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Identification of a Highly Polymorphic Microsatellite VNTR within the Argininosuccinate Synthetase Locus: Exclusion of the Dystonia Gene on 9q32-34 as the Cause of Dopa-responsive Dystonia in a Large Kindred

David J. Kwiatkowski,* Torbjoern G. Nygaard,‖ Deborah E. Schuback,† Scott Perman,* Joel M. Trugman,** Susan B. Bressman,‖ Robert E. Burke,‖ Mitchell F. Brin,‖ Laurie Ozelius,†'§ Xandra O. Breakefield,†'‡ Stanley Fahn,‖ and Patricia L. Kramer#

Departments of *Medicine and †Neurology, Massachusetts General Hospital; and ‡Neuroscience Program and §Department of Genetics, Harvard Medical School, Boston; ‖Dystonia Clinical Research Center and Department of Neurology, Columbia University College of Physicians and Surgeons, New York; #Department of Neurology, Oregon Health Sciences University, Portland; and **Department of Neurology, University of Virginia Health Sciences Center, Charlottesville

Summary

Dopa-responsive dystonia is a clinical variant of idiopathic torsion dystonia that is distinguished from other forms of dystonia by the frequent cooccurrence of parkinsonism, diurnal fluctuation of symptoms, and its dramatic therapeutic response to L-dopa. Linkage of a gene causing classic dystonia in a large non-Jewish kindred (DYT1) and in a group of Ashkenazi Jewish families, to the gelsolin (GSN) and argininosuccinate synthetase (ASS) loci on chromosome 9q32-34, respectively, was recently determined. Here we report the discovery of a highly informative (GT)n repeat VNTR polymorphism within the ASS locus. Analysis of a large kindred with dopa-responsive dystonia, using this new polymorphism and conventional RFLPs for the 9q32-34 region, excludes loci in this region as a cause of this form of dystonia. This provides proof of genetic heterogeneity between classic idiopathic torsion dystonia and dopa-responsive dystonia.

Introduction

Torsion dystonia is a syndrome characterized by sustained, involuntary muscle contractions, frequently causing twisting and repetitive movements or abnormal postures (Fahn 1988). Dystonia can occur as a manifestation of a large number of neurologic disorders. The occurrence of dystonia in the absence of known disease (e.g., Wilson disease), or identifiable biochemical or pathologic disorder, is classified as idiopathic (primary) torsion dystonia (ITD).

ITD occurs in both hereditary and nonhereditary (sporadic) forms. Inherited forms of the condition are generally autosomal dominant in inheritance (Zeman and Dyken 1967; Bressman et al. 1989), although an X-linked form has been described in the Philippines (Lee et al. 1976; Fahn and Moskowitz 1988) and an autosomal recessive form has been reported in Spanish gypsies (Gimenez-Roldan et al. 1988). ITD occurs with a higher frequency in Ashkenazi Jews (1/15,000) (Zilber et al. 1984) than in the non-Jewish population (1/160,000). Disease expression is variable, with children most often developing severe, disabling, generalized dystonia and adults more often having restricted, segmental, or focal dystonia. Penetrance estimates vary, from .30–.40 among Ashkenazi Jews (Bressman et al. 1989; Pauls and Korczyn 1990; Risch et al. 1990) to as high as .75 in non-Jewish kindreds (Eldridge 1970; Ozelius et al. 1989).

A recent classification scheme for ITD recognizes clinical variants that differ from classic ITD by their specific clinical features (paroxysmal dystonia, myoclonic dys-

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Address for correspondence and reprints: David J. Kwiatkowski, Hematology Unit, Massachusetts General Hospital, 8 West, Building 149, Thirteenth Street, Charlestown, MA 02129.

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tonia, and diurnal dystonia) or their pharmacologic responsiveness (e.g., alcohol or levodopa) (Fahn 1989). Dopa-responsive dystonia (DRD) represents one of these variants. DRD is a hereditary dystonia distinguished from ITD by the frequent cooccurrence of parkinsonism (rigidity, bradykinesia, postural instability, and, rarely, rest tremor), diurnal fluctuation of symptom severity, and dramatic responsiveness to treatment with L-dopa (Segawa et al. 1976; Deonna 1986; Nygaard et al. 1986).

We previously established genetic linkage between a gene for dystonia and DNA markers in the 9q32-34 region in a large non-Jewish kindred (Ozelius et al. 1989) and in a group of Jewish families (Kramer et al. 1990). In the non-Jewish family, strongest evidence for linkage was obtained between the gene for dystonia (DYT1) and the gelsolin (GSN) locus. In the Jewish families, the gene for dystonia was most closely linked to the argininosuccinate synthetase (ASS) locus. Both the GSN and ASS loci have been mapped to 9q32-q34, by both physical (Beaudet et al. 1982; Kwiatkowski et al. 1988) and genetic means, with recombination distance 14 centiMorgans (cM) (Kramer et al. 1990). Therefore, the same gene may be responsible for both of these hereditary forms of dystonia.

Here we have investigated in detail a family with DRD (family S) that initially came to medical attention in 1973 (deYebenes et al. 1988). This family is of mixed English-Welsh origin and consists of over 240 members from 5 generations, of whom 25 have some features of dystonia (Nygaard et al. 1990). Linkage analysis using a newly defined (GT)n microsatellite VNTR polymorphism within the ASS locus, and other polymorphic DNA markers from the 9q32-34 region, excludes the ITD loci as a cause of DRD in family S.

Material and Methods

Neurologic Examination and Family Material

The complete pedigree, standardized examination protocol, and clinical features of family S have recently been described (Nygaard et al. 1990). For this analysis, 110 family members and 14 spouses were examined. Affected status for dystonia was assigned following review of the videotapes by at least two blinded neurologists with specialization in movement disorders (S.B.B., R.E.B., M.F.B.). Disease status assignments consisted of these categories: definite (unequivocal features of dystonia), probable (examination highly suggestive of dystonia), possible (examination abnormal but not diagnostic of dystonia), and no dystonia (Kramer et al. 1990). In addition, demonstration of a response to levodopa therapy was required to assure conformity to the DRD phenotype before an assignment of definite DRD was made. Final assignment of affected status of all possible, probable, and definitely affected members previously identified was made without knowledge of pedigree position or allele status by a second review (by S.F.). Blood was available from nine individuals classified as definite DRD, and 51 other members of the pedigree.

DNA Methods

Blood was obtained by venesection from consenting family members. DNA was prepared from peripheral blood leukocytes or established lymphoblastoid cell lines by standard methods (Ozelius et al. 1989). Authenticity of DNA samples was confirmed by multiple analyses with polymorphic markers from the 9q and other regions, to confirm family relationship status. For Southern blot analyses, DNA was digested to completion, size fractionated by agarose gel electrophoresis, transferred to Genetran nylon membranes, probed, and washed as described previously (Ozelius et al. 1989). DNA probes were labeled with (^{32}P)dATP or (^{32}P)dCTP by the hexanucleotide method (Feinberg and Vogelstein 1983). Gel-purified insert fragments were used for labeling. The following polymorphic marker probes on 9q were used: pMCT136 (*PstI*) for random VNTR locus *D9S10* (q34) (Lathrop et al. 1988); CRI-L659 (*TaqI*) for random locus *D9S26* (q31-34) (Donis-Keller et al. 1987), M1D (*StuI*) for the gelsolin locus (*GSN;* q32-34) (Kwiatkowski et al. 1988, 1989), Lamp92 (*TaqI* and *PvuII*) for the random locus *D9S29* (q31) (Pandolfo et al. 1988), and pAK1B3.25 (*TaqI*) for the adenylate kinase-1 locus (*AK1;* q32-34) (Bech-Hansen et al. 1989; Zuffardi et al. 1989). References for map positions and RFLPs for all loci except *D9S29* are cited (Smith and Simpson 1989).

DNA sequencing was performed by subcloning into the M13mp18 and mp19 cloning vectors, using (^{35}S)-dATP and modified T7 DNA polymerase. All sequence data shown were determined on both strands and have been submitted to GenBank. Analysis of allele status for the ASS (GT)n repeat polymorphism was performed using oligonucleotide primer pairs ASS-A and ASS-B, or ASS-A and ASS-C (see fig. 1), in the PCR to amplify the repeat-containing region. Reaction volumes were 10 μl and contained 0.2 mM dATP, dGTP, dTTP; 2.5 μM dCTP; 4 ng each oligonucleotide; 0.08 μl ^{32}P dCTP (3,000 mCi/mm); and 0.05 μl *Taq* polymerase (Perkin Elmer–Cetus). Thermal controller settings were 94°C for 1.5 min; 25 cycles of 94°C for 1 min,

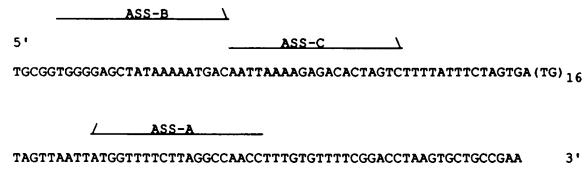

Figure 1 Sequence of the ASS locus clone, ASSg1. The positions of the oligonucleotides synthesized for use in PCR amplification and polymorphism analysis are indicated. The sequence of the ASS-A oligonucleotide is the inverse complement of that shown.

55°C for 1 min, and 82°C for 1 min; 72°C for 9 min. For the pair of oligonucleotides ASS-A and ASS-C, the annealing temperature was reduced to 51°C. Amplified products were analyzed by electrophoresis of a 2-μl aliquot of the preparation on 6% acrylamide, 8 M urea, sequencing-type gels. Gels were dried and exposed to Kodak XAR film for 4–24 h without screen for preparation of autoradiographs.

To standardize readings, the DNA from a single ("control") individual was also amplified and analyzed on each gel. Alleles were designated 0–16, with 0 chosen as the allele with the fewest number of GT repeats we have seen, and the other alleles enumerated 2–16 according to the number of additional bases their amplified fragments contained. Thus, allele 4, for example, contains four additional bases or two additional GT repeats, in comparison with the 0 allele. Allele readings for both RFLPs and the ASS polymorphism were made without knowledge of disease assignment.

The poly GT repeat sequence used for screening genomic clones was obtained from Pharmacia, labeled by the hexanucleotide method, and hybridized to nylon filters to identify GT repeat sequences, by means similar to those used for other DNA probes, as described above.

Linkage Analysis

We classified only those individuals with definite DRD as affected for the linkage analyses. Individuals with definite dystonia, but without proven levodopa responsiveness, and individuals in the other intermediate affected classes for dystonia were coded as "unknown." All individuals rated as having no dystonia were coded as unaffected. Parkinsonism was not considered in the phenotype assignment.

Two-point linkage analyses were performed using the LIPED program, version 3 (Ott 1976; Hodge et al. 1979), which incorporates age correction. Inheritance of DRD in this family is consistent with autosomal dominant transmission of a rare allele (q = .0001) with reduced penetrance. We calculated a penetrance estimate of .35 using the method of Johnson (Johnson et al. 1988), based only on those individuals with definite DRD. Previous reports have suggested there is a sex-related difference in the penetrance of DRD with the number of affected females reported as 2.5 × the number of affected males (Nygaard 1989). This also appears to be the case in the current family. In the absence of any rigorous statistical analysis of the mode of inheritance among families affected with DRD, we conducted pairwise linkage analyses using two different penetrance estimates: (1) .35, calculated on the basis of this family alone; and (2) .05, a very conservative estimate, which essentially restricts the analysis to information from affected individuals. Parameters for the age correction were based on empirical age-at-onset data on the nine affected individuals here. Specifically, penetrance prior to 2 years was set at .0, that between 2 and 12 years increased in a straight line fashion from .0 to .35 (or .05), and that after 12 was set at a constant of .35 (or .05). Allele frequencies for the various markers were based on population frequencies when available; when such information was unavailable, they were estimated by counting alleles in founders and individuals married into this large family.

Multipoint linkage analysis was conducted using the LINKMAP program from the LINKAGE computer package, version 4.7 (Lathrop and Lalouel 1988). Only the most conservative estimate of penetrance .05, was used, and no interference was assumed. The linkage map distance for the markers included in the multipoint analysis was obtained on the basis of the Venezuelan Reference Pedigrees, using the MAPMAKER program version 1.0 (Lander et al. 1987). These data were derived from a large panel of map data we have compiled for the 9q region (L. Ozelius, D. J. Kwiatkowski, J. A. Trofatter, and J. L. Haines, unpublished observations) and are consistent with published maps (Donis-Keller et al. 1987; Lathrop et al. 1988; Northrop et al. 1989).

Results

Identification of a Highly Informative (GT)n Polymorphism within the ASS locus

We screened a panel of ASS genomic clones (Northrop et al. 1989) using a (GT)n dinucleotide repeat sequence in the attempt to find a (GT)n repeat sequence (Litt and Luty 1989; Weber and May 1989) and found that ASSg1, previously described as identifying a *Hin*dIII length-variation RFLP, was strongly positive. We there-

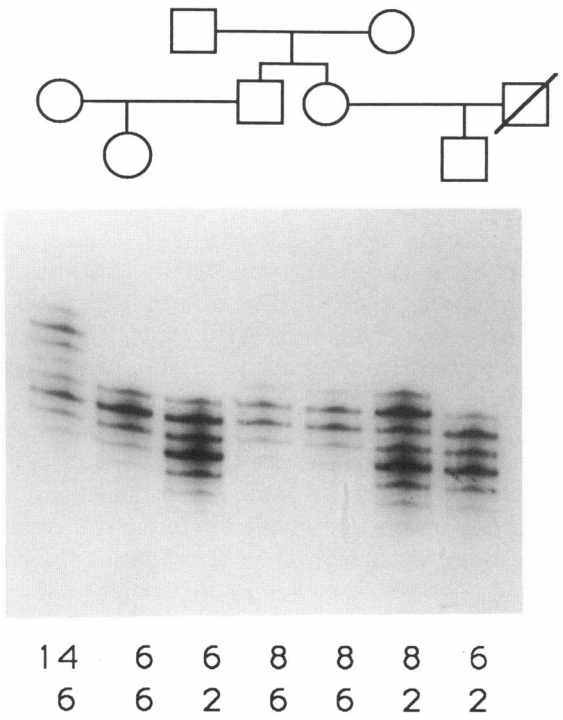

Figure 2 Polymorphism analysis among selected members of family S. Amplification of 5 ng of DNA was performed using oligonucleotides ASS-A and ASS-B. Amplified products were separated on a 6% acrylamide, 8 M urea gel, and an autoradiograph was prepared. Mendelian codominant segregation of alleles is evident. Note that a single darkest band with several fainter surrounding bands results from amplification of a single allele (6 6), whereas two adjacent darkest bands with fainter surrounding bands results from amplification of alleles differing in size by a single repeat (8 6).

fore determined a partial sequence of this clone, as shown in figure 1. To permit PCR amplification of the (GT)n repeat identified in the clone, three oligonucleotide sequences were chosen from the flanking sequence, as indicated, and oligonucleotide primers were synthesized. Pairs of primers were used in PCR amplification of DNA derived from the clone and of human DNA samples. As shown in figure 2, the length of the amplified fragment defined a polymorphism that was seen to be highly informative (table 1), with a total of nine alleles, PIC of .79, and heterozygosity frequency of .81. The polymorphism displayed Mendelian codominant segregation in 100 tested nuclear families (593 individuals tested). No new alleles were generated in 500 meioses observed. Both sets of primer pairs, ASS-A/ASS-B and ASS-A/ASS-C, amplified the repeat region and gave similar allele readings.

Table 1

Allele Designation	Frequency	Length of Product Amplified by ASS-A and ASS-C
16	.03	110
14	.11	108
12	.10	106
10	.09	104
8	.15	102
6	.07	100
4	.10	98
2	.35	96
0	.01	94

NOTE.—PIC was .79; heterozygosity frequency was .81.

Clinical Evaluation of Family S

The portion of the pedigree available for this linkage study is shown in figure 3. There were nine individuals with definite DRD, as assessed by standardized examination. We described seven of these previously (Nygaard et al. 1990); the others (III-11 and V-62) have been upgraded to definite DRD based on reexaminations or review of previously unavailable material, in addition to demonstration of their L-dopa responsiveness.

Linkage Results

Six conventional RFLPs from the 9q32-34 region, and the *ASS* (GT)n polymorphism, were determined on DNA samples from members of family S. Pairwise lod scores between each of these DNA markers and the DRD trait were calculated on the basis of penetrance estimates of both .35 and .05 and are given in table 2. All of these markers provided strong evidence against linkage in this family, with the exception of *AK1* and *GSN*, which were uninformative. Results were similar for both estimates of penetrance. Although the lod scores were somewhat higher (i.e., closer to .0) when penetrance was set at .05, the actual extent of exclusion around the informative markers was virtually the same for both sets of analyses.

Three-point analysis was carried out with the DRD trait and the D9S26 and ASS loci. These two loci were chosen primarily because they were among the most informative markers and they encompass the GSN locus (which was uninformative). They are thus likely to delimit the region of 9q32-34 which contains the dystonia locus (or loci) identified in our previous studies. The addition of other markers strengthened but did not significantly change the results.

Results of the multipoint analysis are presented graphi-

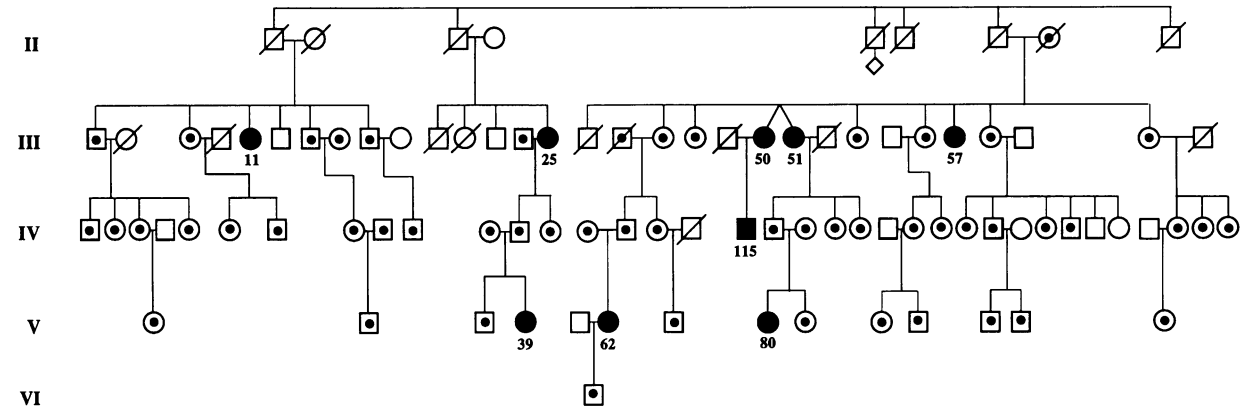

Figure 3 Portion of pedigree of family S with L-dopa-responsive dystonia. The numbers under the affected members refer to the expanded pedigree presented in Nygaard et al. (1990). □ = No definite DRD (includes unaffected possible and probable dystonia, and definite dystonia without proved L-dopa response); ■ = definite DRD; ⊡ or number = DNA sample available; ⊘ = decreased.

cally in figure 4. The lod score is ≤ -2 in a region extending from 5 cM proximal to D9S26, through the D9S26-ASS interval, to 11 cM distal to ASS. If the penetrance is higher than .05, as expected, stronger negative results and a broader area of exclusion are obtained.

Discussion

Our previous studies have demonstrated linkage of the gene causing ITD in a non-Jewish (Ozelius et al. 1989) and several Jewish kindreds (Kramer et al. 1990) to the 9q32-34 region. The most tightly linked mark-

Table 2

Results of Pairwise Linkage Analyses of DRD Gene and Markers of Chromosome 9q

A. Penetrance = .35							
	Lod Score at θ of						
Gene Symbol	.0	.05	.10	.15	.20	.30	.40
D9S29	−6.83	−3.10	−2.13	−1.52	−1.10	−.55	−.22
D9S26	−6.10	−1.89	−1.32	−.99	−.76	−.44	−.20
GSN	.13	.10	.08	.06	.05	.02	.00
AK1	.09	.07	.06	.04	.03	.01	.00
ASS	−9.56	−3.96	−2.72	−1.91	−1.35	−.64	−.23
D9S10	−3.25	−.82	−.36	−.15	−.04	.05	.06
D9S7	−5.38	−1.47	−.73	−.37	−.17	.02	.02
B. Penetrance = .05							
	Lod Score at θ of						
Gene Symbol	.0	.05	.10	.15	.20	.30	.40
D9S29	−6.02	−2.68	−1.81	−1.28	−.92	−.46	−.18
D9S26	−5.74	−1.83	−1.26	−.94	−.72	−.41	−.19
GSN	.14	.11	.09	.07	.05	.02	.01
AK1	.11	.09	.07	.05	.04	.02	.00
ASS	−7.69	−3.10	−2.15	−1.51	−1.07	−.53	−.21
D9S10	−2.28	−.95	−.49	−.27	−.13	.00	.04
D9S7	−4.49	−1.12	−.44	−.13	.02	.08	.01

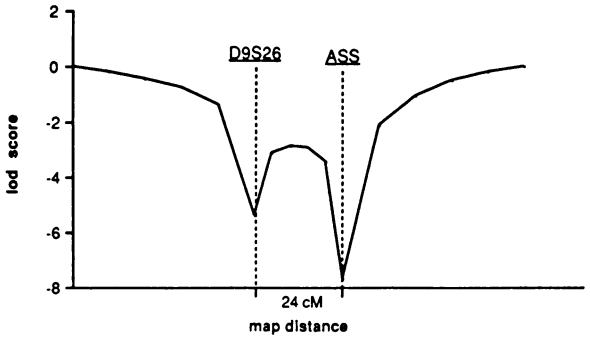

Figure 4 Multipoint analysis of DRD and 9q markers. The location map indicates composite lod scores for DRD at various map positions in a fixed marker map of D9S26 and ASS. Genetic distances between markers are given in centiMorgans and are calculated on the basis of sex-average recombination estimates. Note that the lod score is ≤ -2 in a 34-cM region.

ers in those studies, *GSN* and *ASS*, respectively, are separated by 14 cM, but both populations displayed broad regions ($\geq$10 cM) of positive linkage, suggesting that the same locus (DYT1) may cause susceptibility to ITD in the two populations. The current study provides strong evidence that the DYT1 gene (or other gene in this region) cannot be the cause of DRD in family S. Linkage between DRD and a region of 9q encompassing 40 cM, and containing the most likely site of the causative gene(s) in Jewish and non-Jewish kindreds, was excluded by a lod score of ≤ -2 (odds against linkage of $\geq$100:1). We propose that the locus for DRD in this large family be given the name *DRD*.

The etiology of hereditary dystonia has been difficult to approach by traditional methods of analysis due to the difficulty of obtaining suitable tissue for neurochemical and neuropathological studies. The nosology of ITD is complex (Fahn 1988), due to the occurrence of dystonia in widely separated populations, with clinical features varying in age at onset, tendency to diurnal fluctuation, precise manifestations, and response to pharmacologic therapies. Efforts to determine the responsible locus for each subtype of ITD will be helpful both in understanding their relationships and as initial steps in gene localization and identification.

DRD is distinguished from all other hereditary forms of dystonia on the basis of several clinical features and by its dramatic and sustained responsiveness to L-dopa therapy (Segawa et al. 1976; Nygaard et al. 1988). Although on these grounds one might have predicted that DRD was caused by a gene distinct from that causing classic ITD, the phenotypic similarities between these conditions made it important to directly exclude the possibility of a common causative gene.

Based on clinical observations and the finding of reduced dopamine metabolites in the cerebrospinal fluid, a functional disorder of dopaminergic function has been hypothesized as the cause of DRD (Nygaard 1989). Variation from normal in serum levels of dopamine β-hydroxylase (DBH, the enzyme which converts dopamine to norepinephrine) have been reported among members of family S (deYebenes et al. 1988), as well as in other patients with dystonia (Wooten et al. 1973; Ziegler et al. 1976; Menkes et al. 1987). The gene for DBH has been localized to the 9q34 region by linkage analysis (Wilson et al. 1988) and in situ hybridization (Craig et al. 1988), further suggesting that it is a potential candidate gene for some types of ITD. However, by genetic linkage we have excluded the DBH locus as the cause of DRD in family S and have also excluded this locus in four other forms of dystonia (Schuback et al., in press). The gene for tyrosine hydroxylase, the rate-limiting enzyme for dopamine synthesis, has also been excluded as a cause of DRD in several small families (Fletcher et al. 1989).

The (GT)n polymorphism we have defined within the ASS locus is highly informative and will be useful in analyses of other conditions which localize to 9q32-34, including one form of tuberous sclerosis (Fryer et al. 1987) and nail-patella syndrome (Smith and Simpson 1989). In addition, it should be very useful in genetic counseling among patients and families affected with deficiency of ASS (citrullinemia) (Northrop et al. 1989).

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