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Original Paper

Representational Difference Analysis and Loss of Heterozygosity Studies Detect 3p Deletions in Neuroblastoma

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In an attempt to identify genes involved in neuroblastoma, we scanned neuroblastoma tumour DNAs for homozygous deletions by representational difference analysis (RDA). The RDA produced several difference products, nine of which represented hemizygous deletions located on chromosome 1 or 3. In order to detect deletions, a genomewide loss of heterozygosity (LOH) screening with polymorphic markers was performed. Allelic losses on a number of different chromosomes were detected, mainly in favourable neuroblastomas (stage 1, 2 and 4S). The most frequently deleted region, apart from 1p, was chromosomal region 3p. A more detailed study was made in this region, which showed that 9 out of 58 (16%) tested neuroblastoma tumours showed allelic loss in the same region on chromosome 3p, i.e. 3pter-14.2. Thus, both RDA and LOH studies showed chromosome region 3p as being frequently involved in deletions and/or rearrangements in neuroblastoma tumours. Therefore, it is possible that one or more of the 3p genes implicated in the development of other cancers also play a role in neuroblastoma development and/or progression. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

MANY STUDIES suggest that a putative neuroblastoma tumour suppressor gene, or genes, should be located in chromosome region 1p36 [1]. Advanced neuroblastoma tumours often show deletions and/or translocations involving the short arm of chromosome 1. *MYCN* amplification is another feature often noticed in aggressive neuroblastomas [2]. Several but not all neuroblastomas have deletions on chromosome 1p and/or *MYCN* amplification. Our previous data showed that 26% of neuroblastomas had 1p deletions and 26% *MYCN* amplification [3]. Thus, several tumours, even of advanced stages 3 and 4, do not show these genetic aberrations. It is therefore likely that other factors are involved in the aetiology of neuroblastomas. A few other chromosomal regions, e.g. 4p [4], 11q13–22 [5], 14q11–32 [6] and 17q [7, 8] have been shown to be frequently deleted in some neuroblastomas.

There is a possibility that there could be homozygous deletions in neuroblastoma tumours, like those that have been found in chromosome region 3p in other tumours, for example, in lung [9], breast [10] and colon cancer [11]. A homozygous deletion would be a hot spot in which to look for a tumour suppressor gene, since tumour suppressors are recessive and would be totally inactivated in a homozygous deletion.

In this study, we used a technique called representational difference analysis (RDA). RDA was developed by Lisitsyn and associates [12] as a method to detect the differences between two complex genomes. Its principle is a subtraction hybridisation, which is made possible by reduction of the genomic complexity by amplification of only certain portions of the genome with PCR (polymerase chain reaction). By using tumour DNA as a driver and normal DNA as a tester, DNA segments only present in normal DNA would remain to be amplified after subtraction and hybridisation. From homozygously deleted regions, any DNA fragments amplifiable by PCR would be obtained as difference products.

In the present study we subjected three neuroblastoma tumours to RDA and successfully isolated 10 difference products from two of them. These products were further characterised by sequencing, chromosome localisation and evaluation by PCR versus neuroblastoma tumour DNA. Furthermore, we performed a wide genome loss of heterozygosity scan with polymorphic markers on a subset of

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different staged neuroblastomas to get an overview of the chromosomal regions, in addition to those of chromosome region 1p, commonly deleted in our neuroblastoma tumour material. Both the RDA and the LOH analysis indicated chromosome region 3p as involved in deletions. Therefore a larger, more detailed study was made in this region. The results suggest that genes on 3p could be involved in neuroblastoma tumorigenesis.

MATERIALS AND METHODS

Patient material

Tumour tissue and corresponding normal tissue (fibroblast or blood sample) from 58 different stage neuroblastoma patients were used in the study. The children were diagnosed, staged and evaluated for clinical outcome according to the International Neuroblastoma Staging System criteria (INSS [13, 14]). In the wide genome study, a subset of patients were used; 8 patients with favourable stages 1, 2 and 4S and 9 patients with unfavourable stages 3 and 4. Four patients earlier found to have 1p LOH were included.

For the RDA procedure, three pairs of neuroblastoma stage 4 DNA samples, from tumour and corresponding constitutional tissue, were employed. All three tumours have been extensively analysed [3] and display LOH for polymorphic markers in the 1p region. The three tumours were chosen since LOH studies with PCR-based microsatellite markers indicated that not even a faint signal was present from the deleted allele.

RDA procedure

RDA was performed essentially as described by Lisitsyn and associates [12] with modifications from the detailed laboratory protocol for RDA provided by Lisitsyn on request. Initial digests were performed with Bgl II.

Difference products were restriction-digested, gel-purified and subcloned into the plasmid vector, pIC19R which is a pUC19 derivative. In each series of experiments, at least 96 clones were picked and grown in a 96-well plate and transferred on to nylon membranes to make colony blots. Insert fragments were isolated on a low-melting point agarose gel from digested plasmid DNA and used as hybridisation probes. Redundant clones were eliminated by colony blot hybridisation. To characterise difference products, two serial hybridisation experiments were performed. To exclude non-specific amplification products, an amplicon blot, which has the initial 'tester' and 'driver' amplicon DNA, was used for quick screening of the true difference products. Then the real 'difference' clones in amplicon blot screening were hybridised to the genomic Southern blot for further confirmation and characterisation. The results from the amplicon blot screening were always concordant with those from genomic blots.

Characterisation of difference products

Sequencing. The RDA procedure resulted in 10 difference clones, which were sequenced on an A.L.F. DNA sequencer (Pharmacia, Sweden), the sequencing reactions were made according to the FEBS PRACTICAL COURSE 1993 protocol for sequencing plasmid DNA with T7 DNA polymerase and M13 flourescein primer. The chemicals used for the sequencing reactions were from Pharmacia AutoCycle Sequencing Kit. The lengths of the difference products are shown in Table 1. The sequences are available upon request from the authors.

Possible sequence similarities/homologies to other published sequences and tests for the presence of exon sequences were analysed using FASTA, BLAST and Grail computer libraries.

PCR assay. The sequences were used to design PCR primers (see Table 1), with use of the DNASTAR Primer Select program from Lasergene. A PCR assay was set up for each difference product. Amplifications were carried out in a 20 µl volume containing 125 ng DNA; 15 pmol of each primer; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% (w/v) gelatin; 4.4 nmol of each dATP, dCTP, dGTP, dTTP; and 0.55 U Taq DNA polymerase (Perkin-Elmer/Roche, Branchburg, New Jersey, U.S.A.). Amplifications were performed in a Biometra TRIO Thermoblock. Amplifications were performed with 30 rounds of PCR with the annealing temperatures listed in Table 1.

Chromosome localisation. The PCR assay was used to localise each difference clone to a chromosome, with use of

Table 1. *Experimental data on eight difference products*

Clone	Patient	Difference product length	Chromosome location	Computer search BLAST	PCR product length	Annealing temp. (°C)	PCR primers
1202	55	560 bp	1	H. acidic growth factor, $P = 0.035$	386 bp	49	fp:ATTCCCCGCTGTATTAGTT rp:TGGGGGTGCAGTGGTGTAG
1235	55	528 bp	1		293 bp	58	fp:AGGCCTGGGGTTGCTGGAG rp:TCATGGCCTGGGGAGTGC
5610	174	385 bp	?		144 bp	52	fp:AAGCTTCCTCCACCCATCATTTC rp:AGCTTCTGTAGTTCCGCCTGTTG
5615	174	505 bp	3		357 bp	55	fp:GCTAGGGGTGAAGGGGGTTATT rp:GGTTTGTGCCTGCATTTTCCTT
5635	174	420 bp	3	H.CYP 19,cDNA, $P = 5.7 \times 10^{-14}$	251 bp	58	fp:AGGGGGATGGTGCTAAG rp:GGATTGCCAGGGGAGTATTG
5649	174	319 bp	1	mRNA osteoblast spec. factor, $P = 0.043$	99 bp	56	fp:CCAGCCCGGGACCCTAAAATG rp:AGCCCCTGCCCACTGATG
5651	174	309 bp	3		178 bp	56	fp:AGATGAGAAATTGGGTGAACAGG rp:GAGCGAAGCAGGCAGTGG
5667	174	275 bp	3		142 bp	56	fp:GAAATCACACCCCGAAGAC rp:CTATGGGGATGAGGCTGTGAG

the NIGMS Human/Rodent Somatic Cell Hybrid Mapping Panel #2 from Coriell Cell Repositories (Figure 1).

Detection of deletions using PCR-based polymorphisms. PCR-based polymorphic markers and primer sequences used in the study were derived from the Genethon [15] and CHLC (http://www.chlc.org/) genetic maps. In the wide genome screening, a minimum of one highly polymorphic marker on each chromosome arm was used (data not shown); markers are available on request. The markers used in the chromosome 3 study are listed in Table 2. PCR conditions were according to our previously published procedures [3].

DNA from normal tissue and from corresponding tumour tissue of each patient were run in adjacent lanes and the patterns compared. Three different patterns were obtained. (1) Loss of heterozygosity (LOH); the patients' normal DNA was heterozygous and one of the alleles was missing in the corresponding tumour DNA. (2) No loss of heterozygosity (no LOH); the patients' normal DNA was heterozygous and the corresponding tumour DNA was identical. (3) Non-informative (n.i.); the patient was homozygous for the polymorphism in the normal DNA, making it impossible to detect possible deletion in tumour DNA.

RESULTS

Representational difference analysis

In two of the three neuroblastoma DNA samples used in the RDA experiment, difference products were obtained. The obtained difference products were fragments with lengths ranging from 275 to 595 bp (see Table 1). We sequenced the 10 fragments in both directions (data not shown) and the sequences were compared to other known sequences in the on-line computer libraries FASTA, BLAST and Grail. FASTA and BLAST found similarities to a number of different sequences, but the Grail search for indications of exon sequences did not result in any similarities. The BLAST search with hits with $P < 0.05$ are listed in Table 1.

PCR primers were designed from the 10 sequenced RDA clones and PCR amplification using these primers resulted in PCR products with lengths shown in Table 1. The PCR assay was used to localise the difference products to chromosomes (Figure 1). Four of the products were localised to chromosome 1 (products from patient 55 and 174), and five to chromosome 3 (products from patient 174). Clone 4 could not be localised to a chromosome.

Using the PCR assay from each of the 10 difference products on normal and tumour genomic DNA from corresponding patients, nine of the ten difference products represented hemizygous deletions on chromosomes 1 and 3. Thus, the expected PCR fragment existed in both the normal and in tumour DNA; a homozygous deletion would not have given any signal in the tumour DNA at all.

One of the 10 difference products, with an expected PCR product of 144 bp, could only be found in the normal DNA of patient 174, not in the corresponding tumour. The parents of this patient were also tested and this fragment could not be found in any of them (Figure 2). A number of control DNAs and normal DNA from other neuroblastoma patients were tested as well, but none showed a positive signal for this fragment.

Wide genome study

LOHs were found on several different chromosomes in the neuroblastoma patients, mainly in the low stage (stage 1, 2 and 4S) neuroblastomas. In neuroblastoma stage 1 DNA samples, LOHs were found on five different chromosomes, i.e. 4q, 5q, 8p, 10q and 11p. In stage 2 neuroblastomas, LOHs could be detected in chromosome regions 9q, 10q and 19p. Neuroblastoma stage 4S showed deletions on chromosomes 3p, 4p, 4q, 5p, 8p, 8q, 9q and 11p. In stage 3 tumours, LOHs were found in chromosome regions 3p and 6q. In neuroblastoma stage 4 patients with no deletions on 1p, one LOH was found in chromosome region 14q. The patients with neuroblastoma stage 4 with deletions on 1p also showed one LOH in chromosome region 3p.

Chromosome 3p study

A more detailed study was performed with a larger patient material and more PCR-based polymorphic markers mapped on chromosome 3 were used (Table 2). Nine neuroblastoma tumours of 58 tested (16%) had deletions in chromosomal region 3pter–p14.2. LOH was found in neuroblastomas of all stages: five of 35 (14%) non-metastatic tumours (two stage 1, one stage 2 that later progressed to stage 4, one stage 4S and one stage 3) and four of 23 (17%) tested stage 4 neuroblastoma tumours (Table 2).

One of the samples (174) had a breakpoint between markers *D3S1295* and *D3S1766*, and another tumour (St 102) had a breakpoint between markers *D3S1768* and *D3S1766*. This defines the proximal border of the smallest region of overlap. The distal border is defined by an interstitial deletion in one tumour (189). The deletion breakpoints are between distal markers *D3S1286* and *D3S1768* and proximal markers *D3S1766* and *D3S1598*. Taken together these data give a smallest region of overlap (SRO) of deletion between markers *D3S1286* and *D3S1766*, i.e. 3pter–p14.2 (Figure 3).

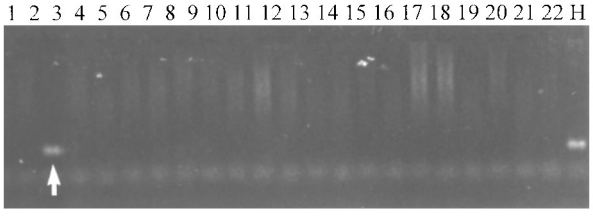

Figure 1. Chromosome localisation of one difference product using a PCR assay against NIGMS human/rodent somatic cell hybrid mapping panel #2. Each hybrid contains one human chromosome indicated above each lane, H is a positive control (human DNA). The arrow indicates a positive signal on chromosome 3.

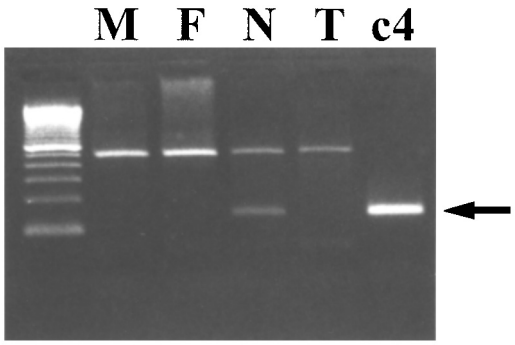

Figure 2. PCR of difference clone 4 against mother (M), father (F), normal (N) and tumour (T) DNA of patient number 174. A plasmid containing the difference product is used as positive control (c4). The arrow indicates the difference product.

Table 2. Clinical parameters and experimental data from patients with neuroblastoma and chromosome 3 deletion

Patient no.	181	St 107	121	162	153	174	189	St 99	St 102
Stage	1	1	2a	4S	3	4	4	4	4
Outcome	N	N	D	N	N	D	A	A	N
Marker									
D3S1270	●	●	●	●	—	●		●	
D3S2387	●	●	●	●	●	●	○	—	●
D3S2405	●	●	●	●	—	●		●	
D3S1286	●	●	●	●	—	●	○	●	●
D3S1768	●	—	—	●	●	●	●	●	●
D3S1295						●			
D3S1766	●	●	●	—	●	○	●	●	○
D3S1598	●	—	—	●	—	○	○	—	—
D3S1769	●	●	○	—	—	○		—	
D3S1764	●	●	○	—	●	○		○	
D3S1754	●	●	—	●	●	○		○	

Patient no: patient identification number. Outcome: N, No evidence of disease; D, dead from disease; A, alive with disease; LOH analysis: black circles = LOH; white circles = No LOH; minus sign = not informative.

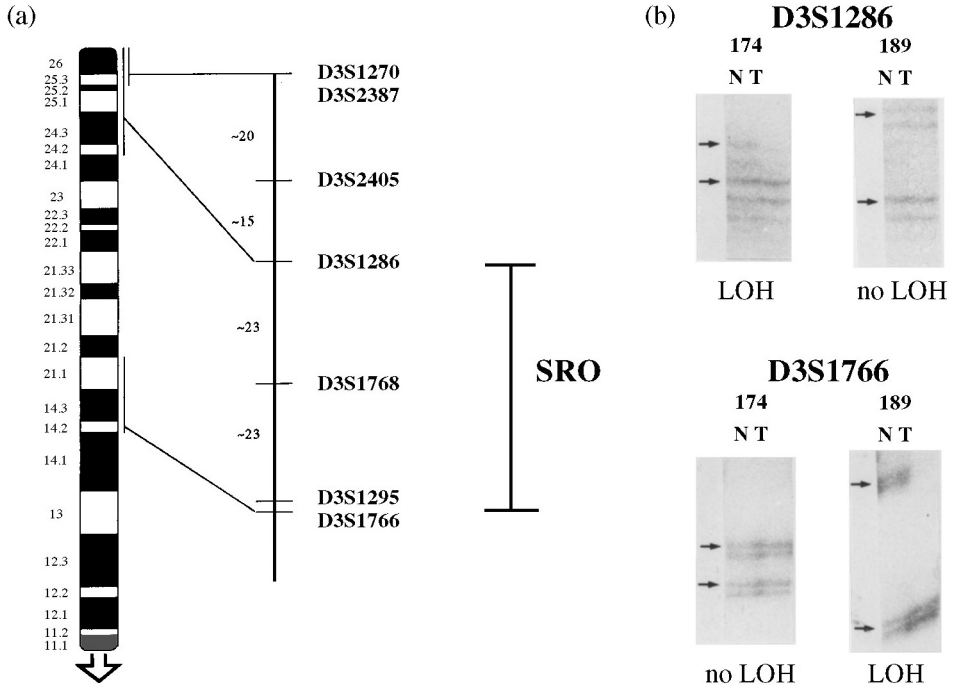

Figure 3. Analysis of 3p LOHs in neuroblastoma tumours. (a) a map of PCR-based markers from the Genethon map and the CHLC map in the chromosome 3p region used in this study, approximate centiMorgan distances are indicated between markers. (b) PCR analysis of normal DNA (N) and corresponding tumour DNA (T) in patients 174 and 189.

DISCUSSION

Recently, at least two ingenious methods have been devised for comparing genomes, i.e. the RDA (representational difference analysis) and the GMS (genomic mismatch scanning) techniques [16]. Briefly, the RDA technique compares 'representative' parts of two genomes and differences between them can be readily detected using a series of enzymatic digests, ligations and rounds of PCR.

We performed RDA on our neuroblastoma material and detected 10 difference products that we have characterised. Nine of the 10 RDA products were results of RFLPs on hemizygously deleted regions in the tumour. This result is not surprising because of the earlier known large deletions on one chromosome 1p copy in the tumour DNA of the tested patients [3]. The patients were chosen because of the unequivocal deletions and total absence of normal DNA. Nevertheless, one new deletion was found on chromosome 3 in one of the patients (pt 174).

One of the difference products (5610) appears to be a *de novo* mutation in the constitutional DNA of the patient since the fragment could not be detected either in the parents or in any of the control DNAs tested. This unique fragment was lost in the tumour DNA. The fragment could be a product of, for instance, a virus infection, although the FASTA, BLAST

and Grail computer searches did not give any results. The insertion might interrupt or damage a gene important for neuroblastoma tumour formation. The fact that the aberrant allele is lost in the tumour is puzzling, but could be explained by a model in which this gene is important for tumour initiation rather than tumour progression. The fact that it was found in a stage 4 neuroblastoma patient and lost in the tumour of the patient is intriguing.

Neuroblastoma is a disease with a number of different stages, from non-metastasising to aggressive metastasising tumours. Generally it is thought that at least one tumour suppressor gene important for neuroblastoma tumour formation is located in chromosome region 1p36 [2], and that *MYC*N amplification plays a role in some of the tumous [2]. Many, but not all neuroblastomas have deletions on chromosome 1p and/or *MYC*N amplification. Thus it is unlikely that this complicated disease can be explained by these two factors alone.

We performed a wide genome study with PCR-based polymorphic markers with a subset of neuroblastoma tumours in order to detect deletions in our material, other than those in chromosome 1p. Interestingly, there were several deletions on a number of different chromosomes in low-stage neuroblastomas (stage 1, 2 and 4S), but only a few in neuroblastoma stages 3 and 4. This is further evidence that low-stage neuroblastomas and higher stage neuroblastomas may be distinct diseases.

Other groups have reported allelic losses in other chromosomal regions apart from 1p, e.g. on chromosomes 4 [4], 11 [5], 14 [6] and 17 [7, 8]. In our material we did not find a high incidence of allelic losses in any of these chromosomes, instead the second most deleted region detected in our study was located in chromosome 3p (16%) after chromosome 1p deletions (26%) [3].

A few cases of chromosome 3 aberrations in neuroblastoma have previously been reported. One patient had a dup 3q syndrome [17] and one fetus with neuroblastoma had a partial dup 3q, unbalanced translocation 3;10 [18]. It appears that in these duplicate syndromes one entire chromosome is often duplicated and the other is missing except for one remaining part, in this case 3q. This part is located as an extra part on another chromosome. This means that in dup 3q, one allele of 3p is missing and the other is duplicated.

Homozygous and heterozygous deletions in this region on chromosome 3 have frequently been detected in a number of different cancers, e.g. lung [9], breast [10] and colon cancer [11]. Tumour suppressor genes important in these cancers have been shown to be located in this region. A mutated tumour suppressor gene can be the cause of a number of different malignancies, depending on the original cell type and what other genes are involved in the particular case. It is possible that one or more of the tumour suppressor genes located on chromosome 3p, already known to be important in other cancers, could also play a role in neuroblastoma aetiology.

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