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Genomewide scans in North American families reveal genetic linkage of essential tremor to a region on chromosome 6p23

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Essential tremor (ET) is the most prevalent adult-onset movement disorder showing evidence of non-random accumulation in some families. ET has previously been mapped to genetic loci on chromosomes 2p and 3q, but no causative genes identified. We conducted genomewide linkage screening with subsequent fine mapping in seven large North American families comprising a total of 325 genotyped individuals that included 65 patients diagnosed as definite ET. Linkage analysis was based on methodology implemented in SimWalk2 and LINKAGE programs. A multigenerational family revealed suggestive linkage to a locus on chromosome 6p23 with maximal nonparametric linkage (NPL) multipoint score 3.281 (P = 0.0005) and parametric multipoint log of the odds (LOD) score 2.983. A second family showed positive linkage to the same 6p23 region with a maximal NPL score 2.125 (P = 0.0075) and LOD score 1.265. Haplotype analysis led to the identification of a 600 kb interval shared by both families. Sequencing of coding regions of 15 genes located in the linked region detected numerous sequence variants, some of them predicting a change of the encoded amino acid, but each was also found in controls. Our findings provide evidence for linkage to a novel susceptibility locus on chromosome 6p23. Analysis of additional ET-affected families is needed to confirm linkage and identify the underlying gene.

Keywords: essential tremor; focal dystonia; linkage mapping; chromosome 6p

Abbreviations: ET = essential tremor; LOD = log of the odds; NPL = nonparametric linkage

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Introduction

Essential tremor (ET) is a movement disorder characterized by action tremor (postural and/or kinetic) in both upper limbs and less commonly in the head, face, voice, tongue, trunk or lower limbs (Elble, 2000*a*). Electrophysiologic studies are consistent with a central source of tremorogenic oscillation (Hallett, 1998; Deuschl and Elble, 2000). The typical patient with ET exhibits no neurological abnormalities other than tremor (Elble, 2002). However, patients in some families exhibit mildly expressed associated dystonia (Lou and Jankovic, 1991; Koller *et al.*, 1994). It has long been a subject of discussion whether tremor-dystonia is a variant of ET or a result of co-inheritance of both conditions (Elble, 2002; Jankovic, 2002). Patients with ET may exhibit mild cerebellar signs (Helmchen *et al.*, 2003).

The estimated prevalence of ET in North America is 1.7% in the general population and at least 4% in individuals older than 40 years of age (Hubble *et al.*, 1989; Louis *et al.*, 1995, 2003*b*). Estimates of the total number of patients in the United States are between 4 and 5 million (International Tremor Association, 1995). Tremor affects simple motor

tasks such as writing, eating, speaking and other activities of daily living, and in some cases progresses to tremor-induced functional disability (Busenbark *et al.*, 1991; Louis *et al.*, 2001*a*). Treatment of ET had limited success (Zesiewicz *et al.*, 2005).

Convincing evidence has been presented for non-random accumulation of ET patients in some families. Tremor is found in 22.5% of first-degree relatives of ET patients compared to 5.6% of first-degree relatives of control individuals (Louis *et al.*, 2001*b*). Monozygotic twins develop ET at a rate of 60 versus 27% in dizygotic twins (Tanner *et al.*, 2001), and in a recent study the numbers were 93 versus 29% (Lorenz *et al.*, 2004). The proposed pattern of ET inheritance is autosomal dominant (Bain *et al.*, 1994), but there are concerns regarding the methodology of such determinations, and in fact other types of inheritance such as complex multigenic models cannot be excluded (Louis and Ottman, 1996; Jankovic *et al.*, 1997).

The search for ET susceptibility loci has proved difficult. Among the frequently cited reasons for failure are diagnostic uncertainties, unclear inheritance patterns, incomplete penetrance. Large pedigrees may include cases possibly caused by environmental factors (Louis *et al.*, 2001*c*). The age of disease onset is variable and increasing with advancing age, therefore the parents are often unavailable for genotyping. In addition, a strong genetic heterogeneity is suspected among the ET pedigrees. Each of these circumstances and their combinations may cause false positive linkage signals or conceal true linkage. A number of investigations in British and North American families were unsuccessful in mapping ET genes (Findley, 2000).

Nevertheless, two chromosomal regions linked to familial ET have been identified by genetic linkage analysis. A 10 cM genomewide scan in 16 Icelandic pedigrees with 75 affected individuals resulted in identification of a candidate region on chromosome 3q13. The multipoint log of the odds (LOD) score assuming an autosomal dominant model was 3.71, and the nonparametric linkage (NPL) score 4.70, $P < 6.4 \times 10^{-6}$ (Gulcher *et al.*, 1997). Linkage to this same locus was assumed but not proven in four Tajik families showing a combined two-point LOD score of 2.05 at marker D31278 (Illarioshkin *et al.*, 2000). Analysis of several other ET families excluded linkage to this locus (Abbruzzese *et al.*, 2001; Kovach *et al.*, 2001). A study of an American family of Eastern European descent detected linkage to a 12.8 cM interval on chromosome 2p22–p25 with a maximum LOD score of 5.92 at marker D2S272 (Higgins *et al.*, 1997). This finding has not been independently confirmed in pedigrees studied by other groups (Illarioshkin *et al.*, 2000; Abbruzzese *et al.*, 2001; Kovach *et al.*, 2001; K. Wilhelmsen, unpublished data). Fine mapping and transcript analysis in the 2p24.1 interval led to the identification of the *HS1-BP3* gene carrying an A265G substitution in members of two ET families (Higgins *et al.*, 2005), but a study of a larger series of affected families and controls found no evidence of co-segregation of the A265G polymorphism with ET (Deng *et al.*, 2005); Shatunov *et al.*, 2005). The proposed causative role of several other genes has also been excluded in subsequent studies: alpha-synuclein and parkin genes (Pigullo *et al.*, 2003, 2004), fragile-X mental retardation type 1 (*FMR1*) gene permutation (Deng *et al.*, 2004; Garcia Arocena *et al.*, 2004), and spinocerebellar ataxia type 12 (*SCA12*) gene (Nicoletti *et al.*, 2002).

We conducted genomewide screening for linkage and performed fine mapping of candidate regions, haplotype analysis and sequencing of positional candidate genes in multigenerational North American families with the goal of finding linked chromosomal regions and genes responsible for genetic susceptibility to ET.

Patients and methods

Ascertainment of patients and families

The total number of genotyped individuals in seven families was 325; of these, 65 were affected with definite ET (Table 1). ET patients were present in 2–4 successive generations of each family. Families were recruited and studied at three collaborating institutions, the Human Motor Control Section, NINDS, NIH; the Department of Neurology, Baylor College of Medicine; and the Department of Neurology, Southern Illinois University School of Medicine. The study was approved by the Institutional Review Boards of each participating institution, and informed consent was signed by each participant. Family members available for the study underwent neurological evaluation by at least one of the co-authors of this report specializing in movement disorders. Uniform diagnostic criteria were established at a Collaborative Essential Tremor Consortium meeting held at the National Institutes of Health, Bethesda, Maryland in July 1996 (Brin and Koller, 1998).

Each patient was classified for both the diagnosis and the disease-severity status. The diagnostic classification included possible ET, probable ET, and definite ET, reflecting the level of uncertainty with the diagnosis. Tremor severity was scored at grade zero (no tremor), one (minimal), two (visible, not disabling), three (moderate, disabling), and four (severe tremor). Patients having bilateral tremor of prolonged prior duration (at least 5 years) and severity grades three or four with secondary causes of tremor excluded were considered as 'definite ET' (unambiguous clinical diagnosis). Patients with the definite ET diagnosis in families EL, IL and VG were additionally studied by accelerometry and surface electromyography to measure hand tremor and motor unit entrainment in the extensor carpi radialis brevis (Hallett, 1998; Elble, 2000*b*). The definite ET diagnosis was considered strongly confirmed in patients with visible tremor in their handwriting or Archimedes spirals documented with digitizing tablet analysis (Elble *et al.*, 1990) or if they exhibited frequency invariant motor unit entrainment at 4–8 Hz in their forearm electromyogram (Louis and Pullman, 2001). The current study was based on genetic analysis of patients with the unambiguous ('definite ET') clinical diagnosis that were coded 'affected'. To minimize the risk that the presence of patients with non-definite diagnosis influences linkage scores, the possible ET and probable ET patients were designated as 'unclassified' and coded as having an 'unknown' phenotype. Mildly or moderately expressed focal dystonia was observed in patients of three studied families while in the other four families there

Table 1 Phenotypic characteristics of patients with essential tremor in seven North American families

Family	Total number of evaluated and genotyped family members	Number of definitely affected	Number of generations in which ET patients were identified and studied	Mean age at disease onset (years)	Tremor with no other neurological abnormalities	Tremor and dystonia
VD	43	14	4	30.2 + 17.8	6	8
EL	46	9	2	39.4 + 23.3	9	
ML	59	7	3	20.9 + 12.4	7	
VG	25	11	4	28.4 + 16.7	7	4
BL	37	6	2	45.5 + 8.0	6	
IL	28	6	2	21.7 + 9.6	6	
PS	87	12	4	28.6 + 13.3	9	3
Total	325	65		30.5 + 16.8	50	15

was action tremor with no other neurological abnormalities (Table 1).

The results of segregation analysis performed in three families (VD, EL and ML) were consistent with an autosomal dominant pattern of inheritance (H.-S. Lee, unpublished data). VD and ML families have been descriptively presented in a previous publication (Jankovic *et al.*, 1997), and genetic analysis for exclusion of known ET candidate regions on chromosomes 2p and 3q was reported in family EL (Kovach *et al.*, 2001), but a genomewide scan has not been previously attempted in any of these seven families. Another disorder, malignant hyperthermia (with four known deaths resulting from the use of inhalational anaesthetics) in family VD showed an inheritance pattern independent from tremor and dystonia. We have identified a novel A2350T missense mutation in the *RYR1* gene as the cause of malignant hyperthermia susceptibility in members of this family (Sambuughin *et al.*, 2001).

Genomewide genotyping

DNA was extracted from blood lymphocytes by the use of the traditional phenol-chloroform extraction technique. Genomewide genotyping was accomplished with markers from the ABI-400 set (ABI Prism® Linkage Mapping Set, version 2.5, MD-10) consisting of fluorescently-labelled PCR primer pairs selected to amplify highly informative 2 bp repeat microsatellite loci and having an average spacing of approximately 10 cM with average heterozygosity 0.785. A total of 374 markers were used on 22 autosomes. Marker positions were verified on the Marshfield Medical Research Foundation genetic framework map (http://www2.marshfieldclinic.org/RESEARCH/GENETICS/Map_Markers/maps/IndexMapFrames.html/). Multiplex reactions were performed to increase efficiency, lower the cost and increase the speed of genomewide screening. PCR was performed according to standard protocols. Amplification products were loaded onto an ABI 373A sequencer or ABI PRISM 3100 DNA Analyser (Applied Biosystems, Foster City, CA, USA). Data were collected and the size of the microsatellite alleles measured using GeneScan ABI 672 software (version 3.1, Applied Biosystems). Files were then imported into Genotyper (version 3.7 NT, Applied Biosystems) to assign genotypes. To assess the reproducibility of genotyping, we implemented a series of quality control checks. Up to 10% of the genotypes were verified by repeated analysis. Allele sizes were standardized by comparison to genotypes of the reference sample CEPH 1347-02 that was included on each gel as internal control. Genotyping was performed blind to clinical status.

Fine mapping

In chromosomal regions showing positive linkage, additional follow-up markers were chosen from the deCODE high resolution genetic map of the human genome (Kong *et al.*, 2002). Priority was given to markers with highest heterozygosity. 'Mistyping' option of SimWalk2 package was used for checking for genotyping errors at the fine mapping stage. Markers showing significant probability of mistyping were removed from analysis. Power of linkage in genomewide and fine-map studies was classified according to published criteria (Lander and Kruglyak, 1995).

Statistical analysis

Tests for Mendelian consistency of intrafamilial relationships and testing for genotyping errors were carried out with the use of the error-checking algorithm implemented in Mega2 version 3.0 software package (http://watson.hgen.pitt.edu/mega2.html; Mukhopadhyay *et al.*, 2005). Identified errors were corrected by removing the marker from the entire family. Allele frequencies for each marker were estimated from all pedigrees by taking into account all genotyped individuals disregarding relationships and affection status and were automatically calculated by Mega2. To take advantage of the power provided by large pedigrees, initial tests for linkage were performed by using nonparametric ('model-free') multipoint linkage analysis. Nonparametric analysis is preferable in pedigrees with a complicated disease transmission model because this type of analysis does not require specification of the inheritance pattern, disease allele frequency, penetrance rate and to a certain extent tolerates phenotype definition errors.

Methodology implemented in the SimWalk2 (version 2.91) program was chosen for multipoint nonparametric genomewide linkage analysis. This program is based on Markov chain-Monte Carlo sampling and simulated annealing algorithms (Sobel and Lange, 1996; Sobel *et al.*, 2001; Sobel *et al.*, 2002; Lange and Lange, 2004). It examines whether affected relatives share an excessive number of marker alleles identical by descent. Excess sharing of marker alleles among the affected people in a specified chromosomal region suggests that the region harbours a disease susceptibility gene. Importantly, this program allows analysis of large pedigrees in a single run without family-size restrictions and requires a reasonable length of time; it can be successfully used for analysis of monogenic traits with reduced penetrance and high disease allele frequency (Lange and Lange, 2004).

SimWalk2 reports several NPL statistics. Of these, the 'BLOCKS RECESSIVE' (BLOCK-rec) statistic evaluates the number of alleles

descending from both parents to the affected individual and therefore is considered the best for evaluation of recessive inheritance (Lange and Lange, 2004). The 'MAX-TREE DOMINANT' (MAX-dom) statistic takes into account alleles inherited from each parent and uncovers a dominant trait in an extended pedigree. The 'ALL-ADDITIVE' (NPL-all) and 'PAIR-ADDITIVE' (NPL-pair) statistics estimate identical by descent allele sharing between affected individuals; the estimated allele sharing values are averaged over all possible inheritance patterns and then normalized. They were proven to be the best for additive or complex traits. NPL-all and NPL-pair statistics can also be successfully applied to large pedigrees with complicated structures (Lange and Lange, 2004). We used SimWalk2 nonparametric and parametric multipoint methods for analysis of fine mapping results. Data obtained in genomewide and fine mapping analyses were plotted using physical positions specified by the Marshfield genetic database.

We also carried out traditional parametric two-point analysis using the MLINK program of the LINKAGE package (Lathrop *et al.*, 1985), with the assumption of autosomal dominant mode of inheritance (Bain *et al.*, 1994), equal recombination rates for males and females, and expected pathogenic allele frequency 0.8%. Although the latter number is only a fraction of ET prevalence estimated between 1.7 and 4%, it is based on preliminary testing of the proposed model and apparently reflects the fact that ET is genetically heterogeneous in most populations. An age-dependent penetrance curve was calculated with a maximal disease penetrance value of 0.98 at the age equal to or older than 70. An affected-only model was chosen because it minimizes the influence caused by the presence of individuals with mild tremor or those having no tremor at a specific point of time but who may contract the fully expressed disease at a later age. Accordingly, we coded the family members with definite ET diagnosis as 'affected' and those without definite ET, including patients with probable and possible ET diagnosis, as 'unknown'.

Search for causative genes

The most promising region identified as linked to ET susceptibility was searched for genes, and selective genes that appeared to be functionally associated with the known pathogenesis of ET and expressed in the brain were studied. Coding regions of a total of 15 genes were amplified and analysed for identification of sequence variants. Primers used for this purpose were designed for each exon using Primer3 software (Rosen and Skaletsky, 2000) and included flanking splice sequences and at least 50 nt into the introns. Amplification was carried out in a total volume of 20–25 μl with 50 ng of genomic DNA, 0.5 μM of each primer, 125 μM of each dNTP, 1.5 mM of $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM of KCl, and 0.6 units of *Taq* DNA polymerase (Applied Biosystems, Foster City, CA, USA). Amplified DNA fragments were analysed by sequencing using a fluorescent Big DyeTerminator^{TM} Cycle Sequencing protocol on an automated ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). DNA variants initially identified in two affected individuals were subsequently screened by sequencing in all family members. The corresponding allele frequency was determined by using NCBI and other databases. If the variant had not been previously reported, 100 or more North American unrelated controls were tested.

Results

Phenotypic features

The total number of neurologically evaluated and genotyped individuals in 7 pedigrees was 325, of which 65 were diagnosed as definite ET (Table 1). Each patient had action tremor (postural or kinetic) in both upper limbs, and four patients (6%) had a combination of hand and head tremor. All affected members of families EL, ML, BL, and IL had tremor and no other neurological abnormalities. Some affected members of families VD, VG and PS had tremor and dystonia in the form of writer's cramp in 11 patients and cervical dystonia associated with writer's cramp in 4. A single patient of the VD family who had definite writer's cramp in association with tremor estimated as less than definite was included in the study. The mean age of disease onset varied among families from 21 to 45 years.

Analysis of the genomewide scans

We screened 22 autosomes to determine chromosomal regions linked to ET susceptibility. Linkage was estimated by sliding multipoint nonparametric analysis carried out by methods implemented in the SimWalk2 (v 2.91) program. The NPL-all scores resulting from SimWalk2 linkage calculations in seven families are displayed in Fig. 1. NPL-all values exceeding or approaching 2.0 with $P < 0.01$ were observed in four families (summary in Table 2). In the VD family, the only locus of the entire genome that showed a linkage signal above the cut-off value was located in a chromosomal region 6p22.3–p23 (maximal NPL-all score 2.086, $P = 0.0082$, at marker D6S422). A linkage signal was detected at a very close chromosomal location 6p23–p24.3 in the unrelated family EL (maximal NPL-all score 2.036, $P = 0.0092$, at marker D6S309). No other region of the genome showed signs of linkage in the EL family. Analysis of the ML family revealed weak linkage to chromosome 13q13.2 (NPL-all score 1.98, $P = 0.0105$). And lastly, a single-marker linkage signal with an NPL-all score 2.703, $P = 0.0020$ was detected in the VG family at chromosome 11q24.3 region. We were unable to uncover linkage to any chromosomal region in the remaining BL, IL, and PS families.

Fine mapping

After obtaining indications of weak positive linkage in the genomewide scans, we genotyped an additional 14 microsatellite markers on chromosome 6p22.3–p24.3 (D6S477, D6S1574, D6S309, D6S470, D6S429, D6S1653, D6S469, D6S289, D6S1630, D6S1605, D6S1584, D6S274, D6S285, and D6S422), three markers on chromosome 13q13.2 (D13S289, D13S263, and D13S219), and three markers on chromosome 11q24.3 (D11S912, D11S4126, D11S910) to substantiate the results obtained in the genomewide scan. The use of additional markers increased marker density in these areas ~5-to 10-fold. The candidate region on

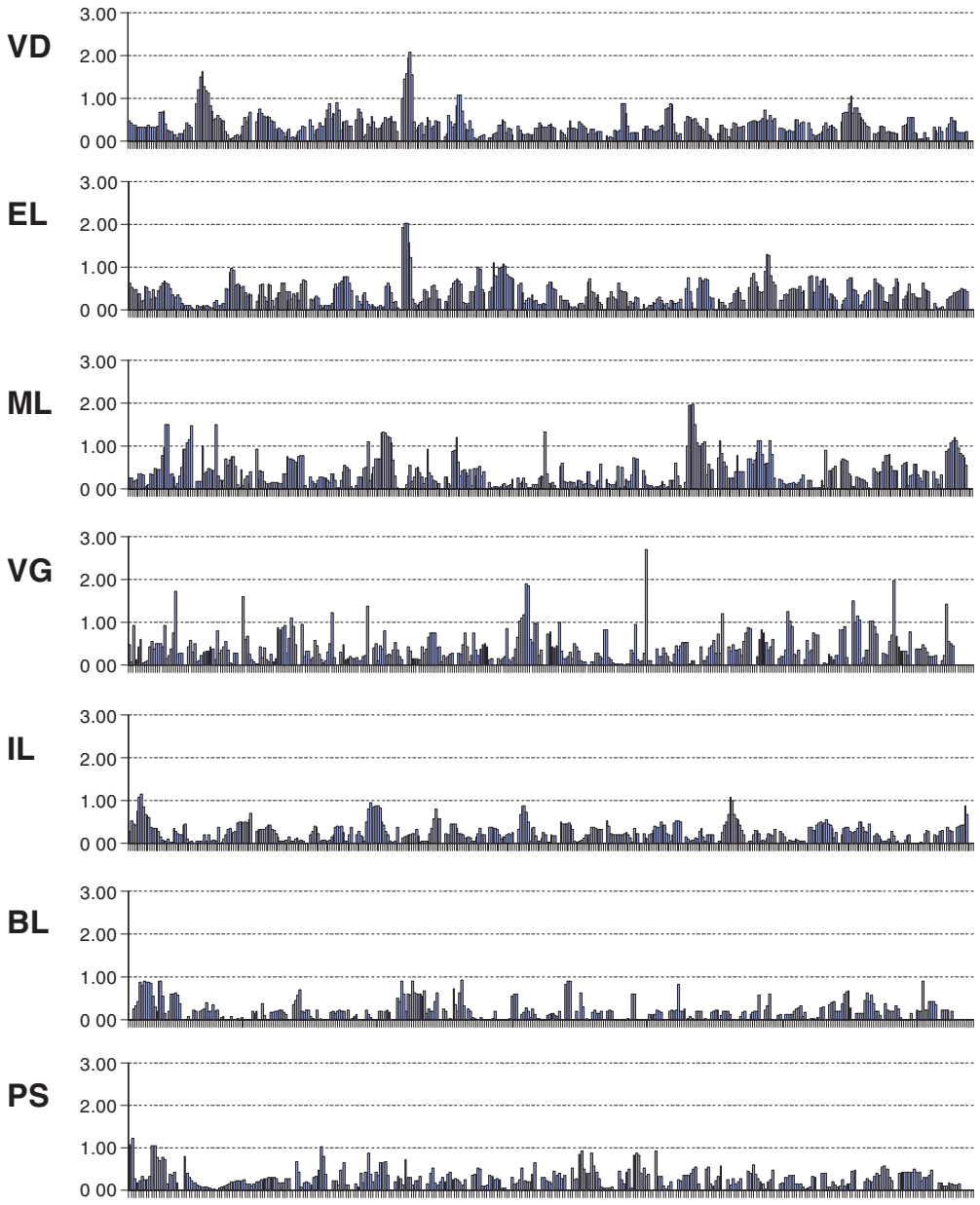

Fig. 1 Results of genomewide nonparametric multipoint linkage analysis (NPL-all scores, Sim Walk 2) in multiplex North American families VD, EL, ML, VG, IL, BL, and PS segregating essential tremor. Genetic distance (in cM) scale for 22 autosomes based on information from the Marshfield meiotic map is plotted on the horizontal axis against NPL-all values on the vertical axis.

Table 2 Summary of nonparametric multipoint analysis of the genomewide scan data (estimated by SimWalk2 program)

Family	NPL-all	*P*-value	Map location	
			Cytogenetic	Marker with maximal score
VD	2.0856	0.0082	6p22.3	D6S422
EL	2.0360	0.0092	6p24.3	D6S309
ML	1.9796	0.0105	13q13.2	D13S263
VG	2.7028	0.0020	11q24.3	D11S912

chromosome 6p withheld this challenge by showing higher linkage scores in the VD family and unchanged scores in the EL family (Table 3). The maximal NPL-all score in the VD family was 3.281, $P = 0.0005$ in a region that includes markers D6S1630-D6S289-D6S469-D6S1605 and located within the 6p23 band (Fig. 2 and Table 3). The fine map of the 6p region in family EL shows maximal NPL-all score 2.125, $P = 0.0075$ at approximately the same region as in the VD family (Fig. 2 and Table 3). This increase of linkage scores on fine mapping in the 6p23 region brings the results in the VD family to the upper limit of the 'suggestive linkage' level and close to the 'significant linkage'

Table 3 Summary of nonparametric (NPL-all scores) and parametric (LOD scores) multipoint analysis of fine mapping data (estimated by SimWalk2 program)

Family	NPL-all	*P*-value	LOD score	Map location	
				Cytogenetic	Marker with maximal score
VD	3.2807	0.0005	2.983	6p23	D6S1630
EL	2.1249	0.0075	1.265	6p23	D6S1630
ML	1.8426	0.0144	1.498	13q13.2	D13S263
VG	1.6099	0.0246	−0.3130	11q24.3	D11S912

criteria requiring NPL score 3.3 (Lander and Kruglyak, 1995). Calculations of linkage in the VD and EL families taken together show an NPL-all score 3.125, $P = 0.0008$ and an even higher MAX-dom score 3.979 with a *P*-value 0.0001 (Fig. 2). Fine mapping at the 13q13.2 region in the ML family and the 11q24.3 region in the VG family showed a decrease of the NPL-all scores, suggesting that the genome-wide scan results in these families were most likely false positive (Table 3).

Parametric multipoint analysis carried out by the SimWalk2 program confirmed the presence of linkage at locus 6p23 with a LOD score 2.983 in the VD family and provided a low positive score of 1.265 in the EL family (Fig. 2). When both families were considered together, the maximal combined LOD score of 4.248 was observed at markers D6S1630 and D6S1605 (Fig. 2). Two-point parametric analysis in the VD and EL families estimated by the MLINK program generated a maximal combined LOD score of 2.70 at marker D6S289 and lower values at other markers in the region (Table 4).

Linkage to previously reported candidate regions on chromosome 3q13.1 (Gulcher *et al.*, 1997) and 2p22–p25 (Higgins *et al.*, 1997) was not evident in any of the studied families. Linkage to the idiopathic torsion dystonia (DYT1) region on chromosome 9q34 and candidate adult-onset dystonia regions DYT5,6,7 and DYT11 through 15 were also excluded in each studied family.

Haplotype analysis

The purpose of haplotype analysis was to additionally evaluate the reliability of detected linkages and illustrate specific features of ET pedigrees. Haplotypes were determined by analysis of marker allele segregation in the pedigrees. In the VD family, 10 of 14 patients with definite ET diagnosis had an identical haplotype for 14 consecutive markers between D6S477 and D6S422 shown as filled bars under individual symbols on Fig. 3. Three patients with definite ET did not share this haplotype (III:8, III:9, and III:15). Of these, the sister and brother, III:8 and III:9, were in their late sixties and the age of tremor onset in these individuals was 12 and 31 years later than the average age of onset in the VD family. They are, most likely, phenocopies since none of their seven children developed tremor. A single patient (III:12) shared with other patients the centromeric part of the disease haplotype but showed a recombination between markers D6S470 and D6S429 (Fig. 3). Several family members with the disease-associated haplotype did not have fully expressed ET, including obligate carriers transmitting the disease from their parents to offspring (III:1 and III:4). Among eight definitely affected members of the smaller EL family, one patient did not share the 14-marker disease haplotype (III:1, Fig. 4). Patient III:5 shared only the telomeric part of the haplotype and showed the presence of a recombination within the 6p candidate region between markers D6S470 and D6S429.

Linkage area shared by the VD and EL families

To determine whether the VD and EL families shared a linkage area and to identify this chromosomal segment, we performed genotyping with three additional microsatellite markers (D6S1034, D6S1006, and D6S2434) and nine single nucleotide polymorphism (SNP) markers. SNPs chosen for this analysis were located about 50–100 kb apart. Analysis of the critical region helped to delineate the shared area by finding recombination breakpoints (Fig. 5). The region restricted by the SNP rs9381921 on the telomeric side and the microsatellite marker D6S2434 on the centromeric end was about 600 kb in size. This fragment is located in the 6p23 chromosomal band and extends partly into 6p24.1. Unexpectedly, the shared area is located slightly telomeric in relation to the candidate region identified on the basis of the highest multipoint and two-point linkage scores (Fig. 5). The wider chromosomal region encompassing both the shared fragment and the area with the highest linkage scores measured 6.43 megabases.

Mutation analysis of transcripts in the candidate chromosomal region

The region shared by families VD and EL and the one showing the highest linkage scores were tested for candidate genes. Each known gene located within (TBC1D7, GFOD1, SIRT5, NOL7, RANBP9, LOC441130, and C6orf79) or near (PHACTR1) the 600 kb shared segment was analysed (Fig. 4). Coding regions with flanking intron fragments were amplified and sequenced as described. Several polymorphic gene variants, previously known or unknown, were identified (Table 5).

Since no causative mutations were found in the shared area, we turned to the centromeric region showing the highest linkage scores and containing several meaningful genes. Of the 28 genes, seven located in the region between markers D6S2434 and D6S422 (JARID2, DTNBP1, MYLIP, GMPR, SCA1, CAP2, and NHLRC1) were also analysed by sequencing. Each of the seven genes

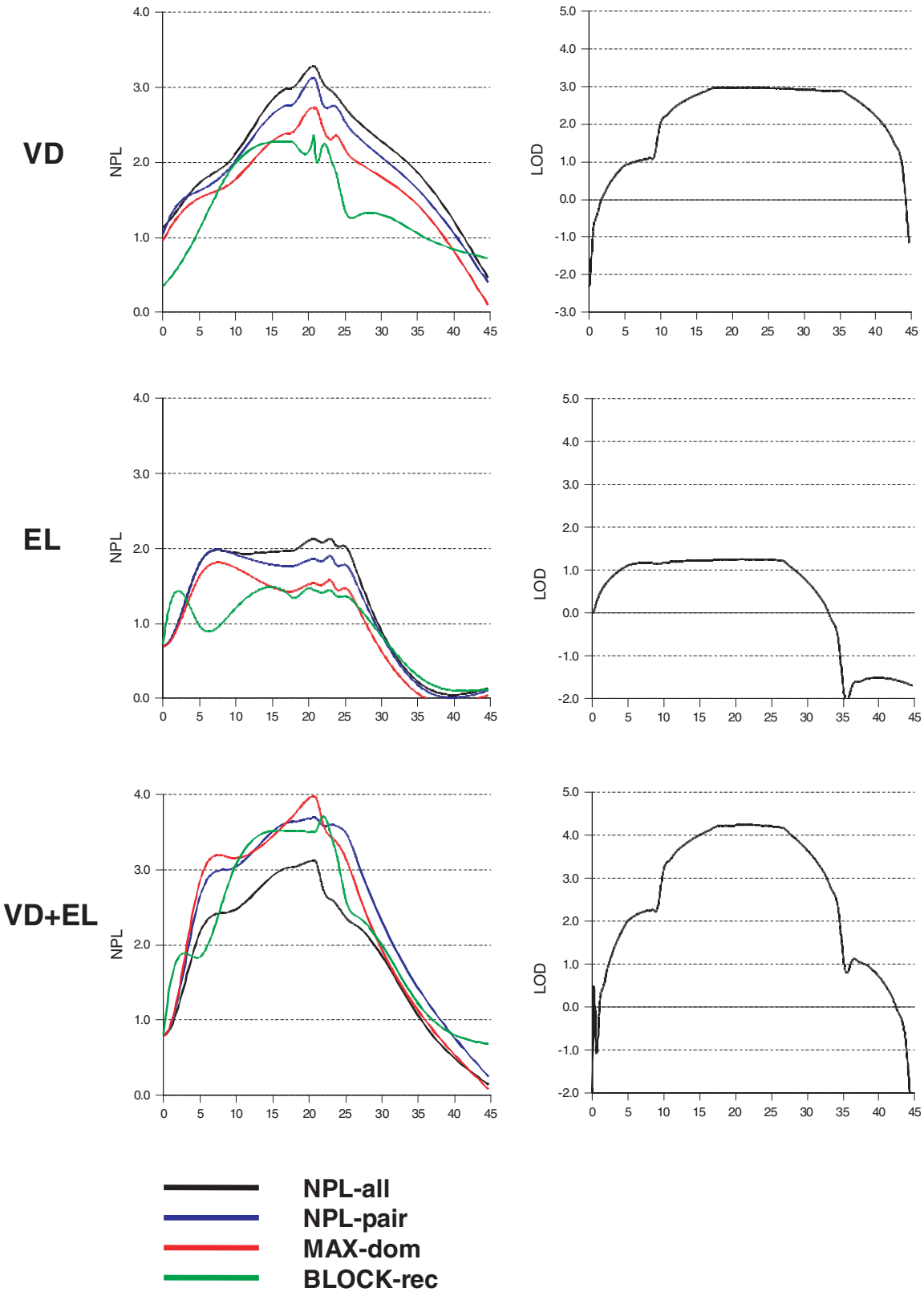

Fig. 2 Results of nonparametric and parametric multipoint analyses of fine-mapping data in families VD and EL calculated by using four SimWalk2 statistics. Fourteen chromosome 6p markers were used in this analysis: D6S477, D6S1574, D6S309, D6S470, D6S429, D6S1653, D6S469, D6S289, D6S1630, D6S1605, D6S274, D6S1584, D6S285, and D6S422. The vertical axis represents NPL or LOD scores against chromosome 6p location points on the horizontal axis (in cM). Calculations were performed for VD, EL, and both VD + EL families analysed together.

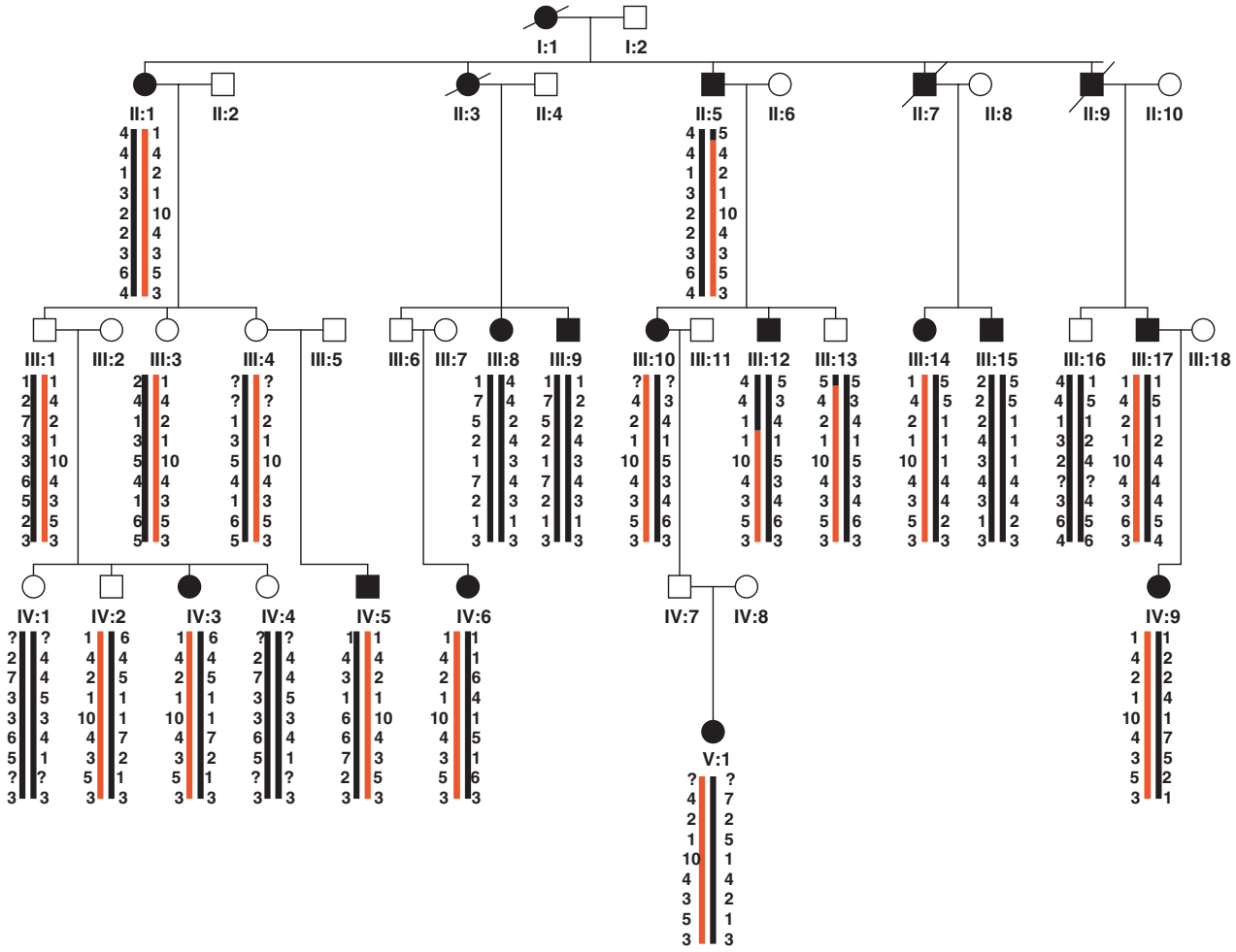

Fig. 3 Abridged pedigree chart of the VD family. Filled symbols: patients with definite ET diagnosis; empty symbols: individuals who did not show fully expressed definite ET or were unavailable for exam. Red bars under symbols: disease-associated haplotype. The haplotype was constructed on the basis of genotyping with nine most informative microsatellite markers. The marker order from telomere to centromere: D6S1574, D6S309, D6S470, D6S429, D6S1653, D6S469, D6S289, D6S1630, and D6S1605. '?' indicates non-critical alleles in some subjects for which genotyping has not been done.

carries out a relevant metabolic function and is expressed in the brain. Mutations in three of these genes are known or suspected to cause human neurodegenerative disease. Single-nucleotide substitutions within the coding region resulting in predicted amino acid change were identified in MYLIP, GMPR and NHLRC1 genes (Table 5). The MYLIP Ile202Leu substitution is a polymorphism with the 202Leu variant frequency of 4 in 180 control chromosomes (0.022), while the MYLIP Asp342Ser polymorphism is more common. The GMPR Ile256Phe and NHLRC1 Leu111Pro substitutions are known common polymorphisms. The results of genotyping of VD family members for these polymorphisms are consistent with allele sharing in the disease-associated haplotype and indicate that the rare MYLIP 202Leu allele co-segregates with the disease. In contrast, the more common 202Ile allele runs with the disease in the EL family. Discordant results were also obtained with the MYLIP Ser342Asn polymorphism: the VD patients show the 342Asp variant segregating with the disease, while EL disease-associated chromosomes carry the 342Ser variant. The results suggest that neither of the identified sequence variants causes ET, but a possibility remains that protein isoforms carrying some of these variations may be functionally impaired and their interaction with other proteins hampered, resulting in biological effects leading to pathological conditions.

Discussion

We attempted to establish genetic linkage in seven North American families with multiple members affected with ET. Although genetic predisposition to ET has long been recognized and the public health significance of this prevalent movement disorder well known, only two previous linkage studies have been reported (Gulcher *et al.*, 1997; Higgins *et al.*, 1997). This is due to significant difficulties that are generally encountered in ET studies: (i) the diagnosis of ET is not always straightforward (Bain *et al.*, 2000), while the

Table 4 Two-point LOD scores for linkage between markers in the 6p candidate region and essential tremor in VD and EL families

Marker	Family	LOD score at recombination fraction				
		Θ = 0.00	Θ = 0.10	Θ = 0.20	Θ = 0.30	Θ = 0.40
D6S1574	VD	−1.61	−0.68	−0.32	−0.16	−0.07
	EL	0.68	1.06	0.85	0.55	0.24
	Total	−0.93	0.38	0.53	0.39	0.17
D6S309	VD	0.08	0.35	0.39	0.30	0.17
	EL	0.55	0.35	0.23	0.14	0.07
	Total	0.63	0.70	0.62	0.44	0.24
D6S470	VD	1.52	1.55	1.13	0.68	0.30
	EL	−0.23	0.22	0.22	0.15	0.07
	Total	1.29	1.77	1.35	0.83	0.37
D6S429	VD	0.44	0.35	0.13	0.00	−0.03
	EL	−0.58	−0.35	−0.17	−0.07	−0.03
	Total	−0.14	0.00	−0.04	−0.07	0.00
D6S1653	VD	1.95	1.48	1.10	0.71	0.33
	EL	−0.13	−0.10	−0.01	0.03	0.03
	Total	1.82	1.38	1.09	0.74	0.36
D6S469	VD	−0.43	−0.57	−0.38	−0.18	−0.04
	EL	0.19	0.19	0.19	0.13	0.06
	Total	−0.24	−0.38	−0.19	−0.05	0.02
D6S289	VD	2.13	1.66	1.21	0.77	0.37
	EL	0.57	0.42	0.35	0.27	0.14
	Total	2.70	2.08	1.56	1.04	0.51
D6S1630	VD	1.88	1.36	0.87	0.46	0.16
	EL	0.46	0.40	0.38	0.28	0.14
	Total	2.34	1.76	1.25	0.74	0.30
D6S1605	VD	1.43	0.90	0.46	0.17	0.02
	EL	−0.75	−0.51	−0.28	−0.14	−0.05
	Total	0.68	0.39	0.18	0.03	−0.03
D6S274	VD	1.36	1.02	0.71	0.43	0.20
	EL	0.15	0.14	0.17	0.13	0.06
	Total	1.51	1.16	0.88	0.56	0.26
D6S1584	VD	1.74	1.23	0.80	0.44	0.17
	EL	0.63	0.50	0.44	0.30	0.14
	Total	2.37	1.73	1.24	0.73	0.31
D6S285	VD	0.89	0.75	0.52	0.29	0.11
	EL	1.59	1.18	0.77	0.42	0.16
	Total	2.48	1.93	1.29	0.71	0.27
D6S422	VD	1.33	1.02	0.77	0.50	0.23
	EL	0.25	0.19	0.21	0.16	0.07
	Total	1.58	1.21	0.98	0.66	0.30

slightest inaccuracy in the identification of disease status may lead to false linkage results; (ii) incomplete penetrance of ET observed in large pedigrees obscures the true pattern of inheritance and prevents the application of appropriate linkage analysis methods (Louis and Ottman, 1996); (iii) the high prevalence of ET reaching 4% makes it likely that two or more disease-causing genes segregate in a single large pedigree (Jankovic *et al.*, 1997); and (iv) the wide range of onset age with increasing prevalence in older individuals may lead to inclusion of non-genetic cases (phenocopies) (Bain *et al.*, 1994; Louis *et al.*, 1995; Wills, 1995). The above circumstances determined the strategy of the current study. Diagnostic criteria were defined at a meeting of a collaborative group of experts (Brin and Koller, 1998; Jankovic, 2002). Since the 'possible' and 'probable' ET diagnoses were poorly defined and caused most disagreements among the experts, only patients with 'definite' ET were included in genetic analysis as affected.

The selection of adequate methods for linkage analysis is another challenge. Parametric methods require knowledge of inheritance pattern, penetrance and phenocopy rates while nonparametric methods were not powerful enough to extract information from a large pedigree. The recently improved SimWalk2 (version 2.91) program based on Markov chain–Monte Carlo sampling has been accommodated for multipoint linkage analysis in pedigrees of any size and is

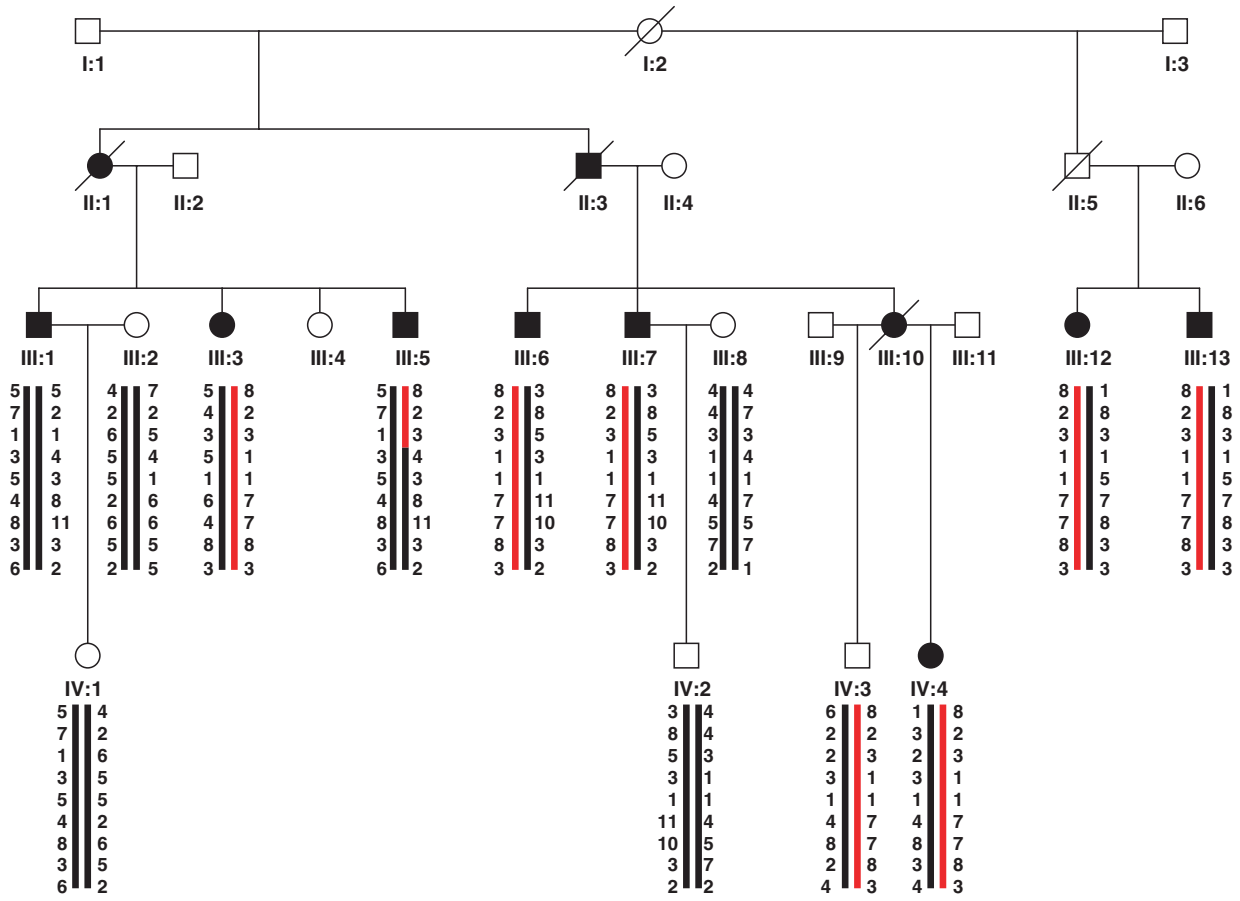

Fig. 4 Abridge pedigree chart of family EL. Designations the same as Fig. 3

suitable for both parametric and model-free nonparametric analysis (Sobel and Lange 1996; Sobel *et al.*, 2001; Sobel *et al.*, 2002; Lange and Lange, 2004). We chose this program as the primary analytical tool because our pedigrees greatly suffered from omission of generations due to incomplete penetrance, the absence of parents and possible presence of phenocopies. The multipoint non-parametric analysis performed by SimWalk2 is especially appropriate in the presence of heterogeneity. Furthermore, the use of a combination of several SimWalk2 statistics provide the best overall chance to detect linkage (Lange and Lange, 2004). In a hope of gaining support for the results of nonparametric analysis, we also used more traditional parametric multipoint and two-point linkage analyses.

We obtained evidence for suggestive linkage between definite ET and markers at a novel susceptibility locus on chromosome 6p23. Linkage to this genomic region was confirmed in family VD by fine mapping and calculations using two different linkage programs. According to the expert recommendations (Lander and Kruglyak, 1995), suggestive linkage cannot be ignored because it may correspond to a major susceptibility locus that is difficult to display due to diagnostic problems and complicated disease transmission model. Most encouraging, the results of analysis of a second studied pedigree showed weak linkage to this same locus. Calculations performed on both families taken together brought the linkage scores close to a significant level.

At the same time, the methodology we used failed to uncover consistent linkage signals at any chromosomal region in five remaining families, implying that more than one ET-associated gene is being segregated in these families. Studies of large multigenerational pedigrees provide significant advantage for genetic analysis but do not guarantee avoiding heterogeneity. Thus, three affected members of the VD family and a single member of the EL family did not share the disease haplotype. There is evidence that these individuals were phenotypically somewhat different from the other affected family members, suggesting that they were phenocopies. Phenocopy rate has not been determined in ET, but a growing number of studies underline the role of environmental factors in ET, as for example beta-carboline alkaloids that are naturally present in the food (Louis *et al.*, 2002) or lead toxicity (Louis *et al.*, 2003*a*).

The search for candidate genes in the 6p23 region did not lead to identification of a causative mutation and was unsuccessful in this sense. But among the identified alleles, there may be some that participate with other still unknown genetic alterations in multifactorial causation of tremor. Thus, the myosin regulatory light chain interacting protein

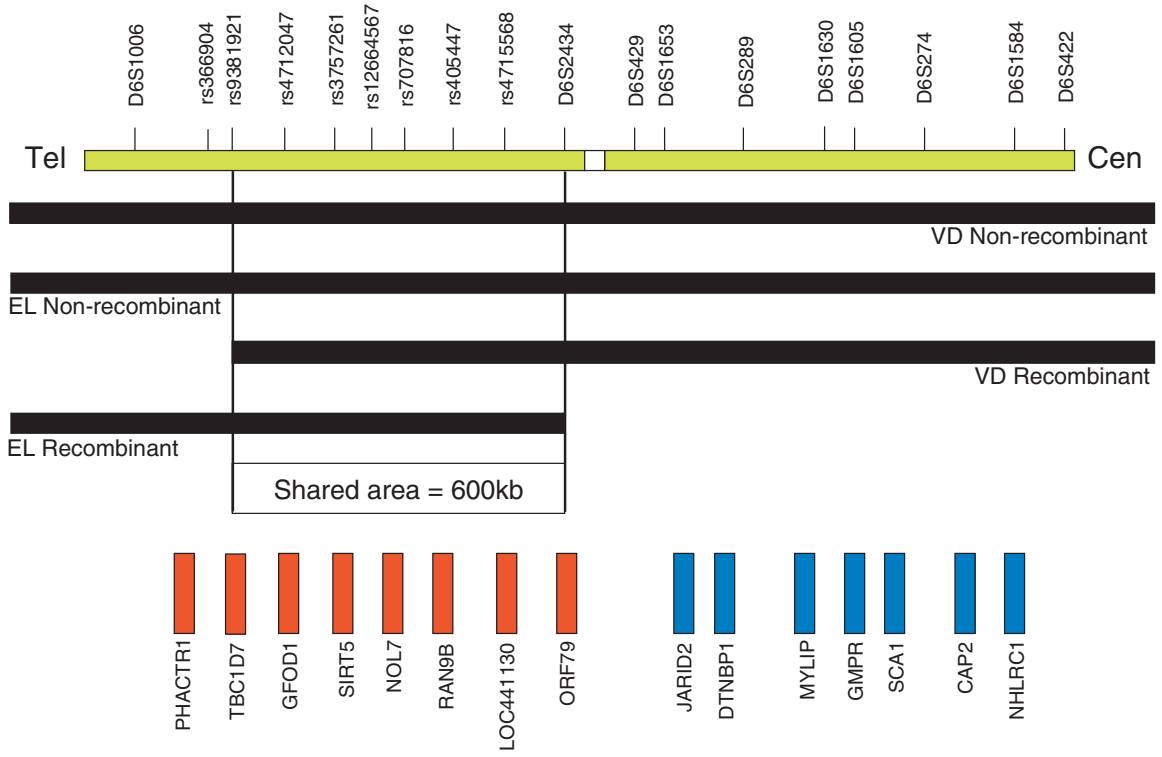

Fig. 5 Analysis of candidate region on chromosome 6p22.3–p24.1. Genotyping with SNPs (rs) and additional microsatellite markers designate a region restricted by recombinations in the VD and EL families (shared area). Eight genes (shown in red) are located within or near the shared 600 kb interval and seven other genes (shown in blue) are located in a larger region between markers D6S2434 and D6S422 containing markers with maximal linkage scores.

(MYLIP) gene showing two amino acid altering polymorphisms is a member of the ezrin-radixin-moesin protein family involved in membrane cytoskeleton interactions and cell dynamics (Bornhauser *et al.*, 2003). MYLIP is expressed in many regions of the developing and adult brain, especially neurons of hippocampus and cerebellum, and was shown to be involved in neurite outgrowth (Olsson *et al.*, 2000). Guanosine monophosphate reductase (GMPR) gene, also possessing coding variations, is involved in thermogenesis (Salvatore *et al.*, 1998), but its function in the nervous system is unknown. Haplotype analysis indicates that the MYLIP gene may still be associated with the risk of ET, even if different phases of the rare polymorphism at codon 202 were present in the linked families. Experimental evaluation of the functional significance of these substitutions would be needed to verify their pathogenic effects.

Although ET is the most common movement disorder, and tremor is a visible sign, there are many disagreements among movement disorder specialists regarding diagnostic criteria of ET (Chouinard *et al.*, 1997). ET overlaps with other neurological disorders such as parkinsonism, dystonia, migraine and peripheral neuropathy (Brin and Koller, 1998; Jankovic, 2002). Major disagreement exists regarding the location and the extent of dystonic movements allowed to be present in patients with ET (Deuschl *et al.*, 1998; Elble, 2002; Jankovic, 2002). In this study, patients expressing either tremor with dystonia or pure tremor possess shared haplotypes in the 6p23 region. It is therefore possible that these syndromes have common pathogenic mechanisms. They may be allelic disorders or result from co-inheritance of separate genes.

Several members of the best studied VD family died from malignant hyperthermia, and many survivors are genetically predisposed to this disorder (Sambuughin *et al.*, 2001). Pedigree analysis indicated that malignant hyperthermia and ET segregated in this family independently, although four family members had both malignant hyperthermia susceptibility and hand tremor. To entertain further a possible connection between malignant hyperthermia and ET, some ET patients have shown good response to calcium channel blockers (Biary *et al.*, 1995) or carbonic anhydrase inhibitors (Busenbark *et al.*, 1993). Calcium channel or other electrolyte metabolism defects may have a role in ET, but only future genetic studies may be able to identify this connection.

In conclusion, we performed genomewide linkage analysis and fine mapping on the largest set of families with ET assembled to date that included various frequently seen phenotypes. Suggestive linkage was identified on chromosome 6p23 in the VD family with positive linkage in a second unrelated family, EL, making this region a novel candidate location presumably containing a gene responsible for ET susceptibility. It will be important

Table 5 Analysis of transcripts located in the 6p candidate region

Gene symbol	Description and possible function	Polymorphisms	Population frequency of the rarer allele
PHACTR1	Phosphatase and actin regulator 1. Binds to actin and PP1. Inhibits PP1 enzymatic activity	None	
TBC1D7	TBC1 domain family, member 7. Function unknown	A/G(Thr136Ala)	Known polymorphism
GFOD1	Glucose-fructose oxidoreductase domain containing protein 1. Has oxido-reductase activity	None	
SIRT5	Silent mating-type information regulation-2 homologue 5 (sirtuin). Regulatory protein with mono-ADP-ribosyltransferase activity	C→T in intron 5 (rs3734674)	0.206
NOL7	Nucleolar protein 7. Function unknown	A→T in intron 7	0.44
RANBP9	RAN binding protein 9. Belongs to RAS family, is essential for translocation of RNA and proteins through nuclear pore complex	C→A in intron	Unknown
LOC441130	Hypothetical gene. Function unknown	C→T in 3′ untranslated region	0.3
C6orf79	Chromosome 6 open reading frame 79. Function unknown	TT deletion in intron	Unknown
JARID2	Jumonji, AT rich interactive domain 2. Regulates transcription, cell growth, central nervous system development	C→G (Thr199Thr) G→A (Leu1093Leu)	Unknown 0.405
DTNBP1 (dysbindin)	Dystrobrevin binding protein 1. Component of dystrophin-associated protein complex. A mutation in DTNBP1 causes Hermansky-Pudlak syndrome; DTNBP1 is reduced in hippocampus of schizophrenic patients	None	
MYLIP	Myosin regulatory light chain interacting protein. Member of ezrin, radixin, moesin (ERM) complex. Inhibits neurite overgrowth	A→C (Ile202Leu) A→G (Ser342Asn)	0.022 known polymorphism
GMPR	Guanosine monophosphate reductase. Catalyses NADPH-dependent reductive deamination of GMP to inosine monophosphate	T→A (Phe256Ile)	0.266
SCA1/ATXN1	Spinocerebellar ataxia type 1. The ATXN1 gene containing an expanded CAG repeat causes SCA1	A→G (Glu-to-Glu)	Unknown
CAP2	Adenylate cyclase-associated protein 2. Interacts with adenyl cyclase and actin	A→G (Lys379Lys)	0.310
NHLRC1	Nhl repeat-containing gene 1. Point and deletion mutations in the NHLRC1 gene cause Lafora disease	T→C (Leu111Pro)	Known polymorphism

to test whether ET maps to the same locus in additional pedigrees.

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