cosmid c102 (CEB15) (Dr G. Vergnaud), YAC clone A230A7 (*ERBB2*) (Dr C. Lengauer), cosmid 7G4 (*NF1*) (Dr S. Watkins).

We gratefully acknowledge the financial support of the "Vereniging voor Kankerbestrijding" "Het Centrum voor de Studie en Behandeling van Gezwelziekten vzw", the European Concerted Action on Molecular Cytogenetics of Solid Tumours (PL920156), and the "Stichting voor Kindergeneeskundig Kankeronderzoek (SKK)".

Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 535–538, 1995
Elsevier Science Ltd
Printed in Great Britain
0959–8049/95 $9.50+0.00

0959-8049(95)00008-9

A Multiplex PCR Assay for Routine Evaluation of Deletion of the Short Arm of Chromosome 1 in Neuroblastoma

G. Schleiermacher, M. Peter, J. Michon, J.-M. Zucker, G. Thomas, H. Magdelénat and O. Delattre

Deletions of the short arm of chromosome 1 (1p) are frequent alterations in neuroblastoma. Although a consensus region of deletion has been mapped to chromosome subband 1p36, recent studies suggest that several distinct loci on this chromosome may be involved in neuroblastoma. Moreover, different patterns of deletion might be associated with different clinical and biological characteristics of the tumours. These findings emphasise the importance of assessing the localisation and the extent of the deletions in neuroblastoma. We developed a technique which allows analysis of loss of heterozygosity at multiple loci on 1p in a single step, making use of a multiplex PCR method. Primers specific for six microsatellite loci mapped in the different regions of interest on 1p were used for simultaneous amplification of DNA, and loss of heterozygosity was determined after separation of the alleles by denaturing polyacrylamide gel electrophoresis. This technique enables a simple analysis of the position and extent of 1p deletions, and can be used for routine evaluation of 1p status in neuroblastoma.

Key words: neuroblastoma, chromosome 1, short arm, deletion, loss of heterozygosity, PCR, microsatellite, prognosis

Eur J Cancer, Vol. 31A, No. 4, pp. 535–538, 1995

INTRODUCTION

NEUROBLASTOMA, a tumour derived from neural crest tissue, is the most frequent extracranial solid tumour in childhood and presents a wide variability of its clinical course. It is characterised by two main genetic alterations: amplification of the oncogene *MYCN* is found in 25–30% of the cases and deletion of the short arm of chromosome 1 (1p), as detected by molecular studies, is observed in 30–40% of tumours [1–3]. Both alterations are detected more frequently in advanced stages of disease and have been associated with a poor outcome [4–7].

Initial molecular studies suggested that deletions encompassed a single region of overlap located in the 1p36.2–3 subband [2, 8]. However, recent observations indicate that, in addition to this locus, other loci on 1p might be involved in the tumorigenesis of neuroblastoma. Indeed, deletions limited to the 1p36.2–3 subband are not associated with *MYCN*, whereas this association is observed for larger deletions [9, 11]. Moreover, preferential loss of maternal alleles has been demonstrated in the former deletions, but not in the latter [12]. These observations suggest that at least two different loci on 1p are involved in tumours. Indication of an additional locus is provided by interstitial deletions which define a second short region of overlap located more proximally on 1p [9]. Finally, preliminary clinical reports suggest that the prognostic information provided by 1p deletion might depend on its size and localisation [10]. These findings emphasise the importance of the determination of the extent and of the chromosome position of deletions in neuroblastoma.

We have developed a method which allows detection of a deletion on 1p in neuroblastoma, determination of its localisation and estimation of its size. This method, which is based on the multiplex PCR amplification of microsatellite loci, enables the determination of these parameters, in a single step, on small tumour samples.

Correspondence to Olivier Delattre.
G. Schleiermacher, M. Peter and H. Magdelénat are at the Laboratoire de Transfert; G. Schleiermacher, J. Michon and J.-M. Zucker are at the Service de Pédiatrie; and G. Thomas and O. Delattre are at the Laboratoire de Génétique des Tumeurs, INSERM 434. Institut Curie, 26 rue d'Ulm, 75231 Paris Cedex 01, France.

MATERIALS AND METHODS

Tissue and blood samples

Tissue samples from neuroblastoma were obtained from the primary site by surgical biopsies, ultrasound-guided fine needle biopsies or from metastatic sites. Tumour samples were shown to contain more than 60% of malignant cells by histological studies. Blood samples were obtained from the same patients.

Analysis of loss of heterozygosity

DNA was isolated from the tumour samples and from blood samples using previously described procedures [13, 14]. For PCR analysis, paired tumour and constitutional DNA were PCR amplified using primers flanking microsatellite loci on chromosome 1p [15]. The reaction was performed in a total volume of 20 μl with 0.3 μM (6 pmol) of each primer, 100 μM of each dNTP, 1.5 mM of $MgCl_2$ and 0.5 U of Taq polymerase (Boehringer Mannheim, Germany). Six pairs of primers described in Table 1 were included in one reaction. In each reaction, 60–80 ng of DNA were used as templates. After initial denaturation at 95°C for 5 min, 35 cycles of PCR amplification were performed. Each cycle consisted of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Terminal extension was carried out at 72°C for 5 min. The amplification products were then denatured in 68% formamide for 5 min. Alleles were separated on a 6% denaturing sequencing gel, containing 7 M urea and 32% formamide. After electrophoresis at 60 W for 5 h, the PCR products were transferred to a Nylon N+ membrane (Amersham International, U.K.). The membranes were prehybridised in a buffer containing 130 mM sodium phosphate, pH 7.4, 10% PEG 6000, 7% SDS, and 250 mM NaCl for 2 h at 42°C. A $(CA)_{12}$ probe that was endlabelled using terminal transferase and the 3′ endlabelling kit (Boehringer Mannheim, Germany) were added directly to the prehybridisation buffer. Hybridisation was performed at 42°C for 2 h. The membranes were washed three times in 2× SSC at room temperature and autoradiography was performed.

RESULTS

We developed a method that allows rapid screening of neuroblastoma samples for the presence of loss of heterozygosity (LOH) in distal, intermediate and proximal regions of chromosome 1p. Paired constitutional and tumour DNA from neuroblastoma samples were used as a template for PCR amplification at various microsatellite loci on chromosome 1p.

In the first series of experiments [9], PCR amplification was carried out using individual sets of primers specific for a single locus on 1p. LOH could be determined after separation of the amplified alleles by denaturing polyacrylamide gel electrophoresis. In order to allow a more rapid analysis of LOH at microsatellite loci, a protocol for a multiplex assay was established. Markers used for this purpose should be highly informative loci, and located in the chromosomal regions of interest. They should be compatible for a simultaneous amplification, and finally the alleles should not overlap in size.

The loci used in this assay have heterozygosities higher than 70% (Table 1). They are dispersed along chromosome 1p with two distal loci, *D1S214* and *D1S199*, mapping to the distal shortest region of overlap. These two loci are located distal to the *ALPL* locus, previously assigned to 1p36.1–p35.1 [16]. Two loci, *D1S200* and *D1S220*, map proximal to the *GLUT* locus in 1p35–p31, and explore the recently described proximal region of deletion [9]. Finally, two loci, *D1S201* and *D1S190*, which enable the analysis of the intermediate region, were included in this assay. The primers specific for these loci resulted in satisfactory DNA amplification in a multiplex assay. Owing to slight variation in the yield of the different amplification products, two exposure times were necessary for good visualisation of the different alleles.

In this combination of microsatellite markers, the alleles detected at these loci do not overlap in size (Table 1). After migration, alleles were arranged from top to bottom in the order *D1S190–D1S220–D1S201–D1S200–D1S214–D1S199* (Table 1 and Figure 1).

Representative results from a tumour with LOH at distal loci (case number 30), at distal and proximal loci (case number 22) and conservation of both alleles at all informative loci (case numbers 66 and 67) are shown in Figure 1.

DISCUSSION

Recent reports suggest that distinct loci on 1p can be deleted in neuroblastoma, and that particular patterns of these deletions

Table 1. *Microsatellite loci used for determination of LOH on chromosome* 1*p in neuroblastoma*

Distance*	Locus symbol	Marker name	Size range of alleles (bp)	Heterozygosity
Telomere				
17 cM				
	D1S214	AFM 147yf8	120–142	0.78
33 cM				
	D1S199	AFM 078yg5	94–116	0.83
16 cM				
	D1S201	AFM 094tb7	186–204	0.73
12 cM				
	D1S190	AFM 046xc11	293–333	0.93
8 cM				
	D1S200	AFM 093xf3	154–178	0.79
7 cM				
	D1S220	AFM 162xg3	231–251	0.82
71 cM				
Centromere				

* From top to bottom, the microsatellite loci are listed in the order of their localisation on chromosome 1p from telomere to centromere, and the genetic distance between them is indicated.

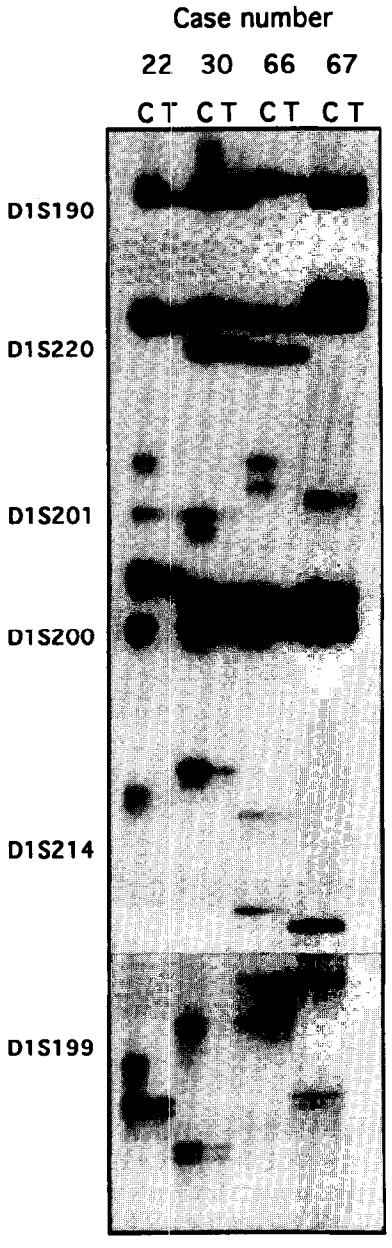

Figure 1. LOH analysis of four neuroblastoma samples by multiplex PCR assay using primers specific for six microsatellite loci on chromosome 1p. C and T indicate constitutional and tumour DNA, respectively. Case 30 has a distal, terminal deletion involving the distal *D1S199* and *D1S201* loci. Case 22 has a large deletion encompassing all informative loci. Cases 66 and 67 show retention of heterozygosity at all informative loci. For visualisation of the *D1S199* alleles, a longer exposure time was necessary.

might be associated with different biological behaviour of tumours. Deletions which are not associated with *MYCN* are of a preferential maternal origin, and are frequently limited to a distal locus on 1p. In contrast, deletions observed together with *MYCN* are frequently larger, demonstrate no preferential parental origin and always encompass a second, more proximal locus [12]. Finally, a third, even more proximal locus is deleted in some neuroblastomas [9]. A recent study suggested that different types of deletions might have different prognostic value [10].

The multiplex PCR assay which we have developed allows the analysis of LOH at these three regions in a single step. The six microsatellite loci included in this assay span a region of approximately 80 cM on 1p. This technique is particularly suitable for small tumour specimens, such as those obtained by fine-needle biopsies, for which only small amounts of DNA are available. Indeed, the PCR method allows LOH analysis of small quantities of DNA since only 60–80 ng of DNA, the amount of DNA contained in 10^4 cells, are required for this analysis. The increasing number of microsatellite loci on chromosome 1p now available and the more precise definition of the loci on 1p altered in neuroblastoma should permit progressive evolution of this test.

Usually, the interpretation of the results is easy, deletion being evidenced by the absence or obvious decrease of one of the two alleles in the tumour DNA. The number of loci showing LOH provides information on the position and extent of the deletion. Replication error which might complicate the analysis occurs with low frequency in neuroblastoma [9, 17]. In some cases, less obvious allelic imbalance does not enable a reliable conclusion. These imbalances can be attributed either to the presence of multiple copies of chromosome 1 in tumour cells or to contamination of the sample by non-tumour cells. This emphasises the need to know the percentage of tumour cells in the sample. Only samples which contain more than 60% of tumour cells can be reliably studied by this method.

The number of copies of chromosome 1 can be appreciated by fluorescent *in situ* hybridisation (FISH) [5, 18, 19]. In fact, it might be advisable to study tumour samples both by FISH and LOH. Indeed, FISH enables the study of individual tumour cells and can detect the presence of additional chromosomal material, and LOH can precisely define the position and extent of the deletions.

Information provided by the genotyping of tumours is becoming increasingly important for the management of patients with cancer. This requires that simple and reliable techniques of analysis are available. Multiplex PCR assays, such as the one described here, should prove useful in routine analysis of tumour samples. In particular, they should enable determination of the prognostic significance of 1p deletions in prospective studies in neuroblastoma as well as in numerous tumours in which 1p deletions have been described [20–25].

1. Brodeur GM, Fong CT. Molecular biology and genetics of human neuroblastoma. *Cancer Genet Cytogenet* 1989, **41**, 153–174.
2. Fong CT, 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.
3. 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. *Nature Genet* 1993, **4**, 187–190.
4. Christiansen H, Lampert F. Tumour karyotype discriminates between good and bad prognostic outcome in neuroblastoma. *Br J Cancer* 1988, **57**, 121–126.
5. 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 Chrom Cancer* 1992, **5**, 141–149.
6. Hayashi Y, Kanda N, Inaba T, *et al.* Cytogenetic findings and prognosis in neuroblastoma with emphasis on marker chromosome 1. *Cancer* 1989, **63**, 126–132.
7. Fong CT, White PS, Peterson K, *et al.* Loss of heterozygosity for chromosomes 1 or 14 defines subsets of advanced neuroblastomas. *Cancer Res* 1992, **52**, 1780–1785.
8. Weith A, Martinsson T, Cziepluch C, *et al.* Neuroblastoma consensus deletion maps to 1p36.1–2. *Genes Chrom Cancer* 1989, **1**, 159–166.

9. Schleiermacher G, Peter M, Michon J, *et al.* Two distinct deleted regions on the short arm of chromosome 1 in neuroblastoma. *Genes Chrom Cancer* 1994, **10**, 275–281.
10. Takeda O, Homma C, Maseki N, *et al.* There may be two tumor suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chrom Cancer* 1994, **10**, 30–39.
11. Cheng NC, Van Roy N, Chan A, *et al.* Deletion mapping in neuroblastoma cell lines suggests two distinct tumour suppressor genes in the 1p35-36 region, only one of which is associated with N-myc amplification. *Oncogene* 1995, **10**, 291–297.
12. Caron H, Peter M, Van Sluis P, *et al.* Evidence for 2 tumour suppressor loci on chromosome bands 1p35-36 involved in neuroblastoma: one probably imprinted, another associated with N-myc amplification. *Hum Mol Genet*, in press.
13. Peter M, Michon J, Vielh P, *et al.* PCR assay for chromosome 1p deletion in small neuroblastoma samples. *Int J Cancer* 1992, **52**, 544–548.
14. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
15. Gyapay G, Morissette J, Vignal A, *et al.* The 1993–94 Généthon human genetic linkage map. *Nature Genet* 1994, **7**, 246–339.
16. Van Roy N, Laureys G, Versteeg R, Opdenakker G, Speleman F. High-resolution fluorescence mapping of 46 DNA markers to the short arm of human chromosome 1. *Genomics* 1993, **18**, 71–78.
17. Gehring M, Berthold F, Schwab M, Amler L. Genetic stability of microsatellites in human neuroblastomas. *Int J Oncol* 1994, **4**, 1043–1045.
18. Stock C, Ambros IM, Mann G, Gadner H, Amann G, Ambros P. Detection of 1p36 deletions in paraffin sections of neuroblastoma tissues. *Genes Chrom Cancer* 1993, **6**, 1–9.
19. Taylor CPF, McGuckin AG, Bown NP, *et al.* Rapid detection of prognostic genetic factors in neuroblastoma using fluorescence *in situ* hybridisation on tumour imprints and bone marrow smears. *Br J Cancer* 1994, **69**, 445–451.
20. Bièche I, Champème MH, Matifas F, Cropp CS, Callahan R, Lidereau R. Two distinct regions involved in 1p deletion in human primary breast cancer. *Cancer Res* 1993, **53**, 1990–1994.
21. Dracopoli NC, Harnett P, Bale SJ, *et al.* Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression. *Proc Natl Acad Sci USA* 1989, **86**, 4616–4618.
22. Flejter WL, Li FP, Antman KH, Testa JR. Recurring loss involving chromosomes 1, 3, and 22 in malignant mesothelioma: possible sites of tumor suppressor genes. *Genes Chrom Cancer* 1989, **1**, 148–154.
23. Leister I, Weith A, Brüderlein S, *et al.* Human colorectal cancer: high frequency of deletions at chromosome 1p35. *Cancer Res* 1990, **50**, 7232–7235.
24. Mathew CGP, Smith BA, Thorpe K, *et al.* Deletion of genes on chromosome 1 in endocrine neoplasia. *Nature* 1987, **328**, 524–526.
25. Simon D, Knowles BB, Weith A. Abnormalities of chromosome 1 and loss of heterozygosity on 1p in primary hepatomas. *Oncogene* 1991, **6**, 765–770.

Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 538–541, 1995

0959–8049/95 $9.50+0.00

0959-8049(95)00037-2

1p36: Every Subband a Suppressor?

R. Versteeg, H. Caron, N.C. Cheng, P. van der Drift, R. Slater, A. Westerveld, P.A. Voûte, O. Delattre, G. Laureys, N. Van Roy and F. Speleman

INTRODUCTION

MORE THAN 18 years ago, the first deletions of the p arm of chromosome 1 were detected in neuroblastomas [1]. Since then, cytogenetic analyses of many tumours and cell lines have revealed that these deletions can vary in size, some being very large, but all typically extending to the telomere. Loss of heterozygosity (LOH) studies later enabled the detection of smaller deletions, and defined the shortest region of overlap (SRO) to band 1p36 [2, 3]. It is, therefore, generally assumed that this region carries a neuroblastoma suppressor gene. However, recent analyses have challenged the simple concept of one single 1p36 neuroblastoma suppressor gene. Here, we review our recent data that suggest that 1p35-36 encodes four genes which may play a role in neuroblastoma tumorigenesis.

Correspondence to R. Versteeg.
R. Versteeg, H. Caron, N.C. Cheng, P. van der Drift, R. Slater and A. Westerveld are at the Institute of Human Genetics, Academic Medical Centre, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam; and H. Caron and P.A. Voûte are at the Department of Paediatric Oncology and Haematology, Emma Kinder Ziekenhuis/Academic Medical Centre, University of Amsterdam, The Netherlands; O. Delattre is at the Laboratoire de Génétique des Tumeurs, Institut Curie, Paris, France; and G. Laureys, N. van Roy and F. Speleman are at the Department of Medical Genetics, University Hospital of Ghent, Belgium.

1p DELETIONS DIFFER IN *MYCN* AMPLIFIED VERSUS SINGLE COPY CASES

LOH of the short arm of chromosome 1 is associated with amplification of the *MYCN* oncogene [2]. We found this association to be very strict, with analysis of 89 neuroblastomas showing that all 18 *MYCN* amplified cases had LOH of 1p [4, 5]. However, nearly 15% of the *MYCN* single copy neuroblastomas had also lost the distal 1p region. We now have convincing evidence that two different 1p suppressor genes are inactivated in *MYCN* amplified and *MYCN* single copy neuroblastomas.

The initial evidence supporting this hypothesis came from our studies on genomic imprinting in neuroblastoma. Genomic imprinting is the phenomenon whereby the two alleles of a gene are differentially expressed, depending on their parental origin. For instance, the *H19* gene on chromosome 11, that is implicated in tumour suppression in rhabdomyosarcoma [6], is expressed from the maternally inherited allele, but not from the paternal allele [7]. Preferential loss of one parental allele of a tumour suppressor region is considered an indication that the suppressor gene in question is imprinted. The allele that is preferentially lost is also the allele that would have been expressed, while the retained allele is the silent one.

We studied the parental origin of 1p36 deletions in neuroblas-