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Original Paper

Delimitation of a Critical Tumour Suppressor Region at Distal 1p in Neuroblastoma Tumours

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We analysed DNA from 68 neuroblastoma tumours for loss of heterozygosity (LOH) on the distal chromosome 1p (1p-LOH) using PCR-based DNA polymorphisms. Fifteen tumours (22%) displayed 1p-LOH. The shortest region of overlap (SRO) for the deletions was defined proximally by marker *D1S244* and distally by marker *D1S80*. The *CDC2L1* locus, located on chromosome 1p36, has been put forward as a neuroblastoma tumour suppressor. We analysed coding regions of the *CDC2L1* gene in a subset of aggressive neuroblastoma tumours with known allelic loss for different 1p-markers. Single-stranded conformation polymorphism, heteroduplex and sequencing analysis of tumour DNA did not reveal any significant changes in the coding region. Using a DNA sequence polymorphism, we showed, in a primary tumour with an interstitial allelic deletion, that this tumour had both alleles of the *CDC2L1* locus retained in the tumour. Thus, we showed that the neuroblastoma tumour suppressor critical region on 1p in our material is defined by loci *D1S244* and *D1S80* and that the *CDC2L1* locus is distal to the critical region. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, tumour suppressor gene, CDC2L1, deletion, 1p36, LOH
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INTRODUCTION

NEUROBLASTOMA IS a neural crest-derived malignancy, usually occurring during infancy and early childhood. Cytogenetic analysis of neuroblastoma tumours and cell lines have revealed several specific alterations. One of these is the presence of cytogenetic signs of gene amplification i.e. double minutes or homogeneously staining regions. Another alteration present in approximately 30% of neuroblastomas is deletions or unbalanced translocations resulting in loss of genetic material from the distal short arm of chromosome 1 (1p) [1–4]. The distal 1p region is, therefore, a candidate position for harbouring one or several neuroblastoma tumour suppressor gene(s). Several groups, including ours, are working towards the localisation, identification and characterisation of such genes involved in the genesis of neuroblastoma tumours.

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Our first aim, therefore, was to try to delimit the critical region for the distal neuroblastoma tumour suppressor gene by analysing the extent of the deletions in the tumours. We therefore performed a detailed loss of heterozygosity (LOH) study on tumours from Swedish neuroblastoma patients. We report here on our continued efforts to try to narrow the critical neuroblastoma tumour suppressor region on 1p using this technique and present the proximal and a distal border for a neuroblastoma tumour suppressor gene based on the Swedish neuroblastoma material, thereby pinpointing it to a 25 cM region within chromosome region 1p36.

The second approach, reported here, was to analyse in detail tumour suppressor candidate genes in primary tumour material, by testing for re-arrangements in coding regions using single-stranded conformation polymorphism (SSCP), heteroduplex (HD) and DNA sequencing analyses. In this study we report on our studies of the *CDC2L1* gene in primary neuroblastoma tumours. This gene was recently put forward as a candidate neuroblastoma tumour suppressor gene [5]. We report that no homozygous re-arrangements

could be detected in the *CDC2L1* gene sequences analysed, and that the *CDC2L1* locus most likely maps distal to the critical neuroblastoma tumour suppressor region determined by us and others.

MATERIALS AND METHODS

Patient material and DNA extraction

Tumour specimens and corresponding normal tissue (fibroblast-biopsy or blood sample) were obtained from 68 children with neuroblastoma of different stages. Most of the patients have been described earlier [6, 7] and the numbering is in accordance with that of the earlier reports. The children were diagnosed, staged and evaluated for clinical outcome according to the International Neuroblastoma Staging System criteria (INSS) [8, 9]. Genomic DNA was extracted from blood, fibroblasts and fresh frozen (−70°C) tissue samples using standard procedures. The tumour cell content of the samples was histologically assessed in adjacent tumour tissue to that used for DNA extraction. For analysis of the *CDC2L1* sequences, DNA from tumour and normal tissue from 9 neuroblastoma patients were used (see Table 1).

Detection of 1p-LOH using PCR-based DNA polymorphisms

The tumours were analysed using the following PCR-based polymorphisms located on the distal 1p-chromosome region: *D1S80* [10], *D1S243*, *D1S468*, *D1S214*, *D1S508*, *D1S244*, *D1S503*, *D1S228*, *D1S200* [11–13], *D1S160*, *D1S170* [14] and *D1S552* [15]. The map of markers in Figure 1 is derived from the Genethon [12] and the CHLC (Cooperative Human Linkage Center, University of Iowa, Iowa City, Iowa, U.S.A.) [15] maps. All primer sequences and polymerase chain reaction (PCR) conditions were performed according to published procedures. The products were resolved on a 6% polyacrylamide sequencing gel. After drying, the gel was exposed to an XAR-5 film (Kodak) overnight at room temperature and the patterns were compared. The different patterns obtained were: (i) loss of heterozygosity (LOH) when the patient was heterozygous and one of the alleles was missing or very weak in the tumour DNA compared to the same allele in constitutional DNA. (ii) No loss of heterozygosity (no-LOH) when the patient's constitutional DNA was heterozygous and the corresponding tumour DNA was identical to the constitutional DNA. (iii) Non-informative (n.i.) when the patient was homozygous for the polymorphism in the constitutional DNA, giving an identical pattern in the tumour DNA. Occasionally we also detected (iv) microsatellite instability [7] when the PCR pattern from tumours display fragments of sizes not present in the constitutional DNA.

SSCP, HD and DNA sequence analysis of CDC2L1 gene sequences

Exons 1–11 of the *CDC2L1* gene were PCR-amplified as described elsewhere [16]. SSCP and HD patterns were analysed simultaneously on the Phast system (Pharmacia, Sweden) using a modified-buffer system (0.2 M Tris - 0.2 M Tricine, pH 8.3). The Phast gels were run according to conditions described [16] and silver-stained (Bio-Rad) as described by the manufacturer. Solid-phase direct DNA sequencing was performed after PCR amplification with the reverse primer biotin labelled. Magnetic separation of the strands was performed using streptavidin-coated Dynabeads M280^{TM} (Dynal, Norway) according to the supplier's recommendation. The DNA sequences were determined using the AutoRead^{TM} kit (Pharmacia, Sweden) with the forward PCR primer fluorescein labelled. The reactions were analysed using the A.L.F.–DNA sequencer (Pharmacia, Sweden).

Ethical approval

The present study was approved as a multicentre study by the ethics committees of the Uppsala University, Karolinska Institute, Stockholm and the University of Gothenburg.

RESULTS

Detection of LOH and mapping of 1p-deletions

Biopsies from neuroblastoma patients ($n = 68$) were subjected to analysis with PCR-based microsatellite STRPs (short-tandem repeat polymorphisms) and one PCR-based VNTR (variable number of tandem repeats) polymorphism

Table 1. *Summary of* 1p *LOH data and CDC2L*1 *exon* 5 *SSCP*

	Patient identification number														
	52	55	68	95	106	121	155	163	169	174	184	189	299	302	316
1p analysis															
D1S80	○	●	●	●	●	–	●	●	●	●	●	–	●	–	●
D1S243	●	●	●	–	–	●	●	–	●	●	●	●	●	–	●
D1S468	–					●						●	–	●	–
D1S160	–	●	–	●	–	●	–	●	●	●	●	●	●	●	●
D1S214	●					–						–	●		–
D1S508	●					●						●	●	–	
D1S244	●	●	●	●	●	○	●	–	●	●	●	○	●	●	●
D1S503	●					–						○			
D1S228	–	●	●	–	●	–	●	●	●	●	–	–	–	–	–
D1S170	●	–	●	–	●	○	●	–	●	●	●	○	●	●	–
D1S552	●	●	–	–	●	○	–	–	–	–	–	○	●	●	–
D1S200	–	●	–	–	●	○	–	○	●	●	●	○	○	○	●
CDC2L1															
Exon 5 SSCP pattern															
Normal	A/B	A/B	B	B		A/B	A	A	A	B					
Tumour	A/B	A	B	B		B	A	A	A	B					

1p analysis, analysis of deletions using a number of PCR based polymorphisms; open circle = no 1p LOH; minus sign = uninformative; solid circle = LOH. Shaded zones indicate presumed areas of LOH in tumours.

(D1S80). 15 of the tested neuroblastomas (22%) displayed LOH for 1p loci (Table 1). Fourteen of the 15 tumours showing 1p LOH were stage 4 tumours. The only child with favourable clinical stage at diagnosis and poor outcome due to progressive disease (case 121; stage 2A) showed LOH for 1p.

Three tumours were found to carry highly informative deletions. Two of these (121 and 189) showed small distal terminal deletions. They displayed LOH for distal markers *D1S243*, *D1S160*, and *D1S508* but not for more proximal markers *D1S244*, *D1S552* and *D1S200*. Another case (52) had an interstitial deletion with both alleles retained for the most distal marker *D1S80*, but LOH for more proximal markers *D1S243*, *D1S244* and *D1S170* (Table 1).

SSCP, HD and DNA sequencing analyses of exons of the CDC2L1 gene

DNA from tumour and corresponding normal tissue of 9 neuroblastoma patients were analysed for aberrant SSCP/HD patterns. All 9 tumours had 1p deletions involving parts of the 1p arm (Table 1). In addition, 4 healthy controls were included in the study. In order to search for mutations in the entire translated region of the *CDC2L1* gene, we analysed exons 1–11 with a combined SSCP/HD technique [16]. Each exon was amplified separately, except for exons 6 and 7 which were amplified together and subsequently separated on the Phast system. For exons 1–4 and 8–11 no difference in SSCP/HD pattern could be detected. In exons 5, 6 and 7, migration differences among different DNAs could be detected (data not shown). In order to evaluate whether this was due to a simple polymorphism, DNAs of 16 additional healthy controls were analysed for these exons. This revealed that all variants found in the neuroblastoma tumour DNAs could also be detected in the DNA of different healthy controls. For both exons 5 and exons 6 and 7, three different migration patterns could be detected, one of them being a combination of the two others. This suggested that the aberrant pattern was due to a common DNA polymorphism in these exons. We designated the three exon 5 patterns as A, B and A/B, respectively (Table 1).

In order to analyse the basis of the different SSCP patterns further, we sequenced the respective DNA from control individuals and from patients displaying the different SSCP patterns. Only differences in one base position were detected. The variation coincided with the different SSCP patterns detected: individuals with pattern A were homozygous (T/T) for a thymidine base in position 75 of the coding region (M88558 in GenBank), while those with pattern B were homozygous for a cytidine (C/C) at this position. Individuals with pattern A/B were heterozygotes (C/T; Figure 2). This is a silent polymorphism since both sequence variants, TAT and TAC, give rise to a tyrosine codon. In the sequence published by Eipers and associates [17] and in that submitted to GenBank, the sequence contains a T in the position in question.

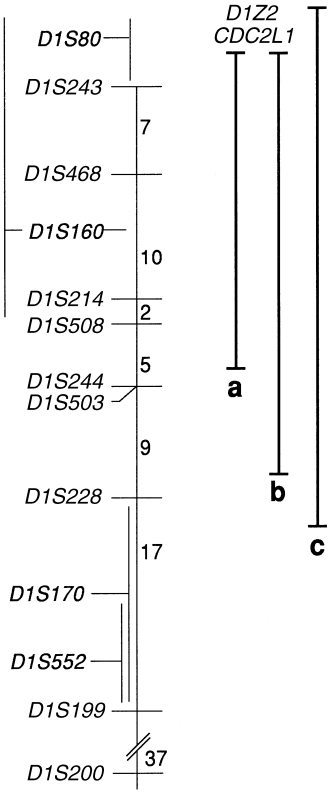

Figure 1. Delimitation of the commonly deleted region in 1p in neuroblastoma. The SRO (shortest region of overlap) of deletions based on the data presented in this study is indicated by bar (a). This SRO is compared with earlier published data of deletions in neuroblastoma tumours and cell lines from White and associates [25], bar (b), and Caron and associates [26], bar (c). The neuroblastoma tumour suppressor consensus region of the previous studies and of our present study is thus defined distally by the loci *D1Z2*, *D1S80* and *CDC2L1* and proximally by loci *D1S244* and *D1S503*.

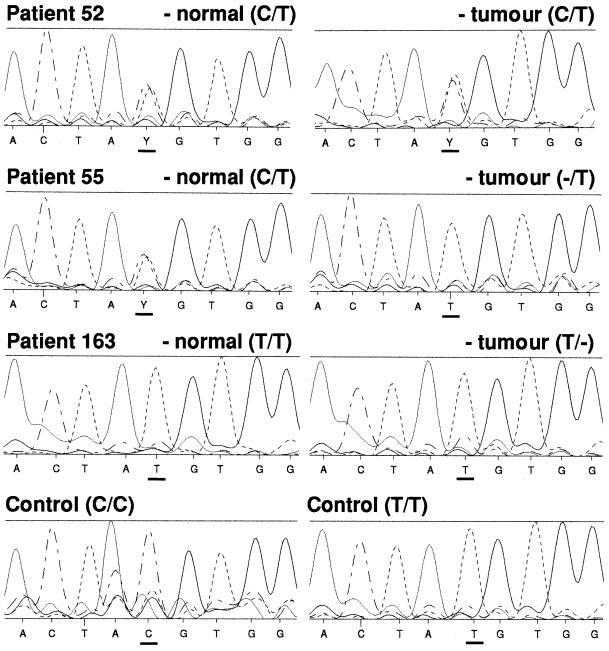

Figure 2. DNA sequence determination of DNA polymorphism in exon 5 of the *CDC2L1* gene. Patient 52 showed a heterozygous pattern (C/T) both in normal and in tumour DNA; patient 55 showed a heterozygous pattern (C/T) in the constitutional DNA and a homozygous pattern (T) due to loss of heterozygosity in the tumour; patient 163 showed a homozygous pattern (T/T) in constitutional DNA giving no information for LOH in the tumour (T/-); two healthy controls displayed a homozygous pattern (C/C or T/T). The DNA base showing polymorphism is underlined in each figure.

Analysis of LOH in exon 5 of the CDC2L1 gene

The finding of a DNA polymorphism in the coding sequence of exon 5 of the *CDC2L1* gene enabled us to analyse the neuroblastoma patients for LOH of this locus. The 1p LOH data on the patients used here are summarised in Table 1. In particular, patient 52 retained both copies of the very distal marker *D1S80*, while several of the more proximal markers showed LOH. Patient 55, in contrast, had LOH for distal markers including marker *D1S80*. We showed by combining SSCP analysis and DNA sequencing that patients 52, 55 and 121 were heterozygous for exon 5 polymorphism in their constitutional DNA (data for 52 and 55 shown in Figure 2). However, in the tumour DNA, patients 55 and 121 displayed LOH for the locus, while patient 52 had retained both alleles (Figure 2 and Table 1). The other tumours were uninformative due to homozygosity of the constitutional DNA (e.g. patient 163, Figure 2).

DISCUSSION

Alterations of the short arm of chromosome 1 (1p) represent the most frequent genetic change in human neuroblastoma tumours [18]. These alterations include deletions and/or unbalanced translocations leading to monosomy of a part of 1p. Characterisation of the region deleted in 1p have been performed first with cytogenetic methods [1, 2, 19] and later using molecular probes, i.e. analysis of LOH [3, 4, 20] and FISH techniques [21, 22]. The size of the region deleted in neuroblastoma tumours varies, but the region 1p36.2–1p36.12 has been implicated as the smallest region most commonly deleted [3, 4]. The high incidence of 1p deletions in aggressive neuroblastoma tumours has been indicative of a gene with neuroblastoma tumour suppressor activity located within the commonly deleted region. Further support for a neuroblastoma tumour suppressor gene in 1p36 are two cases where patients with neuroblastoma carried constitutional aberrations in the 1p36 region [23, 24].

This study presents some approaches our group have used to try to characterise the distal 1p region in neuroblastoma tumours. First, using several markers in the distal 1p region, we screened a group of 68 primary neuroblastoma tumours for 1p LOH (Table 1). LOH for distal 1p could be demonstrated in a subset of neuroblastomas (15/68, 22%). Two cases with distal 1p deletions had their deletion breakpoint as distal as between markers *D1S508* and *D1S244* (cases 121 and 189; Figure 1). The combined information of the 15 cases with 1p deletion therefore gave a consensus deletion region for a tentative neuroblastoma tumour suppressor gene ranging from the proximal marker *D1S244* to the distal *D1S80* (indicated by bar (a) in Figure 1). Several other groups have recently tried to delimit the neuroblastoma tumour suppressor region by analysing large numbers of neuroblastoma tumours and defining the commonly deleted regions. White and associates [25] presented data on a critical deleted region defined distally by marker *D1Z2* and proximally by *D1S228* (this region is indicated by bar (b) in Figure 1). Caron and associates [26], however, had no cases of interstitial deletions in their material, but the proximal border of their commonly deleted region was defined by probe *NN1.3* (region indicated by bar (c) in Figure 1). This is the *GDB* locus *A12M2*, located between marker *D1S228* and *D1S199* [15, 26]. A compilation of these data gives a shortest region of overlap (SRO) for distal 1p deletions defined distally by marker *D1S80*/*D1Z2* and proximally by markers *D1S244*/*D1S503*. This region is likely to be approximately 25 cM using available maps [13, 15].

A number of candidate tumour suppressor genes have been suggested for the 1p region frequently deleted in neuroblastoma tumours. One of these, the *CDC2L1* family of protein kinase genes, localised to the 1p36 region [27], was suggested based on homozygous rearrangements in four different tumour cell lines [5] and on its involvement in apoptosis [28]. We reasoned that if the *CDC2L1* gene is to meet the criteria for a neuroblastoma tumour suppressor gene, there ought to be neuroblastoma tumours with homozygous re-arrangements, such as deletions or translocations, among our patients with 1p LOH. DNA from tumour and corresponding normal tissue of 9 neuroblastoma patients were thus analysed for aberrant SSCP/HD patterns. Several tumours obviously had one copy of the *CDC2L1* locus since the 1p region was deleted, but no re-arrangements or deletions affecting the retained copy could be detected in the 9 neuroblastoma patients and tumours tested. Instead, both alleles of the *CDC2L1* locus appeared to remain intact in patient 52 who has an interstitial deletion in 1p with the very distal tip retained in the tumour. The breakpoint in 1p in this tumour (see Table 1) is between loci *D1S80* and *D1S243*, since both alleles of *D1S80* are retained in the tumour while locus *D1S243* shows LOH. Thus, it is likely that the *CDC2L1* locus is located within the same very distal segment which contains locus *D1S80*. Similar indications of this very distal 1p location of the *CDC2L1* locus were shown by White and associates [25].

In summary, 1p LOH could be detected in a subset of childhood neuroblastoma tumours in the present material (15 of 68, 22%) almost exclusively in tumours showing an unfavourable metastatic stage and poor clinical outcome. The loss of genetic material confined to this chromosomal region in aggressive tumours indicates the position of one or several neuroblastoma tumour suppressor gene(s). Using a panel of genetically mapped PCR-based DNA polymorphisms, the critical neuroblastoma consensus region showing LOH in tumour DNA was defined proximally by the marker *D1S244*/*D1S503* and distally by the marker *D1S80*. The *CDC2L1* locus most likely maps distal to this consensus region deleted in neuroblastoma tumours.

1. Brodeur GM, Sekhon GS, Goldstein MN. Chromosomal aberrations in human neuroblastomas. *Cancer* 1977, **40**, 2256–2263.
2. Gilbert F, Balaban G, Moorhead P, Bianchi D, Schlesinger H. Abnormalities of chromosome 1p in human neuroblastoma tumors and cell lines. *Cancer Genet Cytogenet* 1982, **7**, 33–42.
3. Fong C, Dracopoli NC, White PS, *et al.* Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: Correlation with N-myc amplification. *Proc Natl Acad Sci USA* 1989, **86**, 3753–3757.
4. Weith A, Martinsson T, Cziepluch C, *et al.* Neuroblastoma concensus deletion maps to 1p36.1-2. *Genes Chromosomes Cancer* 1989, **1**, 159–166.
5. Lahti JM, Valentine M, Xiang JL, *et al.* Alterations in the PITSLRE protein kinase gene complex on chromosome 1p36 in childhood neuroblastoma. *Nat Genet* 1994, **1**, 370–375.
6. Hedborg F, Lindgren PG, Johansson I, *et al.* N-myc gene amplification in neuroblastoma: a clinical approach using ultrasound guided cutting needle biopsies collected at diagnosis. *Med Ped Oncol* 1992, **20**, 292–300.
7. Martinsson T, Sjöberg RM, Hedborg T, Kogner P. Deletion of chromosome 1p-loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms. *Cancer Res* 1995, **55**, 5681–5686.

8. Brodeur GM, Seeger RC, Barret A, *et al.* International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. *J Clin Oncol* 1988, **6**, 1874–1881.
9. Brodeur GM, Pritchard J, Berthold F, *et al.* Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993, **11**, 1466–1477.
10. Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC. Analysis of the VNTR locus DIS80 by the PCR followed by high-resolution PAGE. *Am J Hum Genet* 1991, **48**, 137–144.
11. Weissenbach J, Gyapay G, Dib C, *et al.* A 2nd-generation linkage map of the human genome. *Nature* 1992, **359**, 794–801.
12. Gyapay G, Morissette J, Vignal A, *et al.* The 1993–94 Genethon human genetic linkage map. *Nat Genet* 1994, **7**, 246–339.
13. Dib C, Fauré S, Fizames C, *et al.* A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 1996, **380**, 152–154.
14. Engelstein M, Hudson TJ, Lane JM, *et al.* A PCR-based linkage map of human chromosome 1. *Genomics* 1993, **15**, 251–258.
15. Buetow KH, Weber JL, Ludwigsen S, *et al.* Integrated human genome-wide maps constructed using the CEPH reference panel. *Nat Genet* 1994, **6**, 391–393.
16. Sjöberg RM, Hallstensson K, Nordling M, *et al.* Analysis of location and integrity of the human PITSLRE ($p58^{cdc2Ll}$) genes in neuroblastoma cell genomes: evidence against involvement in tumorigenesis. *Int J Oncol* 1996, **8**, 1137–1142.
17. Eipers PG, Lahti JM, Kidd VJ. Structure and expression of the human $p58^{clk\text{-}1}$ protein kinase chromosomal gene. *Genomics* 1992, **13**, 613–621.
18. Brodeur GM, Green AA, Hayes FA, *et al.* Cytogenetic features of human neuroblastomas and cell lines. *Cancer Res* 1981, **41**, 4678–4686.
19. Christiansen H, Schestag J, Christiansen NM, Grzeschik KH, Lampert F. Clinical impact of chromosome 1 aberrations in neuroblastoma—a metaphase and interphase cytogenetic study. *Genes Chromosomes Cancer* 1992, **5**, 141–149.
20. Caron H, van Sluis P, van Hoeve M, *et al.* Allelic loss of chromosome 1p36 in neuroblastoma is of preferential maternal origin and correlates with N-myc amplification. *Nat Genet* 1993, **4**, 187–190.
21. Zink D, Weith A, Martinsson T, Schwab M. Analysis of chromosome band 1p36 alterations by chromosomal *in situ* suppression hybridization with a microclone DNA bank. *Genes Chromosomes Cancer* 1991, **3**, 407–410.
22. Stock C, Ambros IM, Mann G, *et al.* Detection of 1p36 deletions in paraffin sections of neuroblastoma tissues. *Genes Chromosomes Cancer* 1993, **6**, 1–9.
23. Laureys G, Speleman F, Opdenakker G, Benoit Y, Leroy J. Constitutional translocation t(1;17)(p36;q12-21) in a patient with neuroblastoma. *Genes Chromosomes Cancer* 1990, **2**, 252–254.
24. Biegel JA, White PS, Marshall HN, *et al.* Constitutional 1p36 deletion in a child with neuroblastoma. *Am J Hum Genet* 1993, **52**, 176–182.
25. White PS, Maris JM, Beltinger C, *et al.* A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. *Proc Natl Acad Sci USA* 1995, **92**, 5520–5524.
26. Caron H, Peter M, van Sluis P, *et al.* Evidence for two tumour suppressor loci on chromosomal bands 1p35–36 involved in neuroblastoma: one probably imprinted, another associated with N-myc amplification. *Hum Mol Genet* 1995, **4**, 535–539.
27. Eipers PG, Barnoski BL, Han J, Carroll AJ, Kidd VJ. Localization of the expressed human p58 protein kinase chromosomal gene to chromosome 1p36 and a highly related sequence to chromosome 15. *Genomics* 1991, **11**, 621–629.
28. Lahti JM, Xiang JL, Heath LS, Campana D, Kidd VJ. PITSLRE protein kinase activity is associated with apoptosis. *Mol Cell Biol* 1995, **15**, 1–11.

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