Electronic letter

No evidence of allelic heterogeneity in the *DYT1* gene of European patients with early onset torsion dystonia

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EDITOR—Torsion dystonia is a movement disorder characterised by sustained involuntary muscle contractions, frequently causing twisting and repetitive movements or abnormal postures.[1] Primary torsion dystonia (PTD) occurs either in a familial or sporadic pattern with dystonia as the sole phenotypic manifestation with the exception that tremor can be present as well. Early onset, generalised torsion dystonia is the most severe form of hereditary dystonia, and the most prevalent form is the result of mutation in the *DYT1* (*TOR1A)* gene on chromosome 9q34.[2] Inheritance follows an autosomal dominant mode of transmission with reduced penetrance (30-40%),[3] and there is a particularly high prevalence in Ashkenazi Jews (AJ) as a result of a founder effect and genetic drift.[4] Early onset primary dystonia resulting from *DYT1* usually starts in an arm or a leg at a mean age of 12.5 years (this can range, however, from 4 to 44 years).[5] More than 60-70% of cases have progression to generalised dystonia involving limb and axial muscles, but the cranial muscles are only involved in 11-18% of cases.[6] The causative mutation has been identified as a 3 bp deletion (946delGAG) in the coding sequence of the *DYT1* gene, resulting in loss of one of a pair of glutamic acid residues near the C-terminus of the encoded protein, torsinA.[7] Presumably, deletion of this amino acid results in a critical change in the function of the gene product that leads to clinical signs of dystonia.

Currently, this mutation is the only sequence change found to be associated with the disease state, regardless of ethnic origin, both as an inherited[7] or a de novo deletion.[8] The ΔGAG in the heterozygous state accounts for 50-60% of non-Jewish (NJ) subjects[9–11] and over 90% of AJ subjects[5] with early, limb onset generalised dystonia. In contrast to the AJ population, analysis of haplotypes in the NJ population suggests no founder effect but multiple events resulting in independent occurrences of the same recurrent mutation, a situation which is highly unusual in clinical genetics.

The high proportion of European PTD patients with early limb onset phenotype who do not carry the mutation (40%) may represent either allelic or locus heterogeneity in dystonia. Whether other changes within the *DYT1* gene lead to dystonia or some other phenotype still remains unknown, as patients with early onset PTD have been tested only for the 946delGAG mutation in recent studies.[9–13] Only one previous study reported screening for new *DYT1* mutations using a genomic DNA approach in American dystonic patients.[14] The main purpose of this study was to assess if non-Jewish European patients with early onset torsion dystonia without the deletion had other mutations in the *DYT1* gene or anomalies in other genes. Additional mutations in the *DYT1* gene were screened by transcript analysis to allow detection of base substitutions as well as rearrangements resulting from splicing defects, or heterozygous exon deletion (or duplication) not detected by simple PCR amplification.

Patients, methods, and results

Most of the families in our series were ascertained through probands with early onset generalised dystonia diagnosed and treated by deep brain stimulation[15] in the Department of Paediatric Neurosurgery at the Montpellier University Hospital. Additional patients were referred from Neuropaediatrics or Clinical Genetics services. Therefore, our series of patients did not represent a random sample of PTD, but mostly included patients with severe, generalised PTD and/or early onset, in infancy or childhood. The diagnosis of PTD was established according to current criteria.[16] A total of 35 patients were selected for the study (10 familial index cases and 25 sporadic cases). All had onset of symptoms before the age of 24. A very early onset (⩽5 years) group of 11 NJ patients was observed. All the patients were French except one of Turkish origin; two patients had one AJ parent. All families gave informed consent before participating in the study.

The cohort of 35 probands was first examined for the recurrent 946delGAG mutation in the *DYT1* gene. PCR products were generated from genomic DNA with primers 6419 and H48, and the 200 bp product was digested with *Bse*RI of which one cutting site is abolished by the GAG deleted sequence.[7] Taken together, 14 subjects (14/35, 40%) were

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positive for the GAG deletion in the *DYT1* gene, including the two patients whose fathers were of AJ origin. Fifty seven percent (8/14) were males. The mean age at onset of the group was 8.8 years (SD 2.56) (range 6-15 years). The term "typical *DYT1* phenotype" is used generally for a selected group of patients characterised by early, limb onset, generalised dystonia without spread to the cranial muscles such as the face, pharynx, or tongue.[6 16] Any patient who conformed to this phenotype showed the GAG deletion, whereas only two patients (2/17 = 11.8%) with early, limb onset, generalised PTD spreading to cranial muscles tested positive for the *DYT1* mutation. The mutation was also not found in patients with focal dystonia nor in any patient with onset of symptoms in the neck, trunk, or face. Furthermore, all patients with early childhood onset (≤5 years) were non-carriers. This finding agrees with recent published guidelines for diagnostic testing, which states that the optimal classification rule to predict carriers in the NJ population uses for criteria an age at onset between 6 and 16 years.[17]

Nine patients (64%) had at least one relative affected by PTD (table 1). It is noteworthy that in three cases (patients 2, 3, and 13), the affected relatives suffering from either torticollis or writer's cramp had not received a diagnosis of PTD until they underwent detailed clinical examination by neurologists as part of this study. A wide spectrum of symptoms that can overlap with other forms of dystonia was observed in the affected relatives, and disease severity varied considerably within the same family (table 1). Moreover, the father of patient 7 was found to have blepharospasm, an atypical clinical presentation of the *DYT1* mutation. *DYT1* related dystonia does not commonly affect facial muscles as the first site of onset, and such a phenotype has been rarely reported in *DYT1* carriers.[5] This patient is currently treated with botulinum toxin.

All available affected subjects were screened for the 946delGAG mutation in these nine families and were found to carry the *DYT1* mutation (table 1). Asymptomatic *DYT1* carriers were also identified in families of patients 2, 3, 4, and 8, which was consistent with the assumption of autosomal dominant inheritance with reduced penetrance (table 1). Genotypes were determined for four polymorphic markers (D9S2160, D9S2161, D9S63, and D9S2162) surrounding the *DYT1* gene in all carriers of the deletion and available relatives. Products were analysed by GeneScan analysis on an ABI 377 sequencer with 672 software. No common haplotype could be established between *DYT1* carriers, indicating that the GAG deletion has arisen repeatedly but independently in the studied population.

The remaining five positive deletion patients were apparently sporadic cases. In one case, both parents were available for genetic testing and the mutation was found in the unaffected patient's mother. For each of the four other cases (including the two patients of AJ ethnic background), only the mother could be analysed, and was shown to be GAG deletion negative; the father should therefore be tested to determine if these sporadic cases result from either de novo mutation or low penetrance of the mutant gene.

Twenty one patients (21/35 = 60%) with early onset PTD did not carry the GAG deletion. Many of these early onset non-carriers share other clinical features with carriers, including limb onset and a tendency to spread to other body regions. We investigated the possibility of other mutations in *DYT1* producing an early onset phenotype in these patients by screening *DYT1* transcripts from peripheral blood lymphocyte RNA for additional mutations. New blood samples for RNA isolation were obtained in 16 of the 21 non-deleted patients (table 1). None of them was of AJ origin. The age at onset varied more widely in this group compared with the *DYT1* positive group (mean age of 7.4 years (SD 5.81)). Among the 16 patients under investigation (table 1), 14 had generalised disease with involvement of cervicocranial muscles. Ten of them had limb onset whereas three of them had onset in the trunk, neck, or face (cases 30, 31, and 32). In case 33, the first symptom was dysarthria. Patients 34 and 35 developed dystonia of the right upper limb, but no further spread of dystonia had occurred at the time of examination. All but one (patient 34) were sporadic cases. Familial presentation of dystonia in case 34 should be noted. The sister of this patient was reported to have transient idiopathic focal dystonia at the age of 3 years, which resolved rapidly within five months. She is currently aged 8 years and does not present any sign of dystonia.

An 1108 bp region including the 998 bp of the coding sequence was PCR amplified from cDNA derived from lymphocytes using the Access RT-PCR system (Promega, Madison, WI) and specific gene primers (5'-CGCGG TCGGCGCGAGAACAA-3', forward, and 5'-GTGGAAGGACTGAGTGTTGTTT-3', reverse). The PCR products were then submitted to a second round of amplification generating two overlapping fragments by using the following primers (5'-GCAGGGTGGCGCGGGT CC-3' and 5'-AAGGCTTGATGGCATCTA TGAG-3' for fragment 1) and (5'-TGTACAA GGATCAGTTACAGTTGTG-3' and 5'-TGT TTCTTTTCCAACTCCAGGC-3' for fragment 2). Both fragments were screened for heterozygous mutations by sequence analysis.

We detected no nucleotide change that would alter the amino acid sequence of the protein in any of the 16 patients analysed. In addition, we did not find any deletion (or duplication) of a single exon or aberrantly spliced transcripts indicative of splicing mutations. In five out of the 16 patients who were examined, amplification of fragment 1 showed a smaller band in addition to the normal PCR product (fig 1). Sequencing of the smaller band disclosed the removal of Ala60-Lys148, encoded by exon 2, which was consistent with skipping of this exon in lymphocytes. This previously undescribed alternatively spliced form of *DYT1* transcripts resulting in loss of frame was also observed in three normal controls.

Table 1 Clinical, familial, and molecular data of the 35 index cases with early onset PTD tested for mutations in the DYT1 gene

	Patient (sex)	*Age at onset*	*Site of onset*	*Age at generalisation*	*ΔGAG*	*Family history*	*Clinical features in affected relatives*
Limb onset generalised dystonia without spread to cervicocranial muscles	1 (F)	7 y 6 mth	L foot	9 y 3 mth	+	Yes	Mother: mild generalised dystonia
	2 (F)	6 y 6 mth	R hand	9 y 4 mth	+	Yes	PGF: writer's cramp, 2 unaffected
	3 (M)	7 y	R hand	7 y 7 mth	+	Yes	Father: spasmodic torticollis, 3 unaffected
	4 (M)	15 y	Arm	?	+	Yes	4 generalised, 2 writer's cramp, 5 unaffected
	5 (M)	12 y	R hand	17 y	+	Yes	Mother: generalised dystonia
	6 (F)	9 y	Arm	30 y	+	Yes	Cousin: multifocal dystonia
	7 (M)	7 y	L hand	11 y 8 mth	+	Yes	Father: blepharospasm
	8 (F)	9 y 8 mth	R hand	11 y 8 mth	+	Yes	Brother: focal dystonia; MGF: writer's cramp, 1 unaffected
	9 (M)	9 y	R hand	10 y 6 mth	+	No	
	10 (F)	11 y 6 mth	L foot	12 y 7 mth	+	No	
	11 (M)	7 y 1 mth	R foot	9 y	+	No	
	12 (M)	7 y 9 mth	L foot	10 y	+	No	
Limb onset generalised dystonia with spread to cervicocranial muscles	13 (M)	7 y 11 mth	R hand	10 y 4 mth	+	Yes	Father: writer's cramp
	14 (F)	6 y	L foot	8 y	+	No	
	15* (M)	1 y 1 mth	R hand	1 y 6 mth	−	No	
	16* (F)	18 mth	L foot	3 y 6 mth	−	No	
	17* (F)	2 y	L hand	5 y	−	No	
	18* (F)	2 y 7 mth	Arm	2 y 9 mth	−	No	
	19* (F)	3 y	R foot	3 y 6 mth	−	No	
	20* (F)	3 y 6 mth	L foot	8 y	−	No	
	21* (F)	5 y	R hand	5 y 6 mth	−	No	
	22* (F)	8 y	L hand	14 y	−	No	
	23* (F)	13 y	R hand	14 y 6 mth	−	No	
	24* (M)	10 y	L foot	15 y	−	No	
	25 (F)	4 y	Foot	10 y	−	No	
	26 (M)	9 y	Leg		−	No	
	27 (M)	12 y 6 mth	Arm	14 y	−	No	
	28 (F)	12 y 6 mth	Arm	20 y	−	No	
	29 (F)	3 y 4 mth	Leg	13 y	−	No	
Generalised dystonia with other affected sites	30* (F)	2 y	Trunk, face	2 y 6 mth	−	No	
	31* (M)	13 y	Neck, trunk	18 y	−	No	
	32* (M)	22 y	Neck, trunk	22 y	−	No	
	33* (F)	17 y	Dysarthria	?	−	No	
Focal dystonia (writer's cramp)	34* (M)	2 y 6 mth	R hand	− (aged 11 y)	−	Yes	Sister: transient dystonia at 3 years
	35* (M)	7 y	R hand	− (aged 9 y)	−	No	

PGF: paternal grandfather, MGF: maternal grandfather, *patients analysed for additional mutations by transcript analysis, ?: unknown.

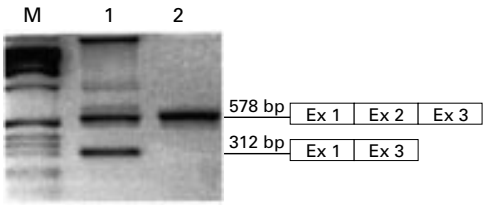

Figure 1 Total RNA from peripheral blood lymphocytes was reverse transcribed and PCR amplified (RT-PCR) using primers located upstream of exon 1 (forward) and in exon 3 (reverse). The products were analysed on a 2.5% agarose gel. The normal product expected for fragment 1 is 578 bp; a shorter fragment (312 bp) was observed in five patients and in three normal controls in addition to the normal fragment. Sequencing of the 312 bp fragment showed that the 266 bp exon 2 was missing, thereby fully accounting for the reduced size of the mutant fragment. M: molecular weight marker (1 kb ladder, Gibco BRL), 1: detection of alternative exon 2 skipping in DYT1 transcripts, 2: normal sized RT-PCR fragment containing exons 1-3.

Moreover, two previously reported polymorphisms (288C/T and 688G/C)[7] in the coding sequence were detected in this group of patients.

Discussion

Our data agree with a previous report by Ozelius *et al*,[14] who failed to identify any new change in the *DYT1 (TOR1A)* gene, and its human homologue *TOR1B*, in 17 patients with features typical of early onset dystonia, by using a DNA based approach. In the present study, an RNA based strategy was used to allow detection of partial gene deletions (or duplications) and inclusions of intronic sequences, in addition to amino acids substitutions. Even though it remains possible that some mutations in untranslated regions could have been missed, these results may indicate that the ΔGAG deletion is the only *DYT1* mutation responsible for early onset PTD.

The predominance of this mutation may reflect an increased frequency of this sequence alteration owing to genetic instability in an imperfect tandem of a 24 bp repeat in the region of the deletion.[8] Also, it has been hypothesised that the inability to identify additional mutations in the *DYT1* gene indicates that the new torsinA protein conformation resulting from the GAG deletion (loss of a glutamic acid residue in the carboxy terminal) is functionally unique. TorsinA bears low but significant homology to the Hsp100/Clp family of ATPase chaperones.[18 19] The recent identification of torsinA as a lumenally associated glycoprotein localised to intracellular membranes of the endoplasmic reticulum and nuclear envelope suggests that this protein serves as a molecular chaperone assisting in the proper folding of secreted and/or membrane proteins.[20] Several molecular mechanisms have been proposed. The mutant form of torsinA may (1) sequester torsinA in inactive complexes, thus acting as a dominant negative, (2) behave as a constitutively active torsinA, thus acting as a dominant positive, and/or (3) possess novel functionality distinct from the biological roles of torsinA.[20]

The identification of a single mutation on affected chromosomes responsible for almost all cases of typical early onset dystonia is remarkable. The GAG deletion would be one of the rare examples of the same recurrent and unique mutation causing a dominantly inherited condition. Other examples include hypokalaemic periodic paralysis,[21] achondroplasia,[22] hypertrophic cardiomyopathy,[23] and the triplet repeat disorders. In all these cases, it appears that the same mutation occurs repeatedly as independent events, whereas other mutations in the same gene cause a different syndrome, have no phenotype, or are incompatible with life.

To date, the *DYT1* gene is the only primary PTD gene identified for which a direct DNA based genetic test is available. Our results confirm a genotype/phenotype correlation in early onset PTD since the 14 *DYT1* positive index cases displayed a characteristic phenotype. The phenotype is marked by onset in a limb, usually before 24 years of age, frequently spreading to other limbs, occasionally to the neck, and rarely to cranial muscles. However, a variable progression of dystonia was observed in familial cases, and atypical features such as blepharospasm may be the only sign. It is still unclear whether these phenotypes represent variable expression of the GAG mutation or the effect of modifying genes or environmental factors. It has been suggested that the GAG deletion in the *DYT1* gene would increase a person's susceptibility to a "second hit" brought on by either genetic or environmental factors.[20] This observation extends the clinical spectrum of *DYT1* associated dystonia and shows the significance of molecular testing in establishing the clinical diagnosis of hereditary dystonic syndromes. As previously stated, *DYT1* diagnostic testing in subjects with atypical clinical features or late onset may be warranted in those having a positive family history of early onset PTD.[12]

In accordance with a previous report,[16] we found no deletion carriers among patients with onset in early childhood (younger than 5 years). The delay of generalisation tends to be shorter (within a few months) in this group of patients compared to *DYT1* patients. According to Fahn *et al*,[16] age is the most important single factor related to the prognosis of idiopathic dystonia. The younger the age at onset, the more likely the dystonia will become severe and also spread to multiple parts of the body .

In conclusion, comprehensive screening of the whole *DYT1* coding sequence in patients with early onset PTD argues for differences in disease causing loci of early onset autosomal dominant dystonia, with additional responsible gene(s) yet to be identified. PTD is a clinically and genetically heterogeneous group of movement disorders. Three other PTD loci have been mapped to date, each associated with a relatively well defined phenotype, although there is substantial phenotypic overlap in individual cases.[5] The *DYT7* locus on chromosome 18p is associated with adult onset focal cervical dystonia,[24] whereas the *DYT6* locus on chromosome 8 is associated with a mixed phenotype (the symptoms range from purely focal to

generalised dystonia with variable age of onset.[25] Recently, a novel PTD locus, *DYT13* on chromosome 1p, has been characterised in a large Italian family with prominent craniocervical and upper limb involvement and mild severity.[26] Other genes involved in the aetiology of dystonia, particularly in early onset generalised PTD, remain to be characterised.

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