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SHORT REPORT

Molecular characterization of an 11q14.3 microdeletion associated with leukodystrophy

Cyril Goizet*[,1,2,3], Isabelle Coupry[1,3], Caroline Rooryck[1], Laurence Taine[2], Virginie Dormoy[1], Didier Lacombe[1,2] and Benoît Arveiler[1,2]

[1]*Laboratoire de Génétique Humaine, Développement et Cancer, Université Victor Segalen Bordeaux 2, F-33076, Bordeaux, France;* [2]*Service de Génétique Médicale, Hôpital Pellegrin-Enfants, Bordeaux, France*

Leukodystrophies represent a heterogeneous group of rare hereditary diseases affecting the central nervous system. The underlying molecular defect remains unknown in almost 50% of cases. We previously assigned a new locus for leukodystrophy of unknown cause to chromosome 11q14.3 by identifying a *de novo* microdeletion in a sporadic case. We now report the precise molecular characterization of this microdeletion. Physical mapping of the region of interest allowed us to identify and analyze candidate gene(s) possibly implicated in leukodystrophy.
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Introduction

Leukodystrophies constitute a heterogeneous group of genetic diseases affecting the white matter of the nervous system. Almost half of the patients have leukodystrophies of unknown cause (LUC).[1,2] We recently described a *de novo* 11q14 microdeletion in a boy with LUC and tyrosinase-negative oculocutaneous albinism (OCA1).[3] The analysis of the *TYR* gene, known to be responsible for OCA1, revealed a del244/245TG mutation on the paternal allele, thus accounting for the OCA phenotype observed in this patient. The 11q14 microdeletion was identified by fluorescence *in situ* hybridization (FISH) analysis using a λ phage containing exon 1 of the *TYR* gene (coding for tyrosinase). A test for hemizygosity using eight polymorphic microsatellite markers (D11S1354, D11S4082, D11S1887, D11S2015, which was incorrectly named D11S1780 in the original report, D11S1367, D11S1342, D11S931 and D11S1358) showed a deleted maternal allele of D11S1367 in the patient. The detection of this chromosomal rearrangement suggested a new chromosomal region that probably contains a gene involved in the pathogenesis of leukodystrophy. We now present the molecular characterization of the limits of this deletion, in order to identify and analyze candidate gene(s) implicated in leukodystrophy.

*Correspondence: Dr C Goizet, Laboratoire de Génétique Humaine, Développement et Cancer, Université Victor Segalen Bordeaux 2, 146, Rue Léo Saignat, 33076 Bordeaux cedex, France. Tel : +33 5 57 57 16 01; Fax : +33 5 56 98 33 48; E-mail : cyril.goizet@chu-bordeaux.fr
[3]These authors contributed equally to this work.
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Materials and methods

Testing for hemizygosity

In all, 11 already known microsatellite markers genetically mapped in the critical interval between D11S1354 and D11S1342 were selected from database searches through the Unified Database (UDB), the Genome Database (GDB) and the Genethon map (Table 1). Six new markers (AP811$(TA)_{14}$, AP3497(TA), AP3497$(CA)_{26}$, AP3497$(CA)_{16}$, AP720$(CA)_{17}$ and AP1815$(TA)_{13}$) located close to the deletion breakpoints were identified by annotation of the contig sequence. PCR procedures are available from the authors on request.

Construction of a BAC contig

To generate a BAC contig, the sequences of the 19 microsatellite markers and six genes described in the

genome databases were used to identify genomic 11q14.3 BAC sequences from this region using Advanced BLAST against the High Throughput Genome sequences division (htg). These BAC sequences were separated into their component contig when the sequence remained unfinished, masked for repetitive sequences and systematically used to identify additional BACs by searching the htg with BLAST. All BLAST alignments above a threshold of 98% identity over at least 2000 bp were manually examined using BLAST 2 Sequences to order the sequence contigs of each BAC, to estimate clone overlaps and to ensure that the patterns of nonmatching sites were compatible with sequencing errors.

Fluorescence *in situ* hybridization

In all, 13 BACs localized in the region of interest close to the TYR locus were used as probes for FISH experiments. The selected BACs were all RPCI-11 human BAC clones obtained from the BACPAC resources (Children's Hospital Oakland Research Institute). DNA was extracted using the Plasmid Midi Kit (Qiagen) following the manufacturer's instructions. The BACs were labelled with digoxigenin-11-dUTP using the Dig-Nick-translation kit (Boehringer Mannheim Inc.). *In situ* hybridization using the labelled BACs was performed as previously described.[3]

PCR amplification and sequencing of candidate genes

Mutation search in three candidate genes, *CTSC* coding for the lysosomal protease cathepsin C, *GRM5* coding for the metabotropic glutamate receptor type 5 (mGluR5) and LOC143680 corresponding to a putative gene (accession number XM_096474) expressed in the brain, was performed by direct sequencing of the entire coding sequences.

Primers used for PCR amplification and sequencing of *CTSC* exons 2–7 were those previously described.[4] Intronic primers were derived in order to amplify and sequence the entire coding region and intron–exon boundaries of *CTSC* exon 1, *GRM5* exons 1–8 and LOC143680. The primers' sequences, PCR and sequencing procedures are available from the authors on request.

Results

Construction of a 5.6 Mb BAC contig in 11q14–q21

Our previous data[3] indicated that the patient with OCA and leukodystrophy presented a deletion encompassing the tyrosinase (TYR) locus and marker D11S1367. Markers D11S1887 and D11S2015 on the proximal side, and D11S931, D11S1358 and D11S1342 on the distal side were not deleted. The size of the region of interest remained very uncertain, since no physical map was available at that time.

Therefore, in order to characterize further the deletion, a contig map of the region was constructed. The sequences of 19 microsatellite markers and of all the genes known to map to the 11q14–q21 region between markers D11S1354 and D11S1342 were used in an Advanced BLAST search against the htg and nr sections. Microsatellite markers were the eight listed in the Introduction section and the additional 11, described in Table 1. Genes already mapped in the region were those coding for cathepsin C (*CTSC*), embryonic ectoderm development (*EED*), homolog of drosophila frizzled number 4 (*FZD4*), metabotropic glutamate receptor type 5 (*GRM5*), malic enzyme type 3 (*ME3*) and tyrosinase (*TYR*).

The sequences of the BAC clones thus identified were in turn compared to one another by BLAST 2 Sequences in order to define clone overlaps, allowing us to construct 'primary contigs'. Sequences of both ends of these contigs were then used in an Advanced BLAST against the htg Database.

Using this strategy, we progressively obtained a contig of 51 BAC clones with only one gap of unknown size that persists between BACs AP003388 and AP003169. The sequence of all of the BACs was annotated (Figure 1). Our data corroborate the physical map that has recently become available on the NCBI Map Viewer while this manuscript was in preparation.

Testing for hemizygosity in the patient

Based on our physical map data, 11 microsatellite markers from the 11q14.1–q14.3 region located between D11S1354 and D11S1342 were selected to search for hemizygosity in our patient. Genotyping results of the patient and his parents are presented in Table 1. These data showed the existence of a deletion for markers D11S1780 and D11S870 on the maternal haplotype.

FISH results

In order to confirm the location of the 11q14.3 deletion breakpoints, 13 BAC clones were selected from our contig and tested by FISH on the patient's chromosomes. The BACs were all localized between the first not deleted microsatellite markers on the proximal and distal sides of the deletion (D11S1887 and D11S4175, respectively) (Figure 1). Results are presented in Table 2. The BACs delimiting this deletion are AP000811 on the centromeric side and AP0001815 on the telomeric side.

Fine mapping of the proximal and distal deletion boundaries

New polymorphic microsatellite markers were then searched for by screening of the sequence of the BACs located at both ends of the deletion with the RepeatMasker server program, in order to refine the hemizygosity

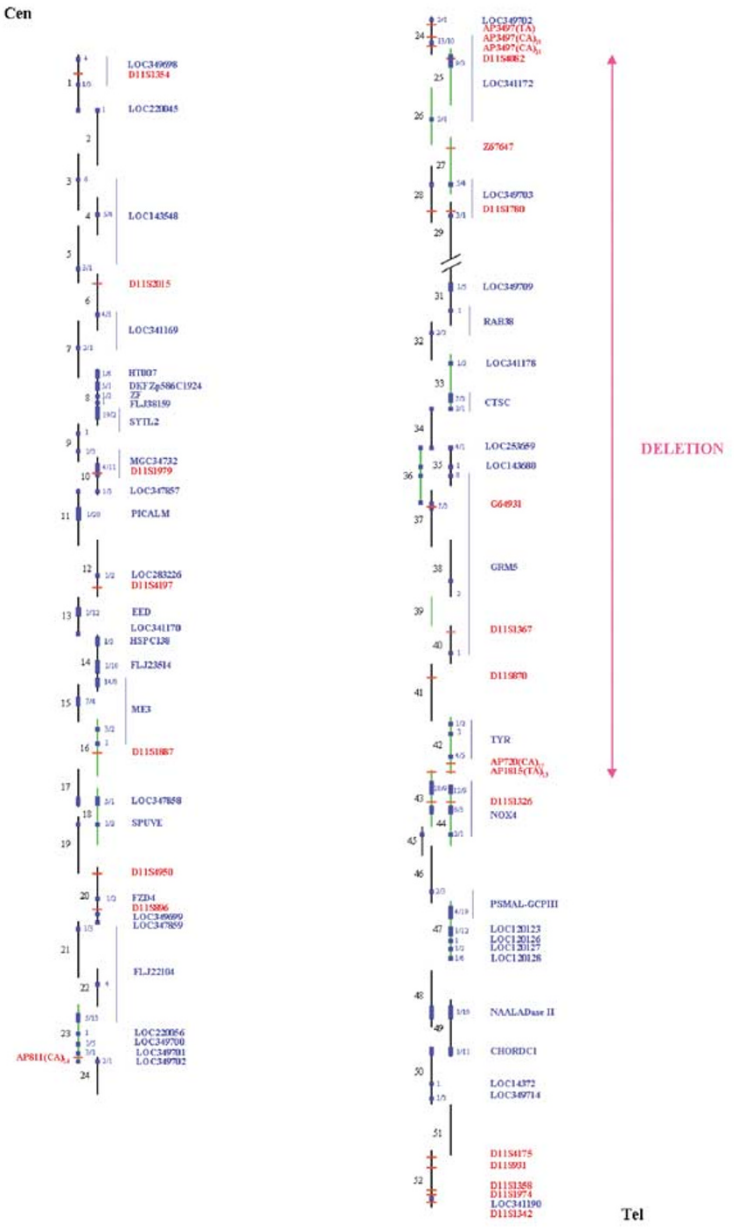

Figure 1 Physical map of chromosome 11q14.3. BACs were ordered from centromere (Cen) to telomere (Tel), and numbered from 1 to 52 (see below). Microsatellite markers are indicated in red, genes (genuine or putative) are in blue and BACs are in black, unless they were tested by FISH (in green). Exons are figured by numbered boxes. The intron–exon structure and direction of transcription of the genes was determined by BLAST 2 Sequences of the cDNA sequences against the genomic sequence. The remaining gap is indicated by a double dashed line. The extent of the deletion is represented by a pink double arrow. (1) AP000857; (2) AP000639; (3) AP003093; (4) AP003095; (5) AP002803; (6) AP003035; (7) AP002878; (8) AP000642; (9) AP000974; (10) AP003128; (11) AP000867; (12) AP003097; (13) AP003084; (14) AP001148; (15) AP002492; (16) AP001831; (17) AP003059; (18) AP002890; (19) AP000654; (20) AP001528; (21) AP002967; (22) AP001102; (23) AP000811; (24) AP003497; (25) AP001784; (26) AP000756; (27) AP000676; (28) AP000749; (29) AP003388; (31) AP003169; (32) AP001642; (33) AC011088; (34) AP000795; (35) AP003120; (36) AC024733; (37) AC026168; (38) AC026201; (39) AP001828; (40) AP000626; (41) AP001482; (42) AP000720; (43) AP001815; (44) AP001927; (45) AP002404; (46) AP003400; (47) AP003122; (48) AP000827; (49) AP000648; (50) AP002364; (51) AP002003; (52) AP000684.

Table 1 Testing for hemizygosity

Microsatellite markers	*Allele size (bp)*			*Comments*
	Father	*Mother*	*Patient*	
D11S1979	253–258	250–253	253	NI
D11S4197	256–256	264–270	256–264	ND
D11S4950	137–137	137–139	137	NI
D11S896	176–178	176–178	176	NI
AP811(CA)$_{14}$	146–160	160–160	160	NI
AP3497(TA)	218–238	217–219	218 —	D
AP3497(CA)$_{26}$	189–189	181–181	189 —	D
AP3497(CA)$_{16}$	180–182	180–182	182	NI
Z67247	230–232	232–232	232	NI
D11S1780	183–191	173–189	191 —	D
G64931	204–204	200–204	204	NI
D11S870	156–162	156–156	162 —	D
AP720(CA)$_{17}$	185–187	187–191	187	NI
AP1815(TA)$_{13}$	164–198	166–194	198 —	D
D11S1326	250–252	250–250	250	NI
D11S4175	151–151	151–182	151–182	ND
D11S1974	118–119	118–119	118–119	ND

Newly identified microsatellite markers are written in bold. Allele sizes are indicated in base pairs (bp) for each marker. ND = not deleted; NI = not informative; D = deleted.
Six novel microsatellite markers were thus identified, and named after the BAC clone they were derived from: AP811(TA)$_{14}$ (heterozygosity: $n = 60$; 80.3%; 10 alleles), AP3497(TA) (heterozygosity: $n = 64$; 85.3%; 16 alleles), AP3497(CA)$_{26}$ (heterozygosity: $n = 58$; 79.4%; 12 alleles), AP3497(CA)$_{16}$ (heterozygosity: $n = 60$; 62.6%, four alleles), AP720(CA)$_{17}$ (heterozygosity: $n = 62$; 76.1%, seven alleles), AP1815(TA)$_{13}$ (heterozygosity: $n = 60$; 84.5%; 14 alleles). All markers were submitted to dbSTS with the accession numbers BV088069, BV088068, BV088067, BV088066, BV088065 and BV088064, respectively.

Table 2 FISH analysis results using RPCI-11 BAC clones on 11q14.3

Accession no.[a]	*BAC clone*	*Genes or microsatellite markers*	*Size (bp)*	*FISH results*
AP001831 [16]	RP11-317J19	ME3, D11S1887	185919	Not deleted
AP002890 [18]	RP11-345O18	LOC347858, SPUVE	184523	Not deleted
AP000811 [23]	RP11-777F6	FLJ22104, LOC220056, LOC349700, LOC349701, LOC349702, AP811(CA)$_{14}$	176953	Not deleted
AP001784 [25]	RP11-137O10	LOC341172 (ex 3–9), D11S4082	112800	**Deleted**
AP000756 [26]	RP11-720D4	LOC341172 (ex 1–2)	171027	**Deleted**
AP000676 [27]	RP11-665E10	LOC349703 (ex 4,5), Z67247	161289	**Deleted**
AC011088 [33]	RP11-292E14	CTSC, LOC341178 (ex 1–3)	164991	**Deleted**
AC024733 [36]	RP11-577L15	GRM5 (ex 7–8), LOC143680, LOC253659 (ex 1–4)	225231	**Deleted**
AP001828 [39]	RP11-243E18	GRM5	137384	**Deleted**
AP000720 [42]	RP11-796A5	TYR, AP720(CA)$_{17}$, AP1815(TA)$_{13}$	147357	**Deleted**
AP001815 [43]	RP11-97D10	NOX4 (ex 13–18), D11S1326, AP1815(TA)$_{13}$	167653	Not deleted
AP001927 [44]	RP11-482J6	NOX4 (ex 1–12), D11S1326	188256	Not deleted
AP003122 [47]	RP11-313I2	PSMAL/GCPIII (ex 4–19), LOC120123, LOC120126, LOC120127, LOC120128	137888	Not deleted

[a]The clone numbers used in Figure 1 are written in brackets.
Clones deleted are in bold type.
The presence of various STSs in the BACs was verified experimentally by PCR: D11S1354 (AC019020), D11S1887 (AP001831), D11S4082 (AP001784), Z67247 (AP000676), *CTSC* exons 4 and 7 (AC011088), *GRM5* exons 7 and 8 (AC024733), *TYR* exons 1–5 (AP000720), D11S1326 (AP001815). In all instances, a specific PCR product of correct size was amplified (data not shown).

analysis in the patient. BACs AP000811 and AP003497, and AP000720 and AP001815, located on the proximal and distal sides respectively, were investigated. Six novel microsatellite markers with CA/GT or TA/AT repeated motifs were identified and tested on 58–62 control chromosomes in order to verify their polymorphic status and to estimate their informativity (Table 1).

Analysis of the patient and his parents with these markers (Table 1) showed that AP3497(TA) and AP3497(CA)$_{26}$ (proximal side) were deleted, indicating that the deletion extended at least toward the proximal end of BAC AP003497. Similarly, marker AP1815(TA)$_{13}$ was also deleted in the patient, suggesting that the distal boundary of the deletion was located within the distal end of BAC AP001815.

Taken together, our data allowed us to localize the centromeric boundary of the deletion between microsatellite AP3497(TA) and the distal end of BAC AP000811, and the telomeric boundary in the proximal part of AP001815 (Figure 1). The deleted chromosomal segment is therefore about 2 Mb in size.

Analysis of candidate genes

The annotation of the whole region's sequence revealed the presence of four known genes (*RAB38*, *CTSC*, *GRM5* and *TYR*) and seven putative genes in the deleted region (Figure 1). *RAB38* is not expressed in the mature brain[4] and was therefore not considered as a candidate gene for leukodystrophy. The *TYR* gene is known to be responsible for OCA observed in this patient.[3] *CTSC* is already known to be involved in Papillon–Lefèvre syndrome,[5] a rare autosomal recessive entity without central nervous impairment, making this gene not a strong candidate. However, since several forms of leukodystrophies are due to lysosomal defects,[1] a mutation search was performed in this gene, knowing that different mutations of the same gene can lead to entirely different phenotypes, for example, mutations of *LMNA* involved in up to seven different disorders (MIM# 150330). *GRM5* was considered as a strong functional candidate. Indeed, mGluR5 belongs to the family of G-protein-coupled receptors involved in responses to glutamate, is expressed in oligodendrocytes and is involved both in myelination and the development of white matter damage.[6] Using direct sequencing, no mutation was identified in the patient in the coding region and at the intron–exon boundaries of either *CTSC* or *GRM5*.

Among the seven putative genes located in the deletion, LOC143680 was the only one to be clearly expressed in the brain. The coding region of this putative gene, derived from three IMAGE clones (AF052171, BC047497, U79242), was analyzed by direct sequencing, but failed to reveal any mutation. Two other putative genes, LOC253659 and LOC341172, were considered as being pseudogenes of GAPDH (MIM# 138400) and HMG4 (MIM# 300193), respectively. Indeed, LOC253659 shares only five out of the nine exons of GAPDH, with a 92% sequence homology. In addition, the predicted structure of LOC341172 suggests the presence of 13 exons, while the HMG gene family members, including HMG4, contain only five exons.

For the last four putative genes, no indication could be found in the various databases that would suggest that they were expressed in the developing and/or mature brain. This will have to be addressed experimentally by either Northern blot or RT-PCR analysis.

Discussion

We molecularly characterized an 11q14.3 microdeletion that was previously identified in a patient with LUC.[3] We constructed a contig of 51 BACs covering the entire region of interest. Only one gap of undetermined size located inside the deleted region between BACs AP003388 and AP003169 (Figure 1) persists in the contig. A screening of BAC libraries using PCR primers recognizing the ends of the two BACs that straddle the gap will be performed in the future. A second region located in a segment 500 kb distal to the telomeric boundary of the deletion remains incompletely characterized, probably due to sequencing difficulties resulting from the fact that this latter region belongs to a chromosomal region located inside an ancient duplication involving an at least 1 Mb large block of DNA on both sides of the centromere in 11p11 and 11q14.[7]

This contig refines the physical map of 11q14.3. We characterized new genetic resources of this region (microsatellite markers and BAC clones) and estimated the extent of the 11q14.3 microdeletion at about 2 Mb. The sequence of the whole region was annotated, revealing the presence of four known genes and of seven putative genes in the deleted region. Mutation search in the coding sequences of the best functional candidate genes, *CTSC* and *GRM5*, and in the brain-expressed putative gene, LOC143680, was negative. Our data almost rule out a recessive mutation of either of these genes as being responsible for the leukodystrophy phenotype. Implication of *CTSC* in the phenotype is unlikely since heterozygous carriers of null alleles of this gene are asymptomatic.[8] A dominant effect by haploinsufficiency remains possible for *GRM5* and LOC143680; in order to investigate this possibility, an extensive mutation search in a population of patients with LUC will be undertaken.

A last hypothesis is that the deletion affects the expression of a gene(s) located outside of the deletion by a position effect. Several examples of this phenomenon have been described, showing that the expression of genes located as far as 900 kb from a chromosomal rearrangement breakpoint can be affected and lead to a disease.[9]

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Electronic databases

UDB: http://bioinformatics.weizmann.ac.il/udb;GDB: www.gdb.org; Advanced BLAST: http://www.ncbi.nlm.nih.gov/BLAST;BLAST 2 Sequences: http://www.ncbi.nlm.nih.gov/gorf/bl2.html;NCBI Map Viewer: http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi; RepeatMasker server: http://woody.embl-heidelberg.de/repeatmask/

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