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Exclusion of malignant hyperthermia susceptibility (MHS) from a putative MHS2 locus on chromosome 17q and of the α1, β1, and γ subunits of the dihydropyridine receptor calcium channel as candidates for the molecular defect

R.Sudbrak, A.Golla[1], K.Hogan[2], P.Powers[2], R.Gregg[3], I.Du Chesne, F.Lehmann-Horn[4] and T.Deufel*

Klinik und Poliklinik für Kinderheilkunde (Allgemeine Kinderheilkunde), Universität Münster, [1]Abteilung Pädiatrische Genetik, Kinderpoliklinik der Universität München, Germany, [2]Department of Anesthesiology, University of Wisconsin, Madison, [3]Department of Pediatrics and Waisman Center on Mental Retardation and Human Development, University of Wisconsin, Madison, USA and [4]Abteilung Angewandte Physiologie der Universität Ulm, Germany

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Malignant hyperthermia (MH) is a potentially lethal pharmacogenetic disease with autosomal dominant inheritance triggered by exposure to commonly used inhalational anaesthetics or depolarising muscle relaxants. A MHS locus has been identified on human chromosome 19q12 – q13.2 and the gene for the skeletal muscle calcium release channel of sarcoplasmic reticulum (ryanodine receptor) (RYR1) is considered a candidate for the molecular defect. However, MH has been shown to be genetically heterogeneous, and in the ensuing search for other MHS genes, a locus on chromosome 17q has been proposed, and the gene for the adult muscle sodium channel (SCN4A) was suggested as a candidate. We performed linkage studies using polymorphic microsatellite markers for subunits of the skeletal muscle dihydropyridine (DHP) receptor, CACNL1A3 mapped to chromosome 1q, as well as CACNLB1 and CACNLG, the latter two localised on chromosome 17q11.2 – q24 in proximity to the proposed MHS2 and the SCN4A loci, and we also included markers for the loci D17S250, D17S579, NM23 (NME1), GH1, and SCN4A from that region. Our results exclude the α1, β1, and γ subunit of the DHP receptor as well as the SCN4A locus as candidates for the molecular defect in MHS for these pedigrees where also the RYR1 on chromosome 19q13.1 has been excluded. A multipoint analysis excludes the disease from the entire 84 cM interval containing the proposed MHS locus on chromosome 17q. These findings in pedigrees where linkage to the MHS1 locus was formally excluded do not give support to the hypothesis of a second MHS locus being localised on chromosome 17q11.2 – q24.

INTRODUCTION

Malignant hyperthermia (MH) is a potentially lethal pharmacogenetic disease with autosomal dominant inheritance (1) that can be triggered in susceptible probands by exposure to commonly used inhalational anaesthetics (e.g. halothane) or depolarising muscle relaxants (e.g. succinylcholine). Patients react with a rapid rise in body temperature, acidosis, hypoxia, masseter or generalised muscle contracture and rhabdomyolysis. To date, the only way to determine susceptibility to MH is with an *in vitro* contracture test (IVCT) performed on a sample of freshly obtained muscle. A standardised protocol for this test has been established by the European Study Group for Malignant Hyperthermia (2, 3) according to which patients are assigned the status of MH susceptible (MHS), MH normal (MHN) or MH equivocal (MHE). Similar protocols have been implemented in North-America (4), but they differ significantly in their diagnostic specificity compared to European criteria, presumably giving higher numbers of false positive results.

Human MHS has been mapped in homology to the animal model of porcine MH to human chromosome 19q12–q13.2 (5) in a region syntenic with the location of porcine MH. The gene for the skeletal muscle calcium release channel of sarcoplasmic reticulum (ryanodine receptor) (RYR1) is also found at this locus (6). Biochemical and electrophysiological studies in swine and humans with MH indicated that the ryanodine receptor is a likely candidate for the molecular defect. This has since been corroborated by the identification of structural changes in that

*To whom correspondence should be addressed at Universitätskinderklinik, Albert-Schweitzer-Straße 33, DW-4400 Münster, Germany

Table 1. Microsatellite repeat polymorphisms

Locus	Primers	Position	Reference
chromosome 1			
CACNL1A3	5′ GCTGAGCTAGCGAGGGGCAGGGT 3′ 5′ CCCAGCAAAAACTGAGTGTGGATG 3′	1q31l-q32	(15)
chromosome 17			
CACNLB1	5′ ACTCCTCATCTGTAGGGTCT 3′ 5′ GAGTCCGCTACCTGAGTGCT 3′	17q11.2-q22	(16)
D17S250	5′ GGAAGAATCAAATAGACAAT 3′ 5′ GCTGGCCATATATATATTTAAACC 3′	17q11.2-q12	(18)
D17S579	5′ AGTCCTGTAGACAAAACCTG 3′ 5′ CAGTTTCATACCAAGTTCCT 3′	17q21	(19)
NME1	5′ TTGACCGGGGTAGAGAACTC 3′ 5′ TCTCAGTACTTCCCGTGACC 3′	17q21.3-q22	(19)
CACNLG	5′ CTGCCTGCGCCCAGACAGCT 3′ 5′ CATCATGCATGTGTATCAGT 3′	17q23	(17)
SCN4A	5′ CTGTCATGATCTGATGGTCA 3′ 5′ TGGGAGTGGATGTGGAGGAGT 3′	17q23	(21)
GH1	5′ TCCAGCCTCGGAGACAGAAT 3′ 5′ AGTCCTTTCTCCAGAGCAGGT 3′	17q23	(20)

gene that segregate with MHS in affected pigs (7, 8) and a subset of human pedigrees (9, 10). More recently, we have reported two unrelated German pedigrees phenotyped according to the European *in vitro* contracture test protocol in whom the MHS trait was excluded from the MHS1 locus on chromosome 19q12−13.2 (11). This and similar findings in several North-American pedigrees (12) indicate the presence of genetic heterogeneity of the MHS trait. In the ensuing search for other MHS genes, a MHS locus on chromosome 17q has been proposed based on linkage studies in North-American and South-African pedigrees (13), and the gene for the adult muscle sodium channel (SCN4A) which maps to this region was suggested as a candidate (14).

The skeletal muscle 1,4-dihydropyridine-sensitive calcium channel (dihydropyridine receptor) is a hetero-pentameric protein involved in excitation-contraction coupling acting both as a voltage sensor and a calcium channel in the transverse tubular system of skeletal muscle with only one isoform of each subunit gene expressed in skeletal muscle. We performed linkage studies using polymorphic microsatellite markers for the $\alpha 1$ subunit (CACNL1A3) mapped to chromosome 1q (15), as well as the skeletal muscle $\beta 1$ subunit (CACNLB1) and the γ subunit (CACNLG). The latter two are both localised on chromosome 17q11.2−q24 (16, 17), in proximity to the proposed MHS2 (13) and the SCN4A loci. In order to further resolve the gene order and to test the possibility that MHS maps into that region, we included markers for a large interval on chromosome 17q comprising the loci D17S250 (18); D17S579 (19); NM23 (NME1) (19); GH1 (20), and SCN4A (21) (markers summarised in Table 1).

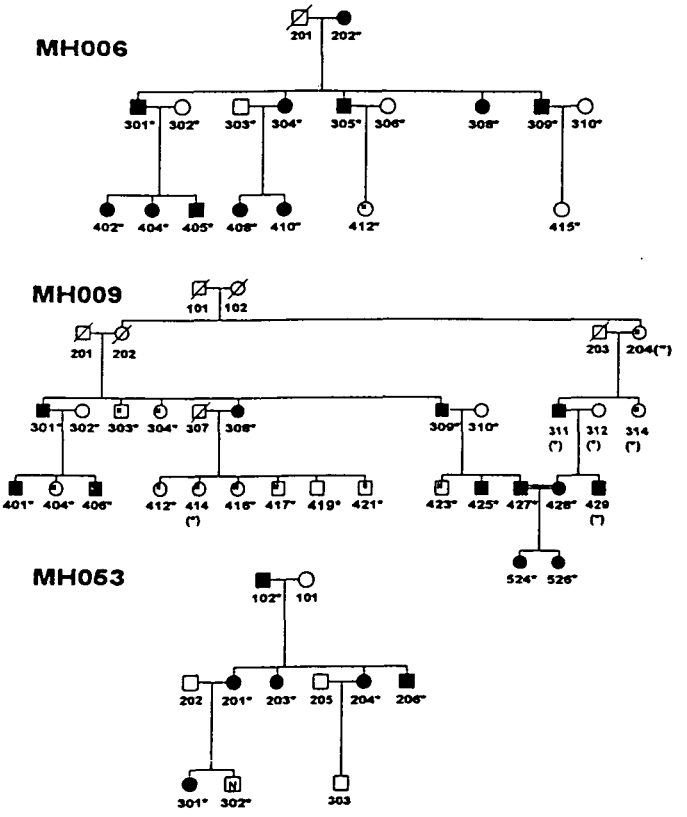

Figure 1. Pedigrees of families MH006, MH009 and MH053 with MHS. The results of the *in vitro* contracture test to define MH phenotype are denoted by black symbols (MHS) and symbols with 'N' (MHN) or 'E' (MHE). Linkage of MHS to the MHS1/RYR1 locus on chromosome 19q has been excluded (for details see Methods section and (11)). An asterisk denotes individuals included in the study. Asterisk in brackets (MH009) indicates individuals sampled but not used for linkage study (see Materials and methods for details).

RESULTS

Exclusion of ($\alpha 1$, $\beta 1$, and γ dihydropyridine receptor subunits as candidates for the molecular defect in MHS

Figure 1 shows the pedigrees MH006, MH009, and MH053 investigated in this study and includes the results of the *in vitro* contracture test results. When typed with the PCR-based microsatellite markers within the CACNL1A3, CACNLB1, and CACNLG genes (Figure 2 a−c) for subunits of the DHP receptor, several recombinations were observed in each of the pedigrees between all of the three markers and MH status. This resulted in cumulated two-point lod scores below −2.0 at 0.0 recombination fraction (Table 2). These findings exclude the $\alpha 1$, $\beta 1$, as well as the γ subunit of the dihydropyridine-sensitive

Table 2. Two-point lod scores for linkage between MHS and markers for the dihydropyridine receptor subunits α1 (CACNL3A1), β1 (CACNLB1), and γ (CACNLG), and the muscle sodium channel (SCN4A)

Locus	Pedigree	z at θ =						
		0.00	0.01	0.05	0.10	0.20	0.30	0.40
CACNL1A3	MH006	−5.29	−4.43	−3.11	−2.26	−1.23	−0.62	−0.23
	MH009	−1.11	−0.95	−0.58	−0.30	+0.01	+0.09	+0.05
	MH053	−0.05	−0.03	+0.01	0.04	+0.05	+0.03	+0.01
	cumulated	−6.45	−5.41	−3.68	−2.52	−1.17	−0.50	−0.17
CACNLB1	MH006	−1.18	−0.90	−0.21	−0.14	−0.34	+0.28	+0.11
	MH009	−2.87	−2.48	−1.64	−1.09	−0.49	−0.19	−0.04
	MH053	−0.73	−0.72	−0.63	−0.45	−0.21	−0.12	−0.09
	cumulated	−4.78	−4.10	−2.48	−1.68	−1.04	−0.03	−0.02
CACNLG	MH006	−0.01	+0.02	+0.07	+0.11	+0.11	+0.07	+0.02
	MH009	−3.32	−2.38	−1.33	−0.83	−0.36	−0.14	−0.03
	MH053	−0.33	−0.29	−0.19	−0.11	−0.03	−0.01	−0.001
	cumulated	−3.66	−2.65	−1.45	−0.83	−0.28	−0.08	−0.01
SCN4A	MH006	−5.04	−3.27	−2.04	−1.21	−0.44	−0.13	−0.02
	MH009	−7.10	−5.56	−3.13	−1.92	−0.82	−0.31	−0.07
	MH053	−0.20	−0.17	−0.08	−0.02	+0.02	+0.02	+0.01
	cumulated	−12.34	−9.00	−5.25	−3.15	−1.24	−0.42	−0.08

calcium channel of skeletal muscle as candidates for the molecular defect in MHS for these pedigrees where also the RYR1 on chromosome 19q13.1 has been excluded (11).

Genetic map of chromosome 17q11.2−q24 including a postulated MHS2 locus and the SCN4A locus

Genetic maps for chromosome 17q containing subsets of the markers used in our studies have been reported (Figure 3). The loci D17S250, D17S579, NME1 (NM23), and GH1, in this order, are included in a genetic map of chromosome 17q12−q23 published previously (19). SCN4A has been reported to be linked to GH1 (22). CACNLB1 has been mapped to chromosome 17q11.2−q22 where its most likely position is between D17S36 and NGFR (16), and CACNLG to chromosome 17q23, showing tight linkage with GH1 (17). Table 3 contains the results of two-point linkage analysis in five pedigrees (MH006, MH009, MH053, MH030 and HPP036; the latter two are unrelated to non-chromosome 19-linked MHS) for the chromosome 17q markers specified in Table 1. Significant lod scores and genetic distances obtained for the markers D17S250, D17S579, NME1, and GH1 are in accord with published data (19). Sex specific differences in recombination frequency were statistically significant only for the intervals D17S579-NME1 (χ^2 = 3.94) and NME1-GH1 (χ^2 = 3.93). Figure 4 summarises multipoint data and depicts the markers in their most likely positions relative to the anchor markers D17S250, D17S579, NME1, and GH1. A maximal lod score of 17.25 for linkage of SCN4A to GH1 was obtained at 0.05 recombination fraction (5.3 cM) which is in contrast to previous reports where no recombinations were observed between these loci. CACNLG is linked to both GH1 and SCN4A with lod scores of 4.02 and 4.15, respectively, at 0.1 recombination fraction (three-point data). Odds against the next likely order NME1-GH1-SCN4A are only 1:18, and thus a definite order cannot be established. The same is true for C-ACNLG where odds against the next likely position distal from GH1 and SCN4A are only 1:19. For CACNLB1, a maximum lod score of 4.72 is obtained at 0.1 recombination fraction for linkage to D17S250, with the position proximal from D17S250 favoured with odds of 16:1 over D17S250-CACNLBl-D17S579, and 237:1 for D17S250-D17S579-CACNLB1. Odds against other positions within the anchor map exceed 1:1000 for all three loci.

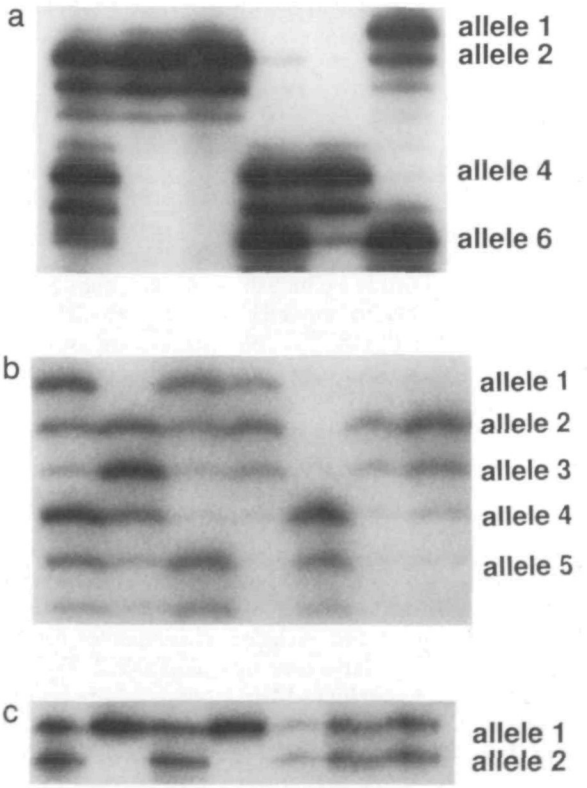

Figure 2. Autoradiographs showing alleles observed for the microsatellite markers CACNL3Al, CACNLB1, and CACNLG for subunits of the dihydropyridine receptor subunits. (See Methods section for deatails.)

Exclusion of MHS from the proposed MHS locus on chromosome 17q

A multipoint analysis based on this refined genetic map excludes MHS in the pedigrees MH006, MH009, and MH053 from the entire interval spanning 84 cM and comprising the loci D19S250, D17S579, NME1 and GH1 (Figure 5). This excludes the proposed MHS locus on chromosome 17q (13) as a candidate

Table 3. Maximum two-point lod scores for linkage and recombination fractions for microsatellite markers on chromosome 17q11.2-q24

θ / z_{max}	D17S250	D17S579	CACNLB1	NME1	CACNLG	SCN4A
D17S250	*	0.06 (0.02–0.14)	0.1 (0.02–0.26)	0.11 (–0.21)		0.26
D17S579	9.80	*	0.084 (0.01–0.23)	0.11 (0.05–0.2)	0.21	0.23
CACNLB1	3.25	4.02	*	0.21	0.2	0.21
NME1	6.33	7.39	(1.49)	*	0.21	0.15 (0.07–0.28)
CACNLG	–	(0.3)	(0.41)	(0.3)	*	0.07 (0.01–0.19)
SCN4A	(1.47)	(2.06)	(1.58)	4.10	4.09	*
GH1	(2.18)	(1.85)	(0.87)	3.15	3.67	15.94

z_{max} (values below 3.0 are not considered significant and are shown in brackets) and recombination fraction at which z_{max} is observed (confidence interval shown in brackets beneath)

in these families. A cumulated two-point lod score of −12.34 at 0.0 recombination fraction was obtained for linkage to the SCN4A locus (Table 2) which therefore is also excluded as a candidate gene in these pedigrees.

DISCUSSION

In contrast to MH in swine, human MH is characterised by substantial genetic heterogeneity. In families in whom linkage to the RYR1 locus on chromosome 19 had been excluded, we investigated genes encoding subunits of the skeletal muscle DHP receptor. Our results firmly exclude three of four genes encoding components of this hetero-oligomer, i.e. the $\alpha 1$, $\beta 1$, and γ subunits, as candidate disease loci. No markers are available at present for the $\alpha 2/\delta$-subunit gene to test if it might be involved in the aetiology of MH.

Two of the dihydropyridine receptor genes, CACNLB1 and CACNLG, map to chromosome 17q (16, 17) in a region where a second MHS locus has been proposed. In those investigations, five pedigrees yielded a cumulative lod score just exceeding 3 for linkage to the marker NM23-H1 for the NME1 locus (13). The same authors have implicated the skeletal muscle sodium channel (SCN4A) as a candidate (14). Both the NME1 and the SCN4A locus, however, are excluded as candidates for MHS in the three pedigrees we have investigated (Table 2, Figure 4), and multipoint analysis excludes MHS from the entire region on chromosome 17q comprising these loci. These results in pedigrees with MHS defined according to the European standard protocol (2, 3) where linkage to the MHS1 locus was formally excluded (11), do not give support to the hypothesis of a second MHS locus being localised in this region.

Our data largely confirms the gene order and genetic distances reported previously (19) for markers D17S250, D17S579, NME1, and GH1. We also integrate the potential candidate loci CACNLB1, CACNLG and SCN4A into the framework of PCR-based microsatellite markers. In contrast to previous reports, we observed recombinations between GH1 and SCN4A, and likewise SCN4A and CACNLG, although a definite order cannot yet be established.

In the absence of markers for more likely candidates, like the gene for the $\alpha 2/\delta$-subunit, the search for new MHS loci will now have to involve the entire genome. Linkage studies in MH are

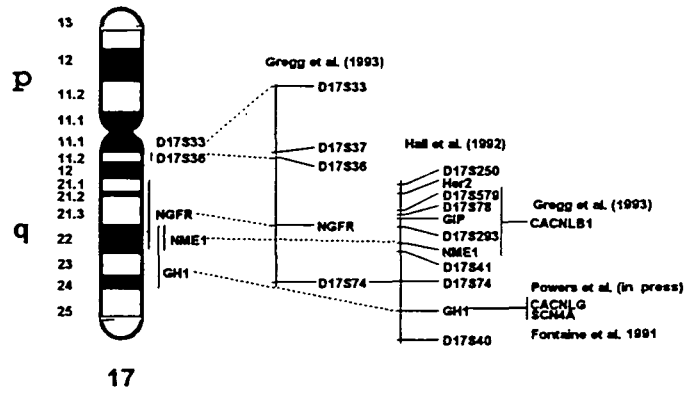

Figure 3. Maps for chromosome 17.

complicated by the difficulty of ascertaining the MHS phenotype. Its diagnosis relies solely on an *in vitro* contracture test for which various protocols have been employed in the past to type patients at risk. The results are certain to vary depending on which IVCT protocol is used. Differences between protocols in the number of false positive and false negative test results must be expected. Protocols such as the one used in many North American studies might detect a higher number of positive probands confounding the validity of the results when these families are used in genetic studies (23). A certain degree of standardisation, along with reasonable diagnostic specificity, has been achieved with the protocol which is followed by the majority of centres in Europe performing the IVC test (2), and there are now available for study several pedigrees with MHS defined according to this protocol and large enough to yield significant lod scores avoiding the pitfalls inherent in the use of cumulative linkage data in a disease that is clearly heterogeneic such as MHS.

MATERIALS AND METHODS

Pedigrees, DNA samples

A detailed description of clinical data, results of the *in vitro* contracture test and genetic studies with markers for the MHS1 locus on chromosome 19q has previously been reported for the pedigrees MH006 and MH009. As described, there is a high probability for the presence of two independent MH mutations

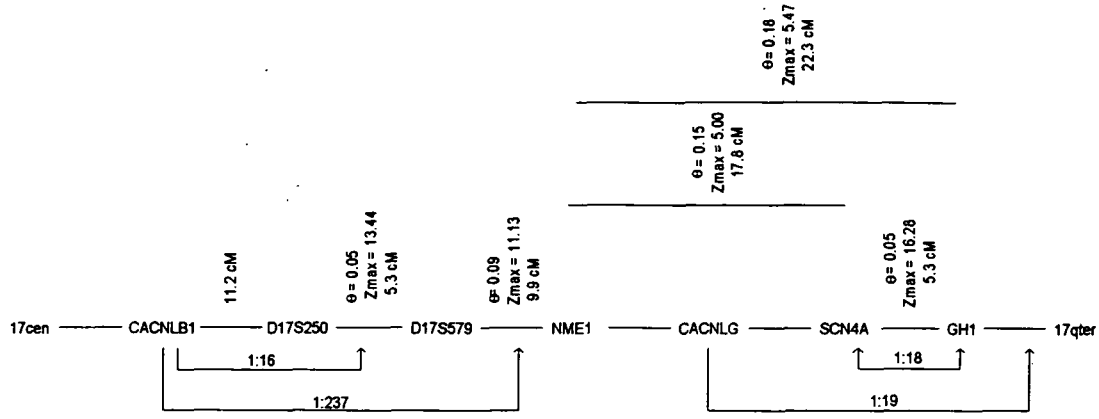

Figure 4. Sex-averaged genetic linkage map for chromosome 17q11.2−q24. The map is based on the order reported by Hall et al. (19) for the markers D17S250, D17S579, NME1, and GH1 and contains the results of linkage analysis in the three MHS pedigrees (MH006, MH009, MH053) described above and another two large pedigrees (MH030, HPP036). Recombination fractions obtained from three-point analysis were converted to centimorgans (cM) using the Haidane mapping function. CACNLB1, CACNLG, and SCN4A are shown in their most likely positions according to ILINK (three-point and four-point analysis) and LINKMAP. Next likely positions relative to the anchor markers are indicated by arrows and odds against this position are given. Odds against any other position exceeded 1:1000. Odds of 1:19 (marked by asterisk) against placement of CACNLG in a position distal from SCN4A/GH1 are obtained only if the order of GH1 and SCN4A is also reversed.

in pedigree MH009, and formal exclusion from the chromosome 19 locus is only possible for the left hand part of the pedigree (11). The right hand branch originating from individual 204 is therefore not included in the linkage analysis for this study. Pedigree MH053 was analysed with the *in vitro* muscle contracture test by D.Olthoff, Leipzig, and linkage to chromosome 19q was excluded by typing with the microsatellite markers for the loci D19S75, D19S190, RYR1, D19S191, D19S47, and APOC2 (data not shown). Genomic DNA was prepared from EDTA whole blood according to methods described elsewhere (24, 25) and amplified by the polymerase chain reaction (PCR) using primers for the microsatellite repeat markers specified (Table 1).

Markers

The following polymorphic microsatellite markers were typed using the primers reported in the reference and listed in Table 1: D17S250 (18), D17S579 (19), NM23-H1 (19), GH1 (20), CACNLIA3 (15), CACNLB1 (16), CACNLG (17), and SCN4A-GA (21). For each primer pair, forward primer sufficient for 20 reactions was end-labelled with ^{32}P-ATP in a total volume of 20 μl containing 100 pmoles oligonucleotide and 10 U T4 polynucleotide kinase (Gibco BRL). Following incubation for 30 minutes at 37°C, the reaction mixture was added to the PCR reagent mix without further purification. PCR reactions were performed in a total volume of 25 μl comprising 200 ng genomic DNA, 200 μM each of dNTPs, PCR buffer (Perkin Elmer/Cetus buffer I), primer labelling mix, and 20 pmoles forward primer, 25 pmoles reverse primer, and 0.5 U Taq-polymerase (AmpliTaq, Perkin Elmer/Cetus). 4 μl of the amplification products were mixed with an equal volume of formamide-loading mix, heated to 90°C for two minutes, and chilled on ice prior to loading onto a denaturing sequencing gel (8% polyacrylamide/7 M urea) and separated under standard conditions.

Autoradiography of the vacuum-dried gel was performed at room temperature for 15−30 hours on radiographic film (X-O-Mat, Kodak). Allele numbers for each marker were assigned based on laboratory standards containing the longest product present in the sample. They do not necessarily conform with numbers given in the original reference.

Data analysis

Two point and multipoint linkage analysis was performed using version 5.1 of the LINKAGE package of programs (26) on a SUN-IPC workstation. Parameters were set as described previously (11). To decrease memory requirements and computation time, genotypes were recoded to 5 alleles for CACNLB1 and GH1, and to 4 alleles for D17S250, D17S579, and NM23, by reducing alleles to those observed in the root family and adjusting those of individuals joining the pedigree by marriage to preserve informativity. Allele frequencies were adjusted accordingly. Pairwise linkage analysis was performed using the MLINK option of the program package to determine sex average lod scores. ILINK was used to estimate sex specific recombination fractions and lod scores as well as for ordering markers in three point analyses. The genetic distances obtained were used in calculating multipoint location scores with LINKMAP in order to exclude MHS from the region of chromosome 17 defined by the marker set. Exclusion was assumed if the lod score did not exceed −2.0.

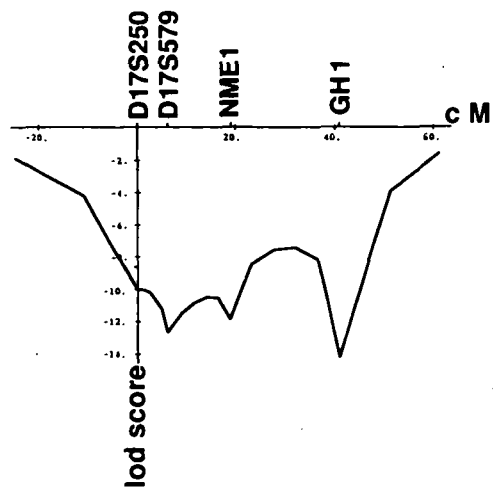

Figure 5. Multi-point location scores excluding MHS in pedigrees MH006, MH009 and MH053 from the proposed MHS locus on chromosome 17q. The location map indicates composite lod scores for MHS at various map positions in a fixed marker map comprising markers D17S250, D17S579, NME1, and GH1. (For details of the markers, see Table 1.) D17S250 is arbitrarily placed at 0 cM, with the rest of the markers at the distances calculated on the basis of sex-average recombination fractions as shown in Table 2. The marker order is that of the genetic map described previously (19). Haidane's mapping function was used to convert recombination fractions to genetic distances given in cM. Note that the lod score is below −2.0 in a 84 cM interval containing the NME1 locus for which linkage to MHS has been claimed (13).

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