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ARTICLE

Giant axonal neuropathy locus refinement to a < 590 kb critical interval

Laurent Cavalier[1], Christiane BenHamida[2], Rim Amouri[2], Samir Belal[2], Pascale Bomont[1], Nadège Lagarde[1], Laëtitia Gressin[3], David Callen[4], Ercan Demir[5], Haluk Topaloglu[5], Pierre Landrieu[6], Catherine Ioos[7], Mongi Ben Hamida[2], Michel Kœnig[1] and Fayçal Hentati[2]

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, CU de Strasbourg, France; [2]Institut National de Neurologie, La Rabta, Tunis, Tunisia; [3]Centre d'Etude du Polymorphisme Humain, Paris, France; [4]Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, North Adelaide, SA, Australia; [5]Hacettepe University Children's Hospital, Ankara, Turkey; [6]Département de Pédiatrie, Service de Neurologie, Hôpital Universitaire de Bicêtre, Kremlin-Bicêtre, France; [7]Service de Pédiatrie, Hôpital Raymond Poincaré, Garches, France

Giant axonal neuropathy (GAN) is a rare autosomal recessive neurodegenerative disorder, characterised clinically by the development of chronic distal polyneuropathy during childhood, mental retardation, kinky or curly hair, skeletal abnormalities and, ultrastructurally, by axons in the central and peripheral nervous systems distended by masses of tightly woven neurofilaments. We recently localised the GAN locus in 16q24.1 to a 5-cM interval between the D16S507 and D16S511 markers by homozygosity mapping in three consanguineous Tunisian families. We have now established a contig-based physical map of the region comprising YACs and BACs where we have placed four genes, ten ESTs, three STSs and two additional microsatellite markers, and where we have identified six new SSCP polymorphisms and six new microsatellite markers. Using these markers, we have refined the position of our previous flanking recombinants. We also identified a shared haplotype between two Tunisian families and a small region of homozygosity in a Turkish family with distant consanguinity, both suggesting the occurrence of historic recombinations and supporting the conclusions based on the phase-known recombinations. Taken together, these results allow us to establish a transcription map of the region, and to narrow down the GAN position to a < 590 kb critical interval, an important step toward the identification of the defective gene. *European Journal of Human Genetics* (2000) **8**, 527–534.

Keywords: giant axon; Bac/Yac contig; homozygosity mapping; linkage disequilibrium; haplotype analysis; *GAN* gene

Introduction

Giant axonal neuropathy (GAN; MIM No. 256850) is a rare autosomal recessive neurodegenerative disorder. Since the initial study,[1,2] fewer than 100 patients have been reported (no prevalence estimate is available). Clinically, GAN patients present with a chronic distal polyneuropathy during childhood, mental retardation, kinky or curly hair and skeletal abnormalities. Ultrastructurally, GAN is characterised by the segmental distension of axons in the central and peripheral nervous systems by masses of tightly woven 8–10 nm neurofilaments.[3] The course of the disease is severe with affected patients having a life expectancy of less than 30 years. However, data collected from a large inbred Tunisian family with slowly progressive evolution suggested a genetic or allelic heterogeneity[4] (MIM No. 256851). Axonal pathologies similar to GAN have been encountered in other conditions.[5] However, despite ultra structural similarities in the accumulation of neurofilaments across the different conditions, the

Correspondence: Michel Kœnig, IGBMC, 1 rue Laurent Fries, BP 163, 67404 Illkirch Cedex, France. Tel: +33 3 8865 3399; Fax: +33 3 8865 3246; E-mail: mkoenig@igbmc.u-strasbg.fr; or Christiane BenHamida, Institut National de Neurologie, La Rabta 1007, Tunis, Tunisia. Tel: +216 1 564 421; Fax: +216 1 565 167; E-mail: faycal.hentati@ms.tn
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type of axon and the site of accumulation within neurons varies greatly. As the pathogenesis of axonal neurofilament accumulation is not known, the identification of the gene(s) involved in the familial forms should help in the understanding of the pathophysiological process of neurofilament accumulation.

We have reported recently, using homozygosity mapping in three consanguineous Tunisian families, that the autosomal recessive *GAN* gene maps precisely to a 5-cM region in chromosome 16q24.1 flanked by markers D16S507 and D16S511.[5] The lod score between the linked haplotype and the disease locus is 14.2 at $\theta_{max} = 0$, and this linkage has been confirmed in five consanguineous families of various origin (Italy, Nova Scotia, Iran and Pakistan), despite a comparative difference in severity between these cases and the Tunisian cases.[6]

We have now established a contig-based physical map of the 5-cM region comprising 27 YACs and 15 BACs, and we placed four genes, ten ESTs, three STSs, and two additional microsatellite markers to generate a transcription map of the locus. We have identified six SSCP polymorphisms in two genes, two ESTs and two STSs, and six new microsatellite markers (one from one EST and five isolated from the BACs). These additional markers localised within the candidate interval were used to investigate linkage disequilibrium in the Tunisian families and to analyse recombinations in GAN families. Taken together, these results allow us to establish a transcription map of the region and to narrow down the GAN locus to a <590 kb critical interval, an important step toward the identification of the defective gene.

Subjects, materials and methods

Subjects

Five Tunisian families with GAN symptoms were available for study. Families I, II and III have been previously described.[4,5] Families IV, V and new branches of families I and III have been recently identified. During childhood, all affected individuals from Tunisian families develop progressive gait instability and suffer frequent falls, distal amyotrophy of the legs, deep sensory loss and abolition of deep tendon reflexes, with normal or curly hair. The affected members of family II present with a slowly progressive form of GAN that is associated with multisystem degeneration without kinky hair.[4] Families I and V are from southern Tunisia, family IV is from central Tunisia, and families II and III are from north-east Tunisia.

A further seven families from more widespread geographic origins (three from France and families from Morocco, USA, Sri Lanka and Turkey) have been recently identified, with one affected individual in each family. Parents of the Turkish family share remote consanguinity. All these families present with classical clinical and histopathological GAN symptoms.

Isolation and analysis of YACs and BACs

YACs and BACs were isolated from the CEPH library[7] by PCR screening using D16S507, D16S3098, D16S505 and D16S511 microsatellite markers[8] located in and flanking the GAN critical region.[5,6]

Mapping of ESTs and STSs

ESTs and STSs sequences were retrieved from public web sites: National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genemap98/), Whitehead Institute Center for Biomedical research (http://www-genome.wi.mit.edu/), Généthon (http://www.genethon.fr), Cooperative of Human Linkage Center (CHLC; http://www.chlc.org/) and, for chromosome 16 only sequences, Los Alamos National Laboratory (LANL; http://www-ls.lanl.gov).[9–17] Primer sequences, expected PCR product sizes and PCR conditions to amplify parts of these sequences were obtained from these web sites. The ESTs and STSs were PCR amplified from genomic DNA isolated from somatic human–mouse hybrid cell lines that contain a single human chromosome 16 fragment[18] and electrophoresed on sequencing gels. ESTs and STSs located on 16q24.1 were PCR amplified from the YAC contig described in this study, and the products analysed on 2% agarose gels. ESTs and STSs were amplified from GAN families by PCR in the presence of ^{32}P-dCTP and analysed by SSCP on large native polyacrylamide gels.[19]

Identification of polymorphic microsatellite repeats

BAC clones were EcoRI digested, transferred to nylon filters and hybridised with di, tri and tetranucleotide repeat probes. Two non-overlapping BACs positive for several STRs were digested with BstY1 or Bg1II and subcloned into the BamHI site of pBluescript II$^{+/-}$ (Stratagene, La Jolla, CA, USA). Filters were screened with $(CA)_{20}$ and $(GGAT)_8$ repeat probes radiolabelled by γ^{32}P-ATP kination. Positive subclones were digested with BstYI and verified by southern hybridisation. The subclones were then sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the Taq cycle sequencing kit (Applied Biosystems) in combination with fluorescent dideoxynucleotides and vector and specific walking primers. The walking primers were hybridised back on to the same EcoRI southern hybridisation filters used for the repeat identification. Microsatellite polymorphisms were typed by PCR amplification of 200 ng of genomic DNA in a 10 μl volume.[20] Allele frequencies were determined from the normal chromosomes of parents and from population-matched unrelated individuals.

Results

Twenty-seven YACs were identified and placed in a single contig extending from D16S3040 to D16S505 (Table 1). Part of the YAC contig has been assembled using information from the primary WhiteHead Institute and LANL physical

Table 1 16q34.1 YAC contig

YACs \ ESTs STSs	d16s3040	d16s3055	d16s507	a109xe9	*138962*	C-MAF	*F22383*	*R00106*	*234766*	*39868*	WI10316	WI3429	*364493*	*193321*	*682397*	PLCγ2	GCSH	KIAA0431/LC8	d16s3098	*347183*	gaat2c08	d16s505	WI10802	*32844*	d16s511
776b7	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
869c11	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
890e11	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
782g11	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
752c6	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
776b6	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
930c3	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
933g7	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
927h5	–	+	+	+	+	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
735b9	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
798g7	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
951h7	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–
728h4	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	+	+	–	–	+	–	–	–	–
775h3	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	+	+	+	–	–	–	–
809c12	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	+	+	+	+	–	–	–	–
884a5	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	–	+	+	+	+	–	–	–	–
890a12	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+	+	–	–	–	–
682d1	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+	+	–	–	–	–
935c6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–
187f5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+	+	–
735e7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–
735d2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–
735a12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
571g8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
606b4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
494e6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–
630c11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–

Presence (+) or absence (–) of ESTs (in italics) and STSs in YACs. a109xe9, AFMa109xe9; PLC, phospholipase Cγ2; GCSH, glycine cleavage human H protein.

maps. Similarly, 15 BACs were identified and placed in a new single contig encompassing D16S3098 and D16S505 (Figure 1). D16S511 is not a part of any contig. The tiling path of the BAC contig is four.

We screened in silico genome databases for chromosome 16q sequences and localised them with respect to 16q24.1 using human–rodent hybrid cell lines (No. CY107, 115 and 121) harbouring breakpoints telomeric to D16S505, between D16S505 and D16S507, and centromeric to D16S507, respectively. Positive sequences were further mapped on the YAC contig. We mapped and ordered ten ESTs and four complete cDNA sequences (from the C-MAF, glycine cleavage human H protein, phospholipase *Cγ2* and *KIAA0431* genes) (Table 1). We also mapped two microsatellite markers (AFMa109xe9 and GAAT2C08) and three STSs (WI10316, WI3429 and WI10802). Six STSs, ESTs or genes (STSWI10316, STSWI3429, EST682397, EST347183, PLCγ2 and *KIAA0431*) appeared to be biallelic polymorphic markers on SSCP gels (Table 2).

We set out to isolate polymorphic markers from the BAC contig. Three $(CA)_n$:$(TG)_n$ and three $(GGAT)_n$:$(ATCC)_n$ new polymorphic repeats were isolated and sequenced. LC1 and LC3 clones contain 14 and 21 uninterrupted AC repeats and LC6, LC2 and LC5 clones contain 9, 11 and 12 uninterrupted GGAT repeats, respectively (Table 2). The position of markers within the BAC contig is shown in Figure 1. LC8 is an 18 AC repeat located in the 3'UTR of the *KIAA0431* gene. Therefore we now have 16 polymorphic markers over a 5-cM region, including ten markers contained in the BAC contig. Such a density allows precise mapping of recombination events as well as for searching linkage disequilibrium among families from restricted populations.

Our previous linkage study revealed two critical recombinations in consanguineous families, between GAN and markers D16S507 (centromeric) and D16S511 (telomeric), respectively (Figure 2). The telomeric recombination occurred in a healthy individual (V2 in family II), who was thought to have received the recombinant chromosome from his mother, who was not informative for D16S507 and more proximal markers.[5] Genotyping with markers GAAT2C08, LC5 and LC6 indicates that this individual is indeed recombinant but that he received the recombinant chromosome from his father. This implies the existence of a second, centromeric recombination (outside the interval shown in Figure 2) and places the distal recombination between GAAT2C08 and D16S505 (LC1, LC2 and LC3 being non-informative; Figure 2). The centromeric recombination occurred in a patient (IV6 in family I) heterozygous for D16S507 and more proximal markers. Markers AFMa109ex9,

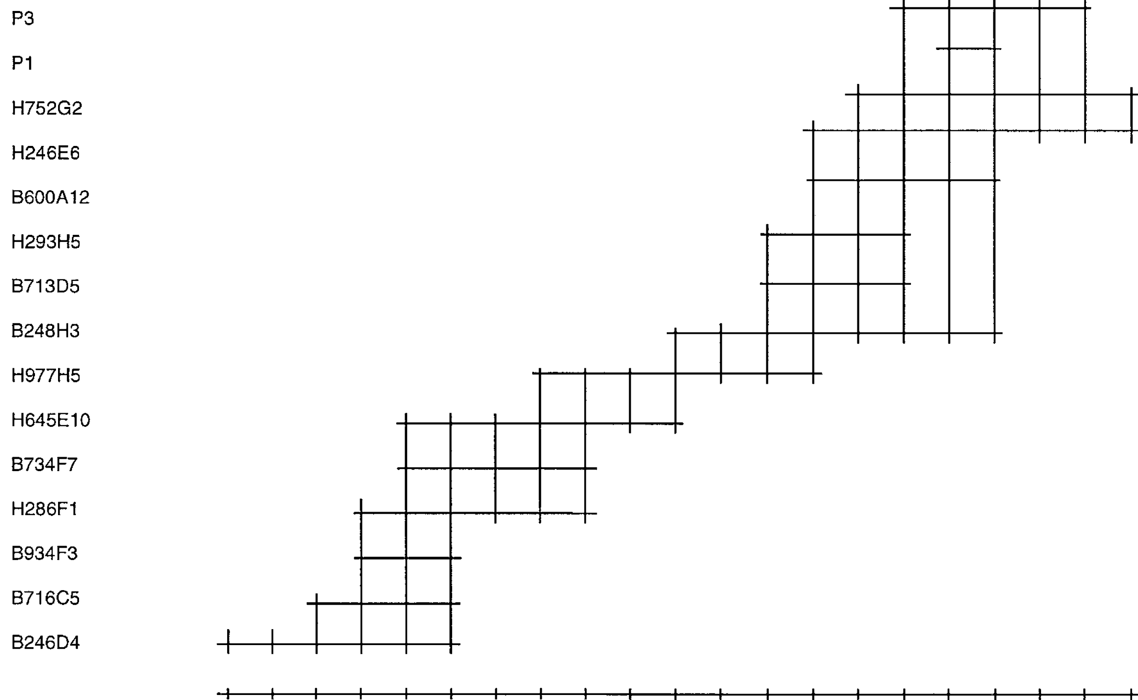

Figure 1 16q24.1 BAC/PAC contig. P1 and P3 are two PACs clones (LLNLP704M0964Q12 and LLNLP704O0764Q12, respectively) available from RZPD-Berlin (http://resource.rzpd.de/clone — details.html). The order between est347183 and d16s3098 is not defined.

Table 2 Polymorphic markers

Markers	*Oligonucleotide sequences*	*PCR conditions*	*PCR fragment*	*Type of repeat*	*No. of alleles*	*Heterozygosity, %*
LC1	gcaaacggacaaactgaatgg/tttgtttaaaggttgactaataatac	56°C	105bp	(CA)14	2	34
LC2	ccaaccctgatctaaaccatt/agcaaggagaggaaacatattc	56°C	116bp	(GGAT)11	4	62
LC3	acggactgataagtatgcaact/tgaagccagttaaatgagtcttg	56°C	340bp	(CA)21	4	48
LC5	gtagctactatatacacaatatgtc/ttcagtgagaagggagatgatc	58°C	245bp	(GGAT)12	6	65
LC6	ccacactgatcttgaactgctg/tgataaagggaatatatgccttt	56°C	275bp	(GGAT)9	4	47
LC8	agttccagtcaagtaggaatca/agctcattaggaattagtagca	56°C	200bp	(CA)18	4	49
AFMa109xe9	aagtgtctggagcagagcct/ccaaatagagagccctgaacataat	60°C	240bp	(CA)[a]	3	27
gaat2C08	ggtagagagactgccccagt/ggtgactgtgaatgtatttaatgc	60°C	279bp	(GAAT)8	2	49
stsWI10316	agcaacgtgtacaacttagtgaag/ggctgggcttttaaacatcc	60°C	175bp	STS	2	42
stsWI3429	atacgctttctgtctgtccca/gctccgtgcttggtcttaag	60°C	251bp	STS	2	48
PLCγ2[b]	gagagaagagagtcagcaacagc/aaggttttcattgctttt	60°C	285bp	gene	2	42
KIAA0431[b]	agatcaacatctcaaactttataata/ccatttcctctggattaaaact	55°C	167bp	gene	2	39
est682397	ttatgctcccagtgaaccc/atccctgtggaatgctgc	55°C	201bp	EST	2	32
est347183	taggttaattaaacagcttaaaga/aattaagtagcacgcactttgtt	56°C	334bp	EST	2	47

[a]not available on public web sites; [b]located in the 3′UTR of these genes.

Figure 2 Pedigrees of Tunisian families. Families I, II and III have been previously described in BenHamida *et al*. Simplified pedigrees of families II and III are shown here. The parents linking the left and right branches of family III are first cousins once removed. They presumably contributed the normal haplotype.

PLCγ2 and LC8 are heterozygous, placing the recombination breakpoint between LC8 and D16S3098 (Figure 2). LC6 and LC5 were not informative in the critical parent.

Nine new families with GAN diagnosis including two families from Tunisia, and seven families of different geographic origin were identified and genotyped with all the markers (Table 3). Homozygosity mapping allowed the confirmation of linkage to the 16q24.1 GAN locus in most of the families. The lod scores in favour of linkage for the Tunisian families IV and V (Figure 2) are 2.05 and 2.11, respectively. Confirmation of linkage in the Sri Lankan and the French n° 3 families is demonstrated by the homozygosity found for all markers tested and is suggestive of non-documented consanguinity in these families (Table 3). In addition, a patient from a remote consanguineous Turkish family was found to be homozygous for at least eight consecutive markers in the linked region (Table 3; the order of the D16S3098 and EST347183 markers is unknown). The first heterozygous marker on the telomeric side is EST347183. Since four of these markers are biallelic and the shared alleles often correspond to the most frequent alleles, the significance of this homozygosity has not yet been established (Tables 2 and 3).

GAN is a very rare disorder (about 100 cases described worldwide) but appears in five large unrelated Tunisian families (17 patients diagnosed). It is therefore plausible that a founder event could account for this clustering of cases, as has already been demonstrated for other autosomal recessive diseases.[21,22] We used our dense set of polymorphic markers in the critical interval to investigate the possibility of linkage disequilibrium between a given haplotype and the disease in the five Tunisian families. Families II and III, which are geographically very closely located, share a common haplotype over ten consecutive markers, from AFMa109xe9 to LC1 (Table 3). The haplotype sharing between families II and III indicates the occurrence of historic recombinations, where LC3 is the closest distal recombinant marker. Taken together with the phase known recombinations, the results define a candidate region for GAN between the LC8 and LC3 markers. This interval is entirely contained in YAC 890a12, which is 590kb in length (Whitehead Institute).

Discussion

In order to narrow down the GAN critical interval, we isolated and placed new polymorphic markers in the 5-cM critical interval between markers D16S507 and D16S511. We further characterised the YAC contig extending from D16S507 to D16S505, spanning a 4-cM nested interval. New YACs and BACs were isolated, adding depth to the contig. YACs 905G3, 903D9, 912D2, 672G5, 936B10, 933H2, 872G9, 822D4 and 693C11 placed immediately distal to this contig on the LANL physical map[11] in fact contain more proximal markers, according to the Généthon map[8] and our own recombination data.[5] D16S511 is therefore not present in the YAC contig and we failed to identify YACs positive for this marker. In the 5-cM interval we mapped 10 contigs of ESTs and four genes (C-MAF, glycine cleavage human H protein, phospholipase Cγ2 and KIAA0431), and used them to determine the overlap of the YACs and BACs and to search for SSCP polymorphisms.

Table 3 Homozygosity mapping and haplotype disequilibrium analysis in GAN families

		Families											
cM	*Markers*	*Tunisia I*	*Tunisia II*	*Tunisia III*	*Tunisia IV*	*Tunisia V*	*France 1*	*France 2*	*France 3*	*Sri Lanka*	*Turkey*	*USA*	*Morocco*
	d16s3073	**9**	**9**	**2**	**9**	nd	nd	nd	nd	nd	nd	nd	nd
	d16s3104	**1**	**3**	**1**	**3**	nd	32	12	nd	nd	nd	nd	33
	d16s507	**8**	**3**	**1**	**3**	33	31	22	nd	**1**	38	nd	89
	a109xe9	**9**	**1**	**1**	**1**	18	11	11	nd	**8**	**1**	11	11
3	stsWI10316	**1**	**1**	**1**	**1**	**1**	12	11	**1**	**1**	**1**	11	11
	stsWI3429	**2**	**1**	**1**	**2**	**2**	21	11	**1**	**2**	**2**	12	11
	est682397	**1**	**1**	nd	nd	nd	11	11	nd	**1**	**1**	nd	11
	PLCγ2	**1**	**1**	**1**	nd	nd	11	21	nd	**1**	**1**	nd	12
	LC8/KIAA0431	**2**	**3**	**3**	**3**	**3**	33	33	**3**	**3**	**2**	33	22
	LC6	**4**	**4**	**4**	**4**	**2**	32	22	**4**	**4**	**4**	44	33
	LC5	**3**	**3**	**3**	**4**	**7**	55	51	**2**	**3**	**2**	23	55
	d16s3098	**2**	**5**	**5**	**3**	**5**	15	55	nd	**4**	**1**	nd	52
	est347183	**2**	**2**	**2**	**2**	**3**	11	12	nd	**2**	21	22	12
	gaat2c08	**2**	**2**	**2**	**1**	**1**	21	11	**2**	**2**	12	11	11
1	LC1	**4**	**1**	**1**	**1**	**4**	14	44	**4**	**4**	41	11	44
	LC3	**4**	**2**	**1**	**2**	**4**	14	44	**4**	**4**	44	44	33
	LC2	**2**	**3**	**2**	**2**	**2**	21	11	**5**	**2**	12	32	nd
	d16s505	**9**	**9**	**9**	**3**	**9**	39	20	**8**	**8**	nd	nd	49
1	d16s511	**10**	**10**	**4/3**	**2**	**0**	13	111	**10**	**1**	nd	nd	24

Allele numbers represent haplotyped genotypes of affected children in each GAN family. Homozygous alleles in consanguineous families appears only once, in bold; homozygosity in 3 French, Sri-Lankese and Turkish families indicate probable ancient consanguinity; nd: not determined.

We identified eight polymorphic microsatellite markers and six biallelic markers, and ordered them on the YAC and BAC contig. These markers, together with the six initial markers tightly linked to GAN, were used to analyse linkage, homozygosity and linkage disequilibrium in a total of 12 families, including the three initial Tunisian families. Analysis of phase known recombination events allowed us to define LC8 and D16S505 as new proximal and distal recombinant markers, respectively. In addition, families II and III share a close geographic origin and an extended haplotype overlapping with the critical region defined by the phase known recombinations. This haplotype sharing is good evidence for a common ancestor who passed the same mutation to both families. Divergence of the haplotype at LC3 and beyond indicates the occurrence of a historical distal recombination. The GAN critical interval is therefore delineated by the LC8 and LC3 markers. Thus, we reduced the GAN interval from the initial 5-cM region to a <590 kb interval contained in a single YAC, representing an important step toward the identification of the *GAN* gene on chromosome 16q24.1

It is puzzling that none of the three other Tunisian GAN haplotypes share a significant segment between them or with the haplotype from families II and III. This might reflect the presence of four different *GAN* mutations in the Tunisian population or that very ancient and close historical recombinations have reduced the shared segment of the remaining families, as has already been shown in another Tunisian recessive disease.[22]

We found homozygosity over an overlapping interval, extending from AFMa109xe9 to LC5, in a Turkish family with remote consanguinity. If this result reflects the occurrence of historical recombination, the GAN locus would be between markers LC8 and EST347183/D16S3098. More markers are needed to establish the possible region of homozygosity in the Turkish family, linkage disequilibrium between the Tunisian families, and to narrow down the recombination breakpoints.

The new critical interval contains the *KIAA0431* gene and EST347183. *KIAA0431* is a 5.5 kb transcript isolated from human brain cDNA libraries and encodes a 667 amino-acid protein of unknown function.[23] The *KIAA0431* gene is partially excluded from the GAN region, since it contains the recombinant LC8 marker in its 3' UTR. However, if the 5' end of the gene is oriented distally, and if the recombination is intragenic, *KIAA0431* may still be a candidate gene for GAN. EST347183 overlaps with 12 other ESTs isolated from a large variety of tissues (brain, heart, colon, testis, uterus, etc), defining a 781 bp contig devoid of open reading frame.[10] We are further addressing the precise localisation of these two candidate genes with respect to the GAN interval.

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