used for the analyses reported here. The first family has 7 affected individuals, 2 of whom also were diagnosed with long-segment Hirschsprung disease [19]. The other 2 families used for these analyses each had 3 affected patients. Pertinent clinical and pedigree information for all 3 families have been previously published [19, 29].

Genotyping

A panel of simple tandem repeat polymorphisms (STRPs) were organised at the candidate regions 1p36, 10q11 (*RET*) and 13q22 (*EDNRB*) for genotyping and LOH studies. The 1p36 STRPs included the dinucleotide repeat polymorphisms *D1S243*, *D1S468*, *D1S214*, *D1S160*, *D1S489* and *D1S507* and the tetranucleotide repeats *D1S1646* and *D1S548* [30]. The 10q11.2 STRPs at the *RET* locus included the dinucleotide repeats *D10S193*, *D10S199*, *D10S1100* (RET A) and *sTCL-2* (RET B) and the tetranucleotide repeat *D10S1217*. Two dinucleotide repeat polymorphisms (*D13S160* and *D13S170*) flank the *EDNRB* gene and were used for genotyping at this locus. All primer sequences, allele sizes and allele frequencies are available either through the Genome database or upon request.

PCR (polymerase chain reaction) amplification was performed in 20 μl volumes with 40 ng of human genomic DNA as a template. Each reaction contained 0.2 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer II and 0.2 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Branchburg, New Jersey, U.S.A.). The sense primer was end-labelled with [γ-^{33}P]dATP using T4 polynucleotide kinase (Promega, Madison, Wisconsin, U.S.A.) prior to PCR. Amplification was performed with a touchdown procedure consisting of a one cycle of denaturation at 95°C for 2 min; 15 cycles of 45 s denaturation at 95°C and an annealing/extension temperature starting at 70°C for 1 min and decreasing by 0.7°C each cycle; 25 cycles of 95°C for 45 s, 55–60°C for 30 s and 72°C for 1 min; one cycle of a 7 min final extension at 72°C. Radiolabelled PCR products were separated in an 8% polyacrylamide/7M urea sequencing gel and autoradiographed at room temperature for 2–4 h.

Linkage analysis

Maximum likelihood linkage analyses were undertaken to compute two-point lod scores for the 1p36, *RET* and *EDNRB* marker loci and the putative neuroblastoma disease locus. These analyses were undertaken using a model that assumed neuroblastoma was attributable to a single biallelic autosomal dominant gene. The population disease allele frequency was

assumed to be 0.0001. Published allele frequencies at each of the polymorphic loci (Genome database) were used for all computations. We also assumed an incomplete penetrance function relating the probability of having developed disease to inheritance of the genetic variant. This penetrance function assumed a 90% probability of manifesting disease by the age of 15 years in variant allele carriers and no chance of developing disease in non-carriers. To strengthen the argument against linkage, we computed lod scores with various combinations of these inferences (disease allele frequency estimates of 0.0001 and 0.001 and disease penetrances of 0.5 and 0.9). All likelihood computations were accomplished using the MLINK and ILINK program packages as implemented in FASTLINK v. 2.3P [31].

LOH analysis

Available corresponding tumour and constitutional DNA pairs were amplified by PCR at each marker locus and run in parallel, as described [30]. LOH was determined when there was at least a 60% decrease in the intensity of one allele in the tumour specimen compared to the constitutional DNA.

RESULTS

We identified 18 kindreds in which neuroblastoma is segregating as an autosomal dominant trait. There are 49 individuals known to be affected with a neuroblastic tumour within these families, for whom complete clinical information is currently available for 44. The largest family has 7 affected individuals, whereas 12 of the families have just 2 affected members (siblings or first cousins). Twenty (45%) patients were diagnosed as infants and 9 are known to have more than one primary tumour. Four patients were adults at the time of diagnosis (20, 30, 44 and 57 years). When these individuals are excluded, the median age at diagnosis was 12.1 months (range < 0–85 months). There is a slight female preponderance among the affected (female:male ratio 1.7:1). Of the 48 patients for whom outcome data are available, 18 died from progressive disease, 23 have no evidence of disease and 7 are currently alive with disease.

Three kindreds considered informative for linkage were chosen for these studies. All available family members were genotyped with a panel of eight STRP markers spanning 1p36. Two-point linkage analysis was performed at each marker locus. These polymorphisms are concentrated at 1p36.2–.3 (Figure 1) and were chosen because they roughly define the consensus region of the hemizygous deletion in neuroblastomas established by LOH studies [4]. The linear order of the STRPs is depicted in Figure 1 and covers 38 centimorgans (cM) of genomic DNA [32]. *D1S507* is 4 cM proximal to the region of consistent loss [4] and *D1S243* is the most telomeric STRP identified on 1p [32].

There was no evidence to support linkage at any 1p36 locus (Figures 2a and 3). Furthermore, there was strong evidence against linkage (lod < −2) at six of the eight chromosome 1p36 markers for θ values of 0 and 0.01 (Figure 3). Evidence against linkage was observed using a variety of allele frequency estimates and penetrance models. In addition, tumour specimens were available for analysis from patients 1001, 1003, 2001, 2002 and 2003 (Figure 2). Each tumour specimen was assayed for LOH at all 1p36 polymorphic loci. At least 6 of the 8 STRPs were informative for each case studied. LOH was not detected in the tumour DNA of any patient and there was no evidence for a replication error phenotype.

Linkage to neuroblastoma predisposition was also tested in these three families at two of the known Hirschsprung disease predisposition loci (Figure 2b). Lod scores of < −2 were found at several polymorphic marker loci in close proximity to both the *RET* and *EDNRB* genes (Figure 3). In addition, there was no LOH in any of the five tumour specimens at either Hirschsprung disease susceptibility locus (average observed heterozygosity for all 7 STRPs used = 0.77).

DISCUSSION

A unique feature of familial neuroblastoma is clinical heterogeneity. The 18 pedigrees we have collected are remarkable for the variability in age at onset and clinical course of the disease, even within a single family. Although there are several examples of the classic presentation of onset in infancy with multifocal disease, many familial patients were diagnosed later in childhood or even during adulthood. The median age at diagnosis of 12.1 months in the affected members reported here is slightly higher than that described previously [1], but is significantly less than the 22 months reported in unselected cases [5]. The heterogeneity of tumour stage and histology, as well as clinical course, is not unlike that observed in sporadic neuroblastomas. It is striking that within a single family one child may have a localised ganglioneuroma cured by surgery alone, whereas another child may have classic Stage 4 disease. Therefore, due to the

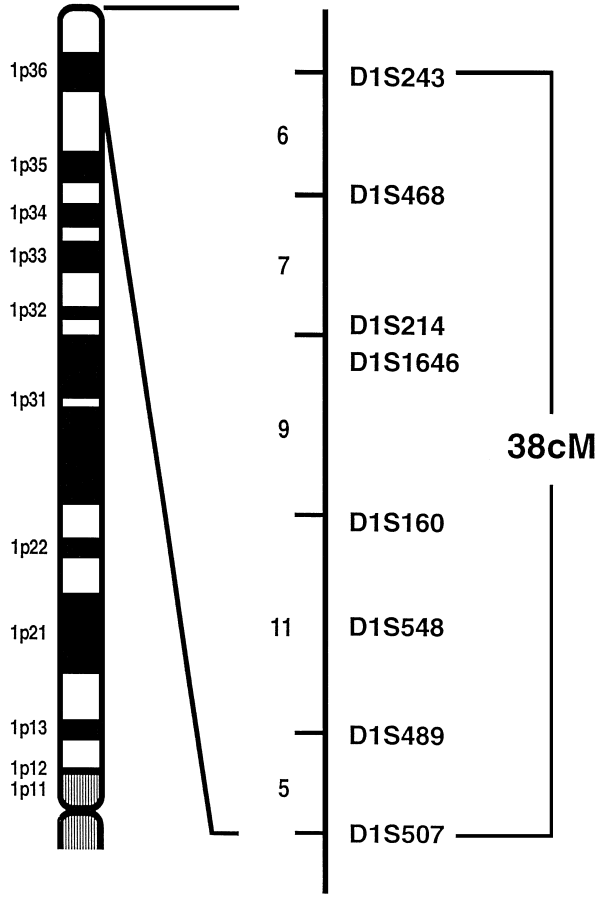

Figure 1. Polymorphic markers at 1p36.2–.3. The cytogenetic map of 1p is displayed on the left, with the polymorphic STRP markers used for this study displayed on the right. The approximate genetic distance between framework markers is indicated [32].

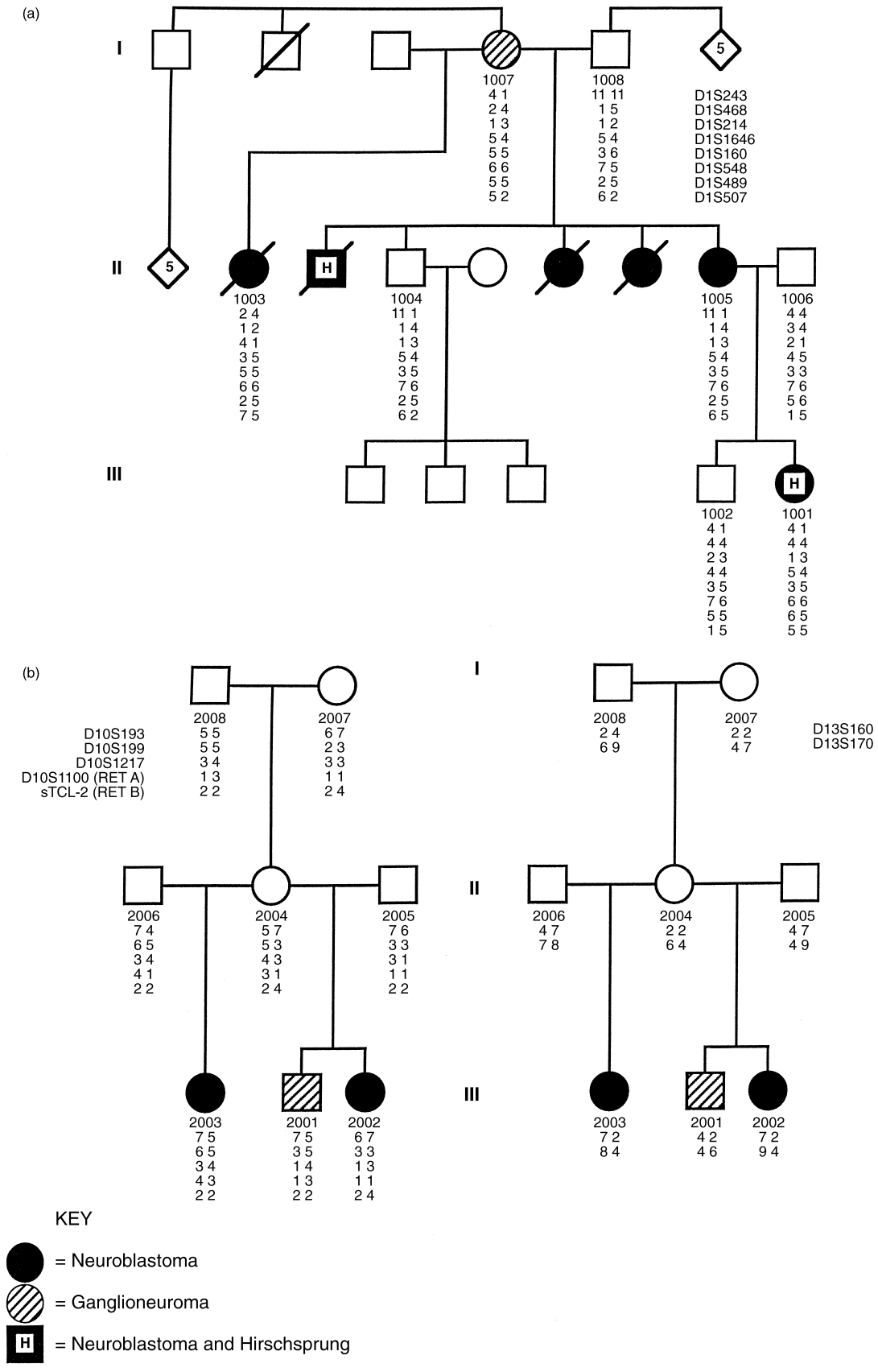

Figure 2. Linkage analysis in kindred 1 at the 1p36 polymorphic *loci* (a) and in kindred 2 at the *RET* and *EDNRB* loci (b). Genotypes arranged into inferred haplotypes are displayed beneath the family members available for study. The linear order of the polymorphic markers used for genotyping are displayed for each *locus* with the generation I haplotypes. Note the random assortment of haplotypes (affected individuals are not sharing a common haplotype) at 1p36 (a) as well as both Hirschsprung disease predisposition *loci* (b, *RET* on the left and *EDNRB* on the right).

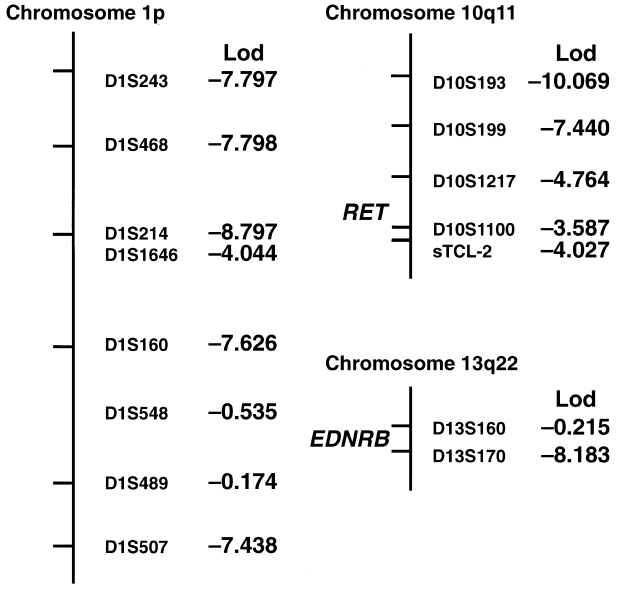

Figure 3. Combined results of two-point lod scores at θ = 0 for chromosome 1p36, 10q11 (*RET*) and 13q22 (*EDNRB*) marker loci. There is no evidence to support linkage at any of the polymorphic marker loci studied, and there is very strong evidence against linkage (lod < −2) at many of the loci.

confounding factors of underdiagnosis on the one hand (small ganglioneuromas or regressing neuroblastomas) and lethality prior to reproductive age on the other, the incidence of germline mutations of a neuroblastoma predisposition gene may be higher than currently recognised. In addition, the clinical data seems to indicate that additional genetic events determine the clinical phenotype of patients with familial neuroblastoma.

Our data strongly suggest that the neuroblastoma predisposition locus is not located within chromosome subbands 1p36.2–.3. We anticipated linkage to this locus because of strong evidence for a neuroblastoma suppressor gene in this region. Indeed, it seemed likely that the responsible gene would be identical to the sporadic neuroblastoma suppressor gene and that linkage analyses would contribute to the ongoing positional cloning efforts in this region. Furthermore, the only reports of constitutional DNA rearrangements predisposing to neuroblastoma involved 1p36, although the 1,17 translocation breakpoint maps proximal to the region studied here [33]. We chose our markers to overlap the consensus region of deletion defined by our LOH studies of sporadic primary neuroblastomas and a neuroblastoma patient with a constitutional 1p36 deletion [4, 6]. It is possible that a more proximal 1p locus is involved in neuroblastoma predisposition, but the high density and statistical power of our linkage calculations make it very unlikely that the predisposition locus maps within 1p36.2–.3, at least in the 3 families studied here.

The occasional association of neuroblastoma with other disorders of neural crest-derived cells may lend insight into molecular pathogenesis. The coincidence of Hirschsprung disease with neuroblastoma in 1 of our families led us to postulate that one of the Hirschsprung predisposition genes would be involved in familial neuroblastoma initiation. However, we again found strong evidence against linkage at both the *RET* and *EDNRB* loci. Linkage analysis is ongoing at the endothelin-3 gene (*EDN3*) locus, which has recently been reported to segregate with Hirschsprung disease and Waardenburg syndrome in 2 families [22, 23].

Our data are consistent with the increasing recognition of the complex nature of neuroblastoma molecular genetics. The 1p36.2–.3 suppressor locus is strongly correlated with a more aggressive tumour phenotype, such as age over 1 year of age, higher stage tumour with frequent *MYCN* amplification and poor survival [12, 30, 34]. Taken together with our evidence that the neuroblastoma predisposition gene does not map to this region, it appears likely that the 1p36.2–.3 suppressor is not involved in neuroblastoma initiation. Alternatively, neuroblastoma predisposition may be genetically heterogeneous and a distal 1p36 gene may be mutated in some families. We are currently extending our experiments to the remaining families at the markers studied here, as well as more proximal 1p loci.

Our strategy for the isolation of a familial neuroblastoma predisposition gene involves completing targeted linkage analyses at selected candidate loci (Table 1). Chromosome bands 14q32 and 11q13-23 have been implicated by LOH studies as harbouring tumour suppressor genes [8–10, 35]. A neuroblastoma suppressor gene may also map to the long arm of chromosome 17. Evidence for this includes the frequent occurrence of translocations involving 17q [11, 12] as well as in the germline of 1 patient who subsequently developed neuroblastoma [7]. In addition, the *NF1* gene maps to 17q11.2 [36]. Finally, gains of the telomeric region of 17q appear to be the most common genetic abnormality detected by comparative genomic hybridisation studies [37]. Linkage analysis is ongoing at each of these three loci.

Other reports within this Special Issue of the *European Journal of Cancer* suggest additional regions of the genome that may be involved in neuroblastoma tumorigenesis, each of which could be considered to contain a candidate locus for linkage analysis. However, if the neuroblastoma predisposition gene does not map to any of the regions listed in Table 1, a genome-wide search may be required. The eventual localisation of the familial neuroblastoma predisposition gene should allow for susceptibility testing of at-risk individuals in these rare families. This is especially important because of the variability in age of onset and clinical course. Furthermore, the subsequent cloning of this gene should lend insight into the initiation of tumorigenesis in both familial and sporadic neuroblastoma patients.

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