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Identification and Expression of Eight Novel Mutations among Non-Jewish Patients with Canavan Disease

Rajinder Kaul,[1] Guang P. Gao,[1,*] Reuben Matalon,[1] Maria Aloya,[1] Quin Su,[1,*] Mei Jin,[1] Anne B. Johnson,[2] Ruud B. H. Schutgens,[3] and J. T. R. Clarke[4]

[1]Research Institute, Miami Children's Hospital, Miami; [2]Department of Pathology, Albert Einstein College of Medicine, Bronx; [3]Department of Pediatrics, University Hospital Amsterdam, Meibergdreef, Amsterdam; and [4]Division of Clinical Genetics, The Hospital for Sick Children, Toronto

Summary

Canavan disease is inherited as an autosomal recessive trait that is caused by the deficiency of aspartoacylase (ASPA). The majority of patients with Canavan disease are from an Ashkenazi Jewish background. Mutations in *ASPA* that lead to loss of enzymatic activity have been identified, and E285A and Y231X are the two predominant mutations that account for 97% of the mutant chromosomes in Ashkenazi Jewish patients. The current study was aimed at finding the molecular basis of Canavan disease in 25 independent patients of non-Jewish background. Eight novel and three previously characterized mutations accounted for 80% (40/50) of mutant chromosomes. The A305E missense mutation accounted for 48% (24/50) of mutant chromosomes in patients of western European descent, while the two predominant Jewish mutations each accounted for a single mutant chromosome. The eight novel mutations identified included 1- and 4-bp deletions (32 ΔT and 876 ΔAGAA, respectively) and I16T, G27R, D114E, G123E, C152Y, and R168C missense mutations. The homozygous 32 ΔT deletion was identified in the only known patient of African-American origin with Canavan disease. The heterozygosity for 876 ΔAGAA mutation was identified in three independent patients from England. Six single-base changes leading to missense mutations were identified in patients from Turkey (D114E, R168C), The Netherlands (I16T), Germany (G27R), Ireland (C152Y), and Canada (G123E). A PCR-based protocol is described that was used to introduce mutations in wild-type cDNA. In vitro expression of mutant cDNA clones demonstrated that all of these mutations led to a deficiency of ASPA and should therefore result in Canavan disease.

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Address for correspondence and reprints: Dr. Rajinder Kaul, 7305 Southwest 123 Street, Miami, FL 33156-5310. E-mail: rkkaulix.netcom.com

*Present address: Institute of Gene Therapy, University of Pennsylvania, Philadelphia

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Introduction

Canavan disease (CD) is an autosomal recessive leukodystrophy characterized by spongy degeneration of white matter. Patients with CD have severe mental retardation, macrocephaly, hypotonia, and head lag (van Bogaert and Bertrand 1949). As the disease progresses, hypotonia gives way to spasticity and patients may develop optic atrophy. The majority of cases of CD are of the infantile form, and the delayed developments are first noted at ~3–6 mo of age (Matalon et al. 1995). The basic biochemical defect in CD is the deficiency of aspartoacylase (ASPA) (Matalon et al. 1988). The enzyme is highly stereospecific toward the hydrolysis of its substrate N-acetylaspartic acid (NAA) to acetate and aspartate (Birnbaum 1955). N-acetylaspartic acid is synthesized exclusively in the brain (Goldstein 1959, 1969). The deficiency of ASPA leads to accumulation of NAA in brain and to its excessive excretion in urine of patients with CD (Matalon et al. 1988, 1995). More than 165 patients with CD have been diagnosed in our laboratory (Matalon et al. 1995). Recently, the cDNA and gene for human ASPA have been cloned (Kaul et al. 1993, 1994*a*). The open-reading frame in human ASPA cDNA predicted a 313–amino acid residue protein and expressed ASPA activity in *Escherichia coli* (Kaul et al. 1993). The cDNA sequence is split into six exons and five introns that span ~25 kb of the human genome. The ASPA-coding sequence is highly conserved across species during evolution (Kaul et al. 1993, 1994*a*). Five different mutations, including the 433 −2(A→G) splice-acceptor site and the Y231X nonsense and C152R, E285A, and A305E missense mutations, have been identified in patients with CD (Kaul et al. 1993, 1994*b*, 1994*c*, 1995). In vitro expression confirmed that these mutations lead to deficiency of ASPA (Kaul et al. 1994*b*, 1995). Among Ashkenazi Jewish patients with CD, E285A missense and Y231X nonsense mutations account for 97% of the 104 mutant chromosomes examined (Kaul et al. 1994*b*; authors' unpublished data). In non-Jewish patients of western European descent, the A305E missense mutation accounted for about half of the mutant chromosomes (Kaul et al. 1994*b*). Eight additional mutations have recently been described in non-

Jewish patients with CD (Shaag et al. 1995); however, the effect of these mutations on ASPA activity has not been demonstrated. We report the identification of eight novel mutations in Canavan patients of non-Jewish background and demonstrate that these mutations lead to the loss of ASPA activity. The eight novel and three previously characterized mutations together accounted for 80% of the 50 independent mutant chromosomes from non-Jewish patients with CD. A preliminary report describing these mutations has been presented elsewhere (Kaul et al. 1994*d*).

Subjects, Material, and Methods

Study Subjects

Mutations leading to CD were analyzed in 25 independent probands of non-Jewish ethnic background. The diagnosis of CD in these probands had earlier been confirmed by excessive excretion of NAA in urine and/or the deficiency of ASPA in their cultured skin fibroblasts. Genomic DNA isolated from lymphocytes or cultured skin fibroblasts and the total cellular RNA from cultured skin fibroblasts were used for the identification and characterization of mutations in the affected individuals. If available, the mutations identified in probands were confirmed in their parents and affected siblings. The frequency of these mutation was determined in the non-Jewish patient pool reported here. However, in families with more than one affected child, only the proband was included for calculating the frequency of these mutations.

Genotype Analysis

The methods used for identification and expression of Canavan mutations were those described earlier (Kaul et al. 1993, 1994*a*, 1994*b*, 1995). In brief, ASPA-specific genomic sequences were amplified by PCR using an exon-specific set of primers. The primer sequences are listed in table 1. The exon-specific primers used were HASP9/HASP1B (exon 1), HASP2A/HASP2B (exon 2), HASP3A/HASP3B (exon 3), and HASP6A/HASPC7 (exon 6). Alternatively, ASPA transcripts were amplified by reverse transcriptase followed by PCR (RT-PCR), as described elsewhere (Kaul et al. 1993). Following amplification of ASPA sequences, mutation analysis was carried out by SSCP and/or the determination of nucleotide sequence. In certain patients, the SSCP analysis either revealed mutation in only one of the *ASPA* alleles or did not reveal an abnormal profile that would be indicative of a base change. In such instances, the nucleotide sequence of all six exons and exon/intron boundaries was determined to rule out the presence of other base changes that could potentially be the cause of their CD. The base changes observed were also confirmed by restriction-endonuclease digestion of the amplified fragments. In the majority of cases, the base change did not result in gain or loss of recognition sequence for any restriction endonuclease. In those cases, a restriction site was introduced in the wild-type (WT) or mutant allele by PCR-directed site-specific mutagenesis (PDSM), as described earlier (Kaul et al. 1994*b*).

In Vitro Mutagenesis and Expression of cDNA

The effect of base change on ASPA activity was determined by expression of mutant cDNAs in COS1 cells (Kaul et al. 1994*b*). The cDNAs were cloned either by RT-PCR amplification of mutant ASPA transcripts isolated from the cultured skin fibroblasts of the patients (Kaul et al. 1994*b*) or by in vitro mutagenesis of WT cDNA by a novel PCR-based mutagenesis protocol outlined in figure 1. The WT cDNA was amplified in two nonoverlapping, adjacent 5′ and 3′ segments. Specific mutation was introduced by using a mutant allele–specific primer during PCR amplification, with the desired base change at its 5′ termini. The 5′ cDNA segment was amplified with sense XHASP18 and the mutant allele–specific antisense primer. The 3′ segment of cDNA was amplified with a phosphorylated WT sense and NHASPC14 antisense primer. The XHASP18 and NHASPC14 primers, as described earlier (Kaul et al. 1993), contain *Xba*I and *Not*I restriction sites, respectively, at their 5′ termini. The two nonoverlapping cDNA segments were ligated, digested with *Xba*I and *Not*I restriction endonuclease and cloned in the pGEUK-C1 expression vector. Alternatively, the ligated product was used as a template to amplify cDNA with XHASP18 and NHASPC14 primers prior to digestion and cloning in pGEUK-C1 expression vector. The cDNA inserts in mutant and WT expression constructs were characterized by determination of their nucleotide sequence. The expression of ASPA activity was determined in COS cell extracts, 72-h posttransfection, as described earlier (Kaul et al. 1994*a*).

Results

Identification and Characterization of Mutations

Mutation analysis was carried out on 25 unrelated non-Jewish patients with CD. Eight novel and three previously characterized mutations were identified in the present study. Nucleotide sequence data describing the eight novel mutations are shown in figure 2. Three point mutations—32 ΔT deletion and 47T→C and 79G→A transitions—were observed in exon 1 from three independent patients. The homozygosity for 32 ΔT deletion was observed in patient AL, of African-American descent, while the heterozygosity for 47T→C and 79G→A transitions were observed in a Dutch (RW) and a German (LW) patient, respectively. The 47T→C transition in patient RW would result in I16T (ATC→ACC) mis-

Table 1

Nucleotide Sequences of the Synthetic Oligonucleotides Used for Amplification of Specific Exons and Exon/Intron Boundaries and for Identification of Specific Mutations, by PDSM

Primer	Sequence[a]	Sense/Antisense
HASP9	5′ CTTCTGAATTGCAGAAATCAG 3′	Sense
HASP1B	5′ CCACTTTCACACAACATCC 3′	Antisense
HASP2A	5′ TATTATCTCAGGCACAGATG 3′	Sense
HASP2B	5′ CAAGTCCTTTGCTGACTTAT 3′	Antisense
HASP3A	5′ AACATACGGTTTTTACCTAAG 3′	Sense
HASP3B	5′ TCTCTGAGTTTCAGCTAGG 3′	Antisense
32TMSE	5′ GTCACATTGCTGAAGAACT*TA 3′	Sense
I16TKPN	5′ AAGAACATATACAAAAGGTTGG*TA 3′	Sense
D114EBST	5′ GATTCCTATGACATTATTTTC*GA 3′	Sense
HASPC16	5′ TCTTCACTGCTCTGGGTT 3′	Antisense
G123ETAQ	5′ CACAACACCACCTCTAACATC*G 3′	Sense
C152YRSA	5′ TTCTCTGGCTCCACTACCG*T 3′	Sense
R168CTAQ	5′ AGGATACTTGGCTATGGAT*C 3′	Antisense

[a] An asterisk (*) indicates the mismatch base introduced in the synthetic primer.

sense mutation. Patient RW carried Y231X nonsense mutation on the other *ASPA* allele. The 79G→A transition in patient LW would cause G27R (GGA→AGA) missense mutation. The second *ASPA* allele in patient LW carried the A305E missense mutation. The 32 ΔT, 47T→C, and 79G→A point mutations in patients and

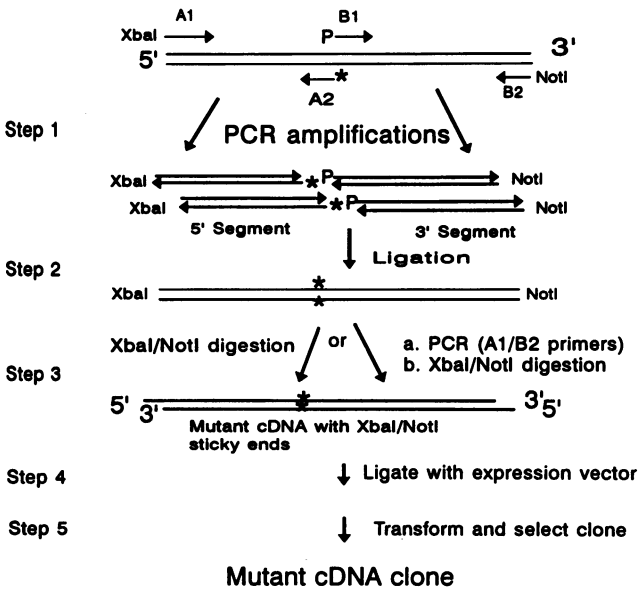
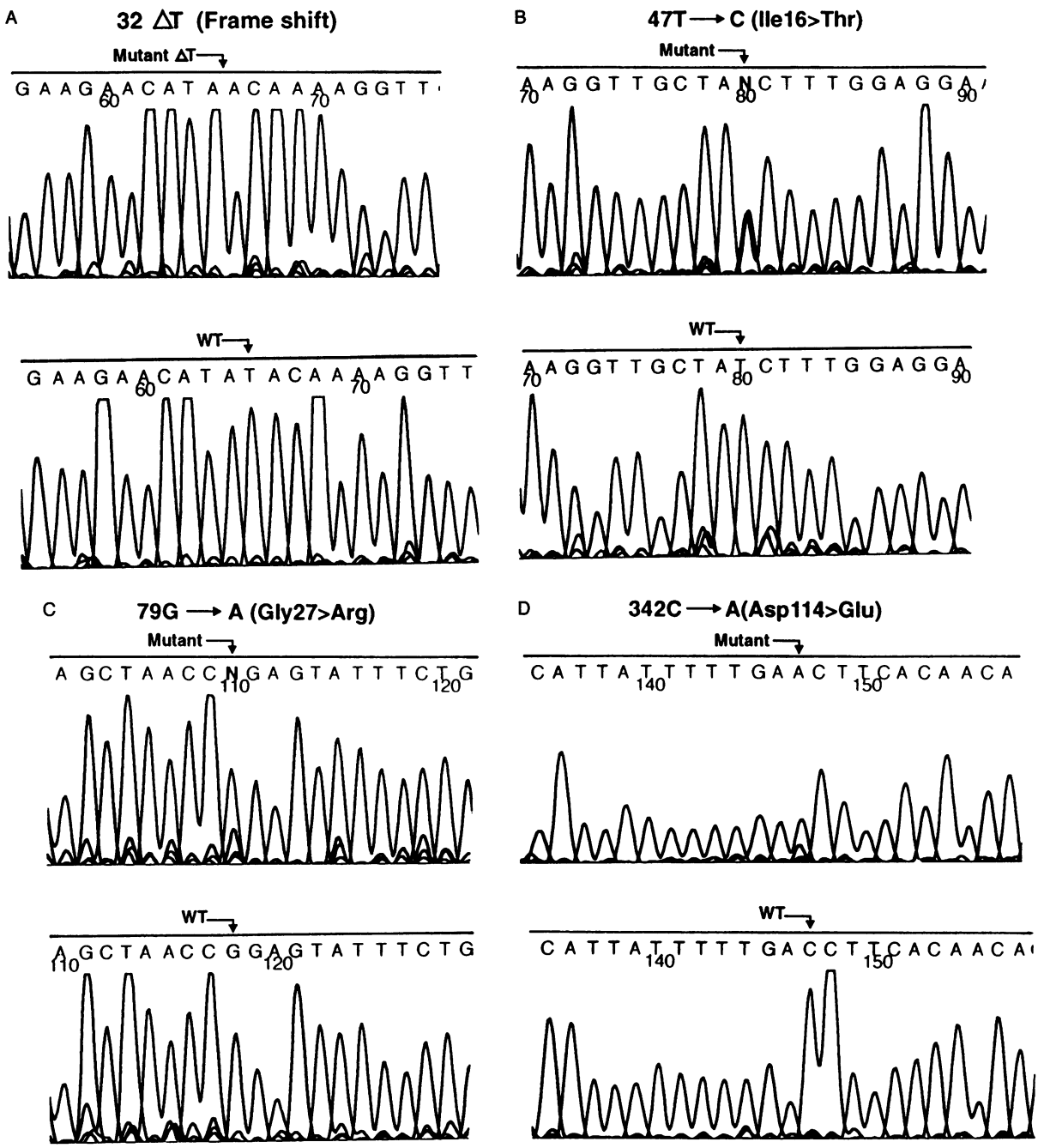


Figure 1 Flowchart of the PCR-based protocol for in vitro mutagenesis of WT cDNA. Primers Al and B2 flank WT cDNA and carry specific restriction-endonuclease linkers to facilitate directional cloning of the amplified product. The desired mismatch at the 5′ termini of primer A2 is indicated by an asterisk (*). B1 is the sense-strand WT primer that is phosphorylated (P) at its 5′ termini prior to PCR amplification. The location of A2 and B1 primers is dictated by the site of mutagenesis in cDNA. The roles of A2 and B1 primers can be reversed if so desired.

their available family members were confirmed by restriction-digestion analysis of the PCR-amplified fragments, and the data are given in table 2.

Two single-base substitutions, 342C→A and 368G→A, leading to D114E (GAC→GAA) and G123E (GGG→GAG) missense mutations, respectively, were observed in exon 2 (see fig. 2). The homozygous D114E mutation was observed in a Turkish patient (DI) with reported consanguinity in the family. The G123E mutation was found in one *ASPA* allele from a Canadian patient (WR) of western European descent, while the second allele in that patient carried the A305E missense mutation. The 455G→A (patients DS) and 502C→T (patients KS) transitions observed in exon 3 would result in C152Y (TGC→TAC) and R168C (CGT→TGT) missense mutations, respectively (see fig. 2). The patient DS is of Irish origin and carried C152Y missense mutation on one *ASPA* allele, while the mutation in other allele remains unknown, and no other base change was observed in coding or exons/intron junction sequences. Patient KS of Turkish origin is the product of a consanguineous marriage and carries the homozygous R168C missense mutation. The R168C missense mutation was also observed in another Canavan allele of unknown ethnic origin. The 342C→A, 368G→A, 455G→A, and 502C→T base changes were confirmed in these patients and their available family members by specific restriction-endonuclease digestion analysis (see table 2).

The SSCP analysis in patient LH, of English origin, revealed that the patient was a compound heterozygote for the A305E missense mutation inherited from the father and another mutation in exon 6 that was of maternal origin (data not shown). Direct sequence analysis of the PCR-amplified exon 6 fragments from the patient LH or



mother were inconclusive. Exon 6–specific PCR-amplified fragments from the patient LH were subcloned, and the paternal or maternal allele–specific inserts were characterized by SSCP analysis (data not shown). Nucleotide sequence analysis of the maternal allele–specific insert revealed a 4-bp AGAA deletion at position 876 (see fig. 2*H*). The 876 ΔAGAA mutation would cause frameshift and premature termination of the translation product following amino acid residue 292. The resulting mutant protein will thus have 299 residues, compared with 313 amino acid residues predicted by the WT cDNA.

The effect of I16T, G27R, D114E, G123E, C152Y, R168C, and 876 ΔAGAA mutations on ASPA activity was tested by in vitro expression of the mutant cDNA constructs in COS1 cells. The data are recorded in table 3. The I16T, D114E, C152Y, R168C, and 876 ΔAGAA mutant ASPA yielded undetectable to <0.5% residual enzyme activity. The G27R mutant ASPA exhibited ~3%, and the G123E mutant ASPA ~26% of the residual enzyme activity. The patient WR with G123E missense mutation did not carry any other base change in the coding or exon/intron boundary sequences in *ASPA*.

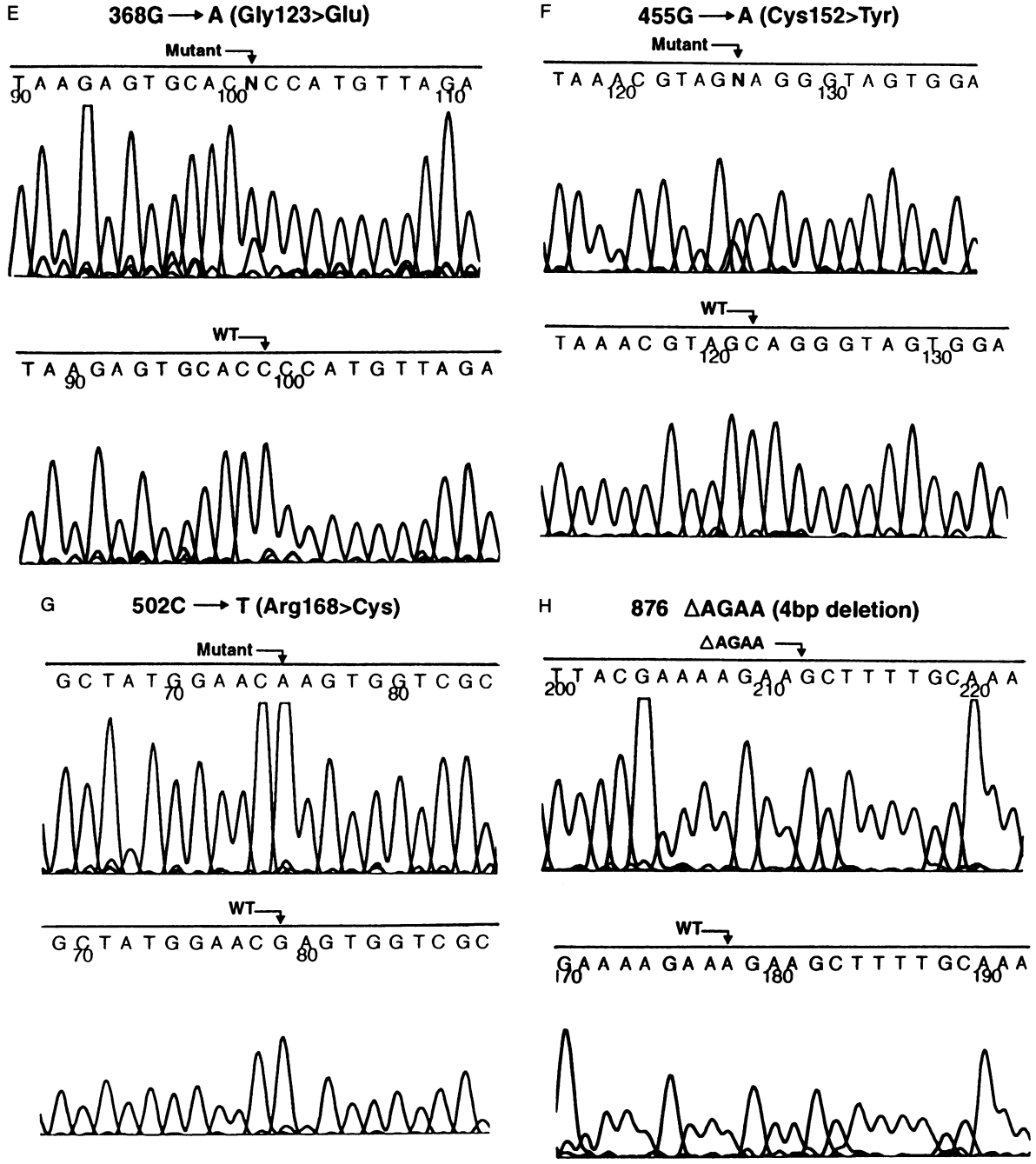


Figure 2 Nucleotide sequence of the PCR-amplified genomic fragments around the observed mutation in patients and the corresponding region from WT normal controls. Exon 1–, 2–, 3–, or 6–specific genomic fragments were amplified, and their nucleotide sequence was determined. *A*, Nucleotide sequence of the sense strand in patient AL around the homozygous 32 ΔT deletion that would lead to frameshift and premature termination of translation product. *B*, Nucleotide sequence of the sense strand in patient RW. The patient carried 47T→C transition (I16T missense mutation) in his paternal chromosome, while the maternal chromosome carried Y231X nonsense mutation. *C*, Nucleotide sequence of the sense strand in patient LW. The heterozygosity for 79G→A transition leading to G27R missense mutation was traced to the paternal chromosome, while the maternal chromosome carried the A305E missense mutation. *D*, Nucleotide sequence of the sense strand in patient DI. The homozygosity for the 342C→A base change (D114E missense mutation) was identified in patient DI, and both parents were carriers for this point mutation. *E*, Nucleotide sequence of the complementary strand around the heterozygous 368G→A base change (G123E missense mutation) in patient WR. The mutation in second chromosome from patient WR carried the A305E missense mutation. *F*, Nucleotide sequence of the complementary strand in patient DS. The heterozygosity for 455G→A base change (C152Y missense mutation) in patient DS was found in the maternal chromosome. The mutation in his paternal chromosome is not known at present, and no other base change was observed in the coding and exon/intron junction sequences. *G*, Nucleotide sequence of the complementary strand in patient KS. The patient is homozygous for 502C→T transition (R168C missense mutation), and both his parents were found to be carriers for this mutation. *H*, Nucleotide sequence of the sense strand in the maternal allele–identified 876 ΔAGAA 4-bp deletion, leading to frameshift and premature termination of the translation product. The paternal allele was found to carry the A305E missense mutation.

Table 2

Restriction-Endonuclease Digestion for Identification of Specific Canavan Mutations

Mutation	PCR Primer Pair	Fragment Size (bp)	Restriction Digestion	Fragment Size (bp)	
				WT	Mutant
32 ΔT	32TMse/HASP1B	284	*Mse*I	202, 82	202, 62, 20
I16T	I16TKpn/HASPC16	154	*Kpn*I	154	129, 25
G27R	HASP9/HASP1B	347	*Msp*I	229, 118	347
D114E	D114EBst/HASP2B	156	*Bst*BI	156	136, 20
G123E	G123ETaq/HASP2B	129	*Taq*I	129	109, 20
C152Y	C152YRsa/R168Taq	87	*Rsa*I	87	67, 20
R168C	C152YRsa/R168Taq	87	*Taq*I	69, 18	87

The 32 ΔT deletion present at the 5′ end of *ASPA* cDNA was not expressed, since it would lead to frameshift and a premature termination of the translation product.

Frequency of Canavan Mutations in Non-Jewish Patients

The data describing the frequency of 11 mutations in 50 independent chromosomes from non-Jewish patients are shown in table 4. The 11 mutations were localized to exons 1, 2, 3, and 6 of the *ASPA* gene and accounted for 80% of the 50 mutant chromosomes. The A305E missense mutation accounted for 48% of the non-Jewish Canavan chromosomes reported in this study. The 876 ΔAGAA and R168C missense mutations each accounted for 6% of the mutant chromosomes. Five other point mutations were found in specific families in single or two chromosomes, yielding a frequency of 2% or 4%, respectively. One chromosome each in two independent patients with CD carried the predominantly Jewish Y231X nonsense and E285A missense mutations.

Discussion

The description of ASPA deficiency and the resulting excessive excretion of NAA in urine is now the hallmark for diagnosis of CD (Matalon et al. 1988, 1995). Cultured fibroblasts are needed for demonstration of the ASPA deficiency in CD and to establish the carrier status of at-risk individuals. The need for cultured fibroblasts makes determination of ASPA activity cumbersome and sometimes even difficult because of the sensitivity of ASPA expression in culture conditions (R. Kaul, unpublished data). The isolation of cDNA and gene for human ASPA have made it possible to identify the molecular basis of CD (Kaul et al. 1993, 1994*a*, 1994*b*, 1994*c*, 1995). Two predominant mutations E285A and Y231X

Table 3

Effect of Mutation on ASPA Activity Observed in Vitro in Transient-Expression System

Mutation	ASPA Activity[a] (mU/mg protein)		Activity Recovered (% of WT control)	Ethnicity
	Mutant	WT		
32 ΔT	Not expressed			African-American
I16T	.03	7.62	.38	Dutch
G27R	.20	6.50	3.07	German
D114E	.02	5.67	.35	Turkish
G123E	.84	3.12	26.90	Canadian
C152Y	.01	5.7	.16	Irish
R168C	None detected	6.90	0	Turkish
876ΔAGAA	None detected	7.62	0	English

[a] WT and mutant cDNA were cloned in SV40 late-promoter-driven pGEUK-C1–expression vector and transfected in COS1 cells by lipofectin-mediated DNA transfection. Expression of ASPA activity was determined in cell extracts 72 h posttransfection. Control COS1 cells alone or transfected with pGEUK-C1 vector DNA alone did not express any detectable ASPA activity. The residual ASPA activity recovered is the mean of at least two independent transfection experiments.

Table 4

ASPA Mutations and Their Frequency in 50 Independent Chromosomes of Non-Jewish Origin

Position	Exon	Mutation	Type	No. of Alleles	Frequency
32 ΔT	1	Frameshift/premature termination	Deletion	2	.04
47T→C	1	I16T	Missense	1	.02
79G→A	1	G27R	Missense	1	.02
342C→A	2	D114E	Missense	2	.04
368G→A	2	G123E	Missense	1	.02
455G→A	3	C152Y	Missense	1	.02
502C→T	3	R168C	Missense	3	.06
693C→A	5	Y231X	Nonsense	1	.02
854A→C	6	E285A	Missense	1	.02
876ΔAGAA	6	Frameshift/premature termination	Deletion	3	.06
914C→A	6	A305E	Missense	24	.48
Total				40	.80

accounted for 97% of the mutant chromosomes among Ashkenazi Jewish patients (Kaul et al. 1994*b*). Both of these mutations are rare in patients of non-Jewish origin. The single chromosome with the Y231X nonsense mutation identified in this study was transmitted by a parent of Jewish origin, while the source of the single E285A mutant chromosome remains unknown.

The 11 Canavan mutations reported here involve ASPA codons that are conserved in bovine and mouse genes (Kaul et al. 1993, 1994*a;* R. Kaul, unpublished data). The mutations reported include small deletions that result in frameshift and premature termination and point mutations that lead to missense or nonsense mutations. The A305E is the most frequently identified Canavan mutation in patients of western European origin, including those of Dutch, English, and German descent (Kaul et al. 1994*b;* Shaag et al. 1995; present study). Epidemiological studies to determine the origin of the A305E mutation in western Europe would be interesting. The 876 ΔAGAA has so far been found only in patients from England. The 455G→A base change leading to the C152Y missense mutation identified in an Irish patient is interesting. In an earlier report, a C152R missense mutation detected in an Arab patient was due to a 454T→C base change at the same codon (Kaul et al. 1995). It was postulated that the C152 residue present in a strong β-sheet structure may be involved in the formation of a disulfide bond necessary for the maintenance of ASPA in a conformationally active state (Kaul et al. 1995).

The majority of the mutations in non-Jewish patients reported so far are private in nature, found only in isolated families. The homozygous 32 ΔT deletion found in an African-American patient is the only known case with CD in this population group. The homozygosity for this apparently private mutation is rather intriguing, since no consanguinity was reported in this family. The parents of this patient were not available for genotype analysis. Expression studies so far suggest that all except the G123E missense mutation should lead to severe Canavan phenotype. The G123E missense mutation with ~25% residual ASPA activity could lead to a milder Canavan phenotype. More data are necessary to define genotype/phenotype correlation in Canavan patients. Eight other Canavan mutations reported in non-Jewish patients (Shaag et al. 1995) are different from the ones described here; however, none of these were characterized by expression analysis. The expression of missense and in-frame deletion mutations is particularly necessary. In our population study of Ashkenazi Jewish individuals, we observed a C310G missense polymorphism that had no effect on ASPA activity as determined by in vitro expression studies (R. Kaul, unpublished data). It is interesting to note that C310 residue in human ASPA is not conserved in bovine (Kaul et al. 1993) or mouse (R. Kaul, unpublished data) enzyme. The complete loss of ASPA activity by an apparently conserved D114E missense change, compared with the recovery of 25% residual ASPA activity with G123E missense mutation, suggests difficulty in predicting the outcome of such mutations.

The wide spectrum of Canavan mutations in non-Jewish patients suggests that biochemical methods must be used for diagnosis in noninformative families. The predominance of E285A and Y231X mutations in Ashkenazi Jewish patients have made it possible to establish a carrier rate of 1:38 (Matalon et al. 1994) to 1:41 (Kronn et al. 1995) among Ashkenazi Jewish individuals from the New York area. A carrier rate of 1:59 has been reported for the E285A mutation alone in Israel (Elpeleg et al. 1994). Since the carrier rate of CD is comparable to that of Tay-Sachs disease in Ashkenazi Jewish people, it would be reasonable to screen this population for the two predominant Canavan mutations. Despite advances

in our understanding of molecular basis of CD, we do not understand the pathophysiology in this disorder. Currently, studies are in progress to create a knockout mouse model for CD in our laboratory. Successful creation of such a model will be helpful for the understanding of the pathophysiology and the exploration of various modes of treatment in CD.

Acknowledgments

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