



Lack of interstitial chromosome 1p deletions in clinically-detected neuroblastoma

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Abstract

Loss of heterozygosity (LOH) of the distal part of the short arm of chromosome 1 in neuroblastoma is a well characterised phenomenon. In addition, previous reports have described interstitial deletions outside the common region of loss on chromosome 1p36, suggesting additional tumour suppressor loci. In this study, we have searched extensively for interstitial 1p deletions in a panel of 67 neuroblastoma samples from clinically-detected cases. We used three VNTR probes and 10 dinucleotide markers from the 1p32-36 regions reported to show interstitial deletions. Fifteen (22%) tumours showed telomeric LOH without evidence for more proximal interstitial deletions. Forty-five tumours showed no LOH or allelic imbalance. Seven (10%) tumours demonstrated allelic imbalance for one or more markers. These tumours were subsequently analysed by fluorescent *in situ* hybridisation (FISH) and flow cytometry. The patterns found in all seven tumours were consistent with copy number changes of the entire chromosome 1, without evidence for interstitial deletions. This study indicates that interstitial deletions of chromosome 1p are rare in clinically-detected neuroblastoma when analysed by a combination of molecular and cytogenetic techniques. © 2002 Published by Elsevier Science Ltd.

Keywords: Neuroblastoma; Chromosome 1p; Interstitial deletions; Allelic imbalance; Loss of heterozygosity; Ploidy

1. Introduction

Neuroblastoma is the most common malignant solid childhood tumour and is derived from the neural crest. Genetic aberrations like amplification of the *N-myc* oncogene (25%) [1] and loss of chromosome 1p (30%) [2,3] are indicators of unfavourable prognosis.

The frequent 1p deletions suggests that this region harbours tumour suppressor gene(s), but so far no neuroblastoma suppressor genes have been identified. The most frequent pattern of chromosome 1p loss is telomeric deletions. Loss of heterozygosity (LOH) studies in neuroblastoma tumours and cell lines have resulted in different smallest regions of overlap, suggesting the presence of at least two suppressor genes [4,5]. Several

1p candidate tumour suppressor genes have been suggested, e.g. *TP73* [6], *DR3* [7], *HRK3* [8] and *AML2* [9]. However, no evidence for consistent mutations have been found in these candidate suppressor genes.

Besides telomeric deletions, Takeda and colleagues [10] reported interstitial deletions extending more proximally at 1p36 with a consensus region flanked by D1S47 and the oncogene *FGR* (Fig. 1). Typically, these deletions were found in tumours from mass screening programmes. The age of these patients was mostly under 1 year and they have a good prognosis. In contrast, patients with large telomeric deletions were mostly over 1 year and had an unfavourable clinical outcome [11]. In another tumour series, two regions of interstitial deletions were observed (see Fig. 1) [12]. In a LOH study, focused at the distal part of chromosome 1, three cases with interstitial deletions were found (Fig. 1) [13]. In addition, Schleiermacher and colleagues reported interstitial deletions in a proximal region, between the 1p34 markers D1S197 and D1S203 [14].

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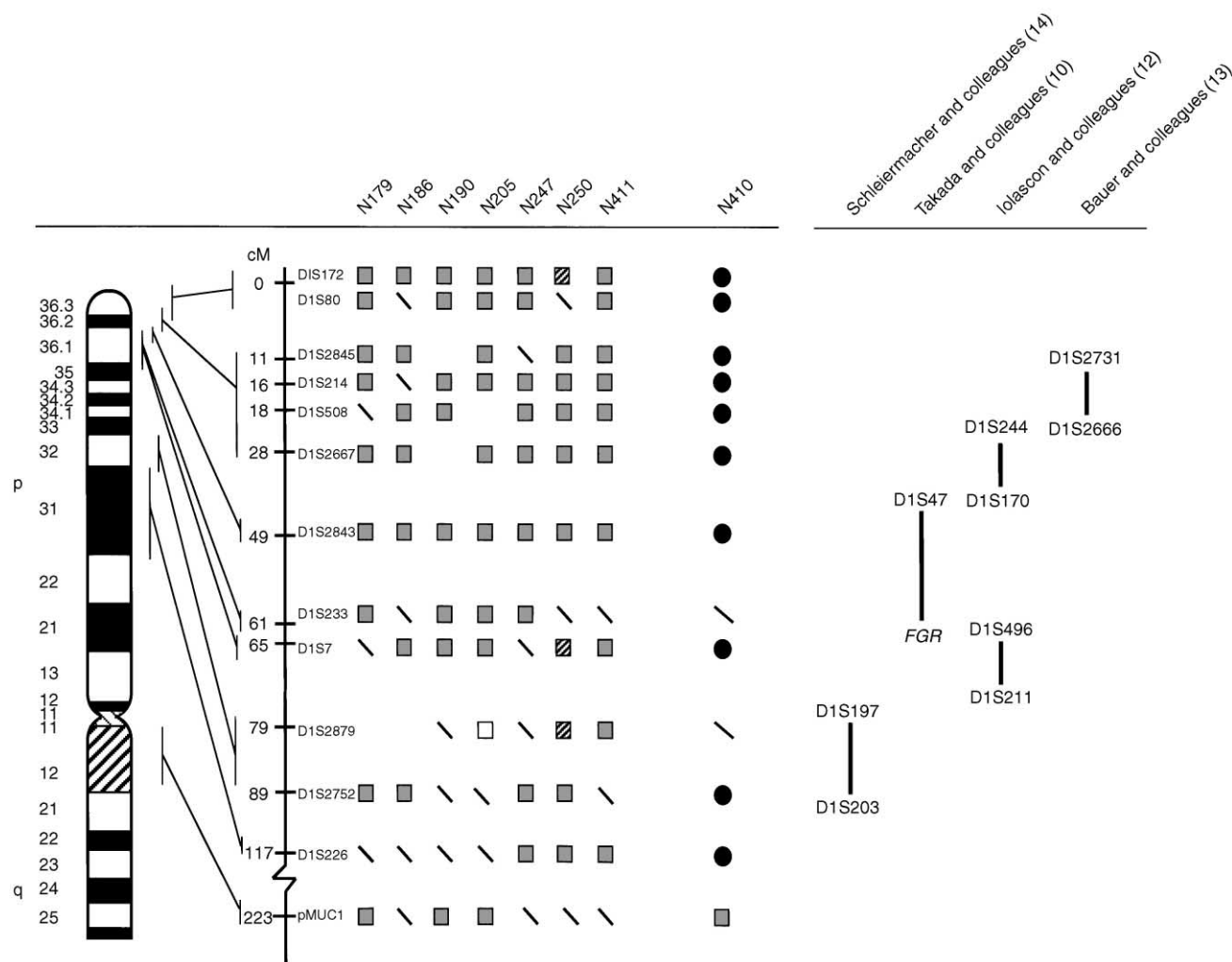


Fig. 1. Overview of chromosome 1p analysis of seven tumours with allelic imbalance and N410 with typical loss of heterozygosity (LOH). Tested polymorphic probes are shown at their cytogenetic and genetic (cM) positions. Filled circles: LOH, open boxes: no LOH, gray boxes: imbalance, dashed boxes: suggestive for interstitial deletions, dash: not informative. On the right side previously reported regions of interstitial deletions are displayed by black bars with flanking markers [10,12–14].

To shed more light on the apparent discrepancies in these reports, we have thoroughly analysed a panel of clinically detected neuroblastomas for the occurrence of interstitial deletions. We applied four high-resolution deletion mappings using restriction fragment length polymorphism (RFLP) analysis by Southern blot and polymerase chain reaction (PCR), flow cytometry and interphase fluorescent *in situ* hybridisation FISH.

2. Patients and methods

2.1. Tumour material

We selected 67 samples of tumour tissues and corresponding blood from our tissue bank. Twelve tumours had N-myc amplification; 34% of the cases were under and 61% of the cases were above the age of 1 year; the percentage of cases with International Neuroblastoma

Staging System (INSS) stages I, II, III, IV and IVs were 5, 12, 13, 45 and 13%, respectively. Tumour samples were collected from the Emma Kinderziekenhuis/Academic Medical Center, Pediatric Oncology Center of the Southern Netherlands, the Sophia Kinderziekenhuis (The Netherlands) and the University Hospital of Ghent (Belgium). Samples were snap-frozen immediately after surgery and stored at -80°C . Only tumour samples containing more than 60% of tumour tissue were used for the analysis.

2.1.1. Southern blot analysis of chromosome 1p

Southern blot analysis was done according to standard procedures. Briefly, filters with *TaqI* digestions of 10 μg DNA tumour and corresponding constitutional DNA were hybridised with the following polymorphic VNTR probes, in distal to proximal order: D1S172/CEB15, D1S7 and pMUC1. All probes are placed on chromosome 1p according to the CEPH map (www.cephb.fr).

2.1.2. PCR analysis of polymorphic chromosome 1p markers

Polymorphic microsatellite repeat loci were amplified simultaneously in the tumour and corresponding constitutional DNA of the patients by using PCR. PCR was performed in a final volume of 15 µl containing 30 ng template DNA; 2.5 pmol of primers; 100 µM deoxynucleotide triphosphate (dNTPs) deoxycytidine triphosphate (dCTP) 5 times lower concentration; standard Boehringer buffer; 0.4 units of *TaqI* polymerase (Boehringer, Germany); [α -32P]dCTP (Amersham, UK). DNA denaturation occurred at 94 °C for 5 min followed by 35 cycles on an automated thermocycler (Gene Amp PCR System 9600, Perkin-Elmer-Cetus), 94 °C for 30 s annealing at 56 °C for 1 min and extension at 72 °C for 1.5 min, followed by a definitive elongation at 72 °C for 7 min. Subsequently, after denaturation for 5 min at 80 °C each sample was loaded on a polyacrylamide gel and electrophoresed. Gels were autoradiographed using Kodak light-sensitive films. The following loci were analysed: D1S2845, D1S80, D1S214, D1S508, D1S2667, D1S2843, D1S233, D1S2879, D1S2752 and D1S226. Primer information is available at the Genome Data Base (<http://www.gdb.org>). This panel of polymorphic probes has been selected by virtue of their ability to detect telomeric and interstitial deletions reported in previous studies [5,10,12,14–19]. Allelic loss was defined as a complete or almost complete disappearance of one allelic band. Cases with significantly decreased intensity of one allele were scored as imbalance.

2.1.3. Ploidy assessment by flow cytometry

DNA ploidy was determined by DNA flow cytometry. Nuclear suspensions were prepared from 50 µm tumour sections by trypsin digestion as described by Dressler and colleagues in Ref. [20]. After staining with propidium iodide, suspensions were filtered and subsequently analysed by fluorescent activated cell sorting (FACS) (Becton Dickinson, USA). Histograms were constructed of each sample after at least 10 000 events were examined.

2.1.4. Interphase FISH

Two-colour interphase FISH was done according to the methods described earlier in Ref. [21]. Briefly, per FISH experiment two probes were combined. Firstly, chromosome 1 centromeric heterochromatic probe pUC1.77 (2 ng/µl) together with telomere probe D1Z2 (p1.79) at position 1p36.3 were hybridised. Secondly, pUC1.77 together with BAC clone RPCI-11-260M10 containing D1S2879 (AC022754) (15 ng/µl) mapping to chromosomal band 1p32 were hybridised. Probe pUC1.77 was directly labelled with CY3 (red), D1Z2 and BAC clone RPCI-11-260M10 indirectly labelled with immunodetection by Avidine-fluorescein isothiocyanate (FITC) (green) was performed. Next, nuclei were counterstained with 20 ng/ml DAPI and embedded in antifade

solution (20 mM DABCO, 1 M Tris-HCl and 90% glycerol). Signals were microscopically analysed using an Olympus microscope and images were stored after digitalisation. For each experiment, a minimum of 100 interphase nuclei were scored.

3. Results

3.1. Molecular analysis

We screened 67 neuroblastomas for chromosome 1p loss by southern blot analysis with the VNTR markers D1S172/CEB15, D1S7(pMS1) and pMUC1. Subsequently, all 67 cases were subjected to deletion mapping using 10 mini- and micro-satellite repeat markers. We classified the allelic patterns according to the ENQUA Group guidelines [22] into three categories: (1) *allelic loss* (i.e. almost complete disappearance of an allelic band); (2) *allelic imbalance* (i.e. significant difference in intensity between allelic bands); (3) *no abnormalities*.

In 45 patients, we found no clear differences between the allelic patterns in the tumour DNA and the matched constitutional DNA (group 3). Clear-cut LOH (group 1) was found in 15/67 cases (22%), (for an example see case N410, Fig. 1). In seven (10%) tumours, we detected a significant decrease in the intensity of an allelic band for one or more 1p markers (group 2). Of the 15 cases with clear-cut LOH, 12 had amplification of *N-myc*. In these cases, the deleted region always included the most telomeric markers D1S172/CEB15 and D1S80, whereas one or more proximal markers were intact without evidence for interstitial deletions. All seven tumours with allelic imbalance had a single copy *N-myc* status. The extent of allelic imbalance varied between the different 1p markers within the same tumour sample (Fig. 1). Some imbalances were highly suggestive for interstitial deletions. This was especially the case for tumour N250 (see Fig. 1, dashed boxes and Fig. 2). In tumour N250, the allelic intensities of the most distal marker D1S172/CEB15 could reflect LOH compared with the flanking marker D1S2845. Likewise, the allelic pattern of the proximal markers D1S7 and D1S2879 are suggestive for interstitial loss (see arrowheads Fig. 2). In tumour N205, the allelic imbalance observed for most 1p markers was not found for D1S2879, which showed equal intensities for both alleles. In order to discriminate between (interstitial) allelic loss and an imbalance of polymorphic 1p alleles as a result of copy number changes of chromosome 1, we performed DNA-content measurements and interphase FISH experiments for all tumours with allelic imbalance.

3.2. Nuclear DNA content

All seven tumours with allelic imbalance showed an aneuploid pattern, indicating the possibility of extra

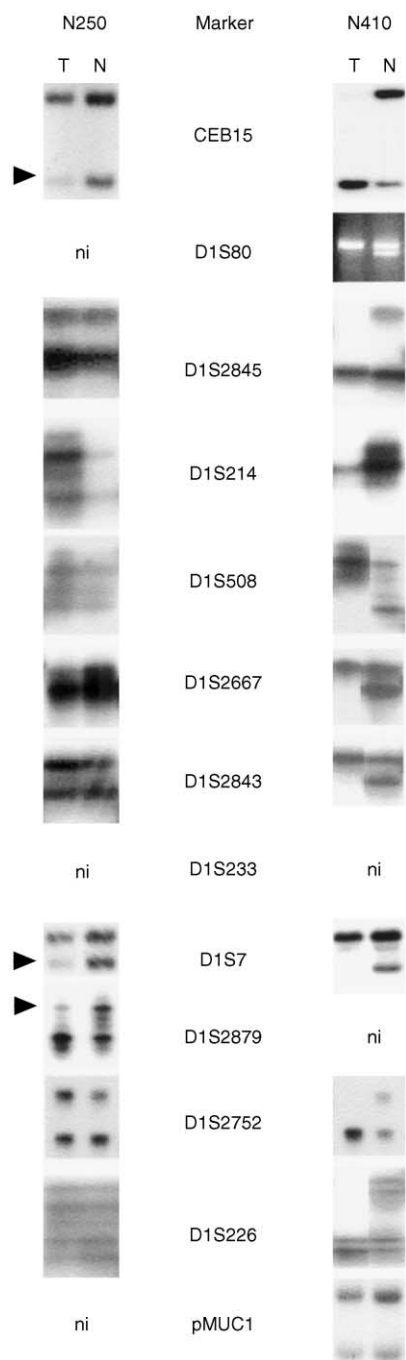


Fig. 2. Southern blots and polymerase chain reaction (PCR) analysis of VNTR probes in tumour N250 and N410. Alleles are depicted from tumours N250 with allelic imbalance and N410 with typical LOH, which are representatives from Fig. 1. Arrowheads: mark substantial allelic imbalance. ni, not informative; T, tumour; N, normal.

copies of chromosome 1, which might explain the observed imbalance (see Table 1). Four tumours showed a near-triploid DNA content. N205 showed a major triploid subpopulation (33%). Tumours N411 and N190 displayed a near-tetraploid DNA content. Unequal copy numbers of the two parental 1p alleles could explain the imbalances for the 1p markers in these cases.

To further substantiate these findings, we performed interphase FISH for chromosome 1p.

3.3. Fluorescence in situ hybridisation

Two-colour FISH analysis was performed with the telomeric 1p36 probe D1Z2 (green), and the chromosome 1 centromere probe pUC1.77 (red). The control tumour N410 with clear-cut 1p loss shows four signals for the chromosome 1 centromere probe and two signals with the 1p36 telomeric probe (Fig. 3).

In Table 1, an overview is given of the interphase nuclei groups if more than 20% was found. Of the seven tumours with allelic imbalances, 6 cases (N179, N186, N190, N205, N247, N250) showed clear trisomy for chromosome 1 without evidence of chromosome 1p loss in the majority of the scored nuclei (see Table 1). A representative example is case N250 (Fig. 3). It shows that the imbalance of D1S172/CEB15 in this tumour is caused by the over-representation of one allele rather than loss of an allele.

In the remaining tumour N411, we found a mixture of nuclei with 32% tetrasomy and 29% trisomy of chromosome 1p. This 1p pattern apparently resulted in an unequal distribution of 1p alleles in the tumour DNA, explaining the imbalance found with the molecular polymorphic 1p markers.

The possible interstitial loss of the marker D1S7 and D1S2879 in N250 and the D1S2879 pattern in N205 were further explored by two-colour interphase FISH with a BAC-containing D1S2879 and the centromere probe. In both N205 and N250, the D1S2879 FISH patterns were identical to the FISH pattern of the telomeric probe D1Z2. In N205, the majority of the nuclei were trisomic for D1S2879 and a large disomic fraction was found. Besides excluding a possible interstitial deletion of this locus, the disomic fraction explains the irregularity of the allelic pattern in this case. In N250, the possible interstitial allelic loss could not be substantiated, as >70% of the nuclei showed a trisomy without deletion of D1S2879.

Taken together, our molecular analysis of 1p supplemented with DNA-index measurements and interphase FISH shows that in all 7 cases, allelic imbalances result from whole chromosome changes and there is no evidence for interstitial deletions in our series. Therefore, in our series of 67 neuroblastomas we found no evidence for interstitial deletions.

4. Discussion

In neuroblastoma telomeric deletions of 1p36 are well recognised, but the existence of interstitial deletions is still under debate. This study contributes to the ongoing discussion and to the question of which techniques have

Table 1

Overview of tumour cell content, flow cytometric analysis and corresponding fluorescent *in situ* hybridisation (FISH) analysis of seven tumours with imbalance and N410 with loss of heterozygosity (LOH)

	N179	N186	N190	N205	N247	N250	N411	N410
Histology	> 80	> 90	100	> 80	> 80	> 60	> 80	> 90
% Tumour cells								
Ploidy								
DI	1.63	1.38	1.86	1.40	1.30	1.65	2.03	1.88
Aneuploid%	80	79	74	35	81	72	76	81
Diploid%	20	21	26	65	19	28	24	19
FISH								
D1Z2:centromere (%)	3:3 (53) 2:2 (23)	3:3 (64)	3:3 (67) 3:2 (25)	3:3 (55) 2:2 (35)	3:3 (59) 2:2 (20)	3:3 (70)	3:3 (29) 4:4 (32)	4:2 (72)
D1S2879PAC:centromere (%)				3:3 (46) 2:2 (42)		3:3 (72)		4:2 (65) 3:2 (25)

Percentages of tumour cell content. Ploidy, DNA index (DI) from flow cytometric analysis; nd, not determined. Fraction of the aneuploid and diploid populations are indicated. FISH, Hybridisation patterns for chromosome 1 found by 2 colour FISH analysis using subtelomeric probe D1Z2 and a chromosome 1 centromere probe (patterns of >20% are shown). Tumour N250 and N205 were subjected to an additional 2 colour FISH using a BAC containing D1S2879 combined with the same centromere probe.

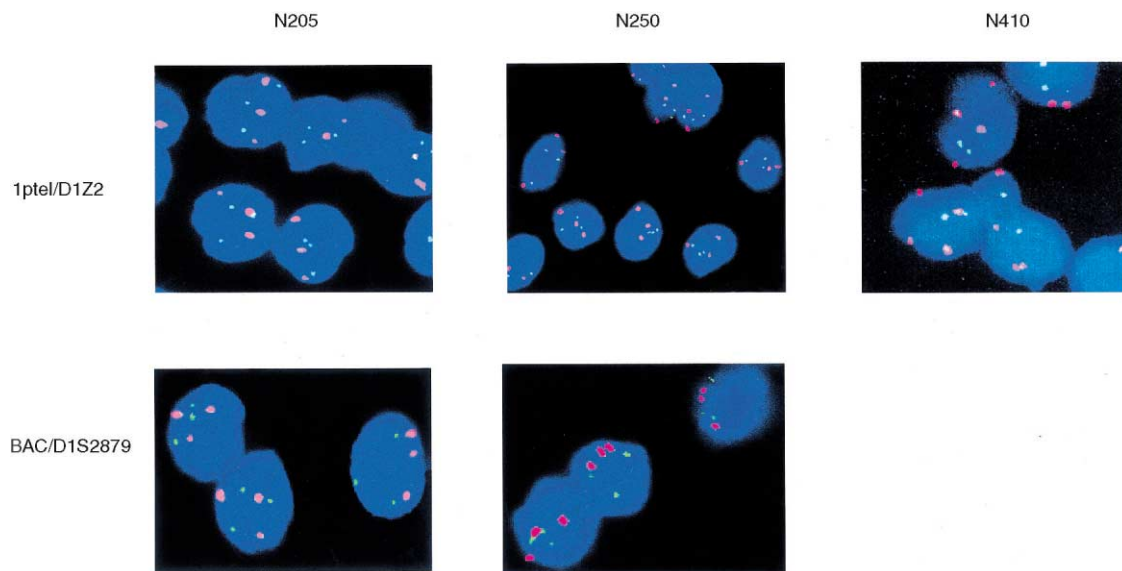


Fig. 3. Representative images of 2 colour fluorescent *in situ* hybridisation (FISH) performed on interphase nuclei of tumour samples N205, N250 and N410; top: D1Z2 (1ptel, green) and pUC1.77 (1p cen, red); bottom: D1S2879 (1p32, green) and pUC1.77 (1p cen, red).

to be applied for unequivocal results. We have studied paired tumour and corresponding normal DNA samples from 67 patients with three VNTRs and 10 CA-repeats markers on chromosome 1p to search for interstitial deletions. 15 (22%) showed telomeric loss and 7 (10%) showed allelic imbalance. All seven tumours with molecular imbalances showed whole chromosome abnormalities for chromosome 1 by interphase FISH, without evidence for (interstitial) deletions. This is in contrast to previous studies [10–14]. Several explanations are possible for this discrepancy. The interstitial deletions reported by the Japanese researchers were

found in a group of neuroblastomas identified in mass screening programmes. These selected tumours are prone to spontaneous regression and have a favourable clinical outcome. Therefore, they might be biologically distinct from clinically-detected cases. Perhaps more importantly, all studies reporting interstitial deletions relied on molecular techniques (PCR and Southern blot) without confirmation by cytogenetic techniques. In our study, we were only able to distinguish between true deletions and allelic imbalances by adding FISH to our molecular polymorphic marker analysis. Confirmation of previously reported interstitial deletions by cytogenetic

and FISH studies would therefore be useful. Our results show that interstitial deletions are a rare event in clinically-detected neuroblastoma.

In the control tumour N410, we showed a two-colour FISH pattern of two subtelomeric and four centromeric signals. This disproportional distribution of subtelomeric and centromeric 1p signals does not necessarily reflect the presence of LOH. The two subtelomeric signals may well have been equally distributed; one remaining paternal and maternal allele. This case demonstrates this analysis alone to be a positive control tumour the importance of molecular techniques like Southern blot and PCR analysis could not detect 1p 20H.

During our study, we encountered several technical pitfalls, rendering the interpretation of allelic patterns in neuroblastoma samples difficult. Infiltration of normal cells, e.g. lymphocytes and fibroblasts can mask LOH. Alleles from the normal cells give rise to a background signal, which can disguise allelic loss. Second, whole chromosome copy number abnormalities (e.g. trisomy) can give rise to unbalanced intensities of alleles in the tumour compared with the constitutional DNA. These banding patterns, resulting from an overrepresentation of chromosome 1, can mimic allelic loss. To avoid these pitfalls, we implemented the following stepwise protocol for deletion analysis. First, histological analysis was used to select tumour samples with >80% of tumour cells. Second, we scored the allelic patterns for every tumour and divided them into three groups; no abnormalities, clear-cut LOH and allelic imbalance. Finally, we used interphase FISH for the allelic imbalance cases to distinguish between allelic loss by deletion and whole chromosome changes leading to allelic imbalance. We also used DNA flow cytometry analysis to obtain the ploidy status of the tumour. However, in our experience, ploidy measurement provides only circumstantial evidence to discriminate between whole chromosome abnormalities and structural abnormalities of chromosome 1p.

The prognostic value of the loss of chromosome 1p in clinically-detected neuroblastoma has been well established [2,3]. Currently, large multicentre prospective studies are ongoing (e.g. ESIOP/LNESC 1) to test the clinical usefulness of biological prognostic factors, like 1p loss. Our study illustrates that deletion studies of 1p by molecular techniques only can yield ambiguous results. Hence, we feel that characterising chromosome 1p in neuroblastoma for clinical purposes should consist of a combination of molecular analysis (LOH studies) and cytogenetics such as FISH, at least for allelic imbalance cases. This is completely in line with the proposal guidelines of the ESIOP/LNESC biology subcommittee [22]. The combination of molecular techniques and cytogenetic analysis allows the reliable identification of chromosome 1p deletions both for clinical purposes, as well as for deletion mapping studies for the identification of neuroblastoma tumour suppressor genes.

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