MUTATION ANALYSIS OF THE ASPARTOACYLASE GENE IN NON-JEWISH PATIENTS WITH CANAVAN DISEASE

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1. INTRODUCTION

Canavan disease (OMIM # 271900) is an inherited leukodystrophy caused by deficiency of aspartoacylase (ASPA) (Matalon, et al., 1988) a cytosolic enzyme found in oligodendrocytes (Madhavarao, et al., 2004). It hydrolyzes *N*-acetylaspartic acid (NAA) to acetate and aspartic acid. In Canavan disease (CD), NAA concentrations in brain increase causing vacuolization in the lower layers of the cerebral cortex and subcortical white matter with intramyelinic swelling and myelin loss. Symptoms of this rare antosomal recessive disease appear within two to four months of birth, consisting of poor head control, truncal hypotonia, developmental delay, and lack of visual tracking. Subsequently, progressive macrocephaly, limb spasticity and seizures develop. With gastrostomy feedings and close nursing care, some of these children will remain interactive for a number of years although they are unable to sit alone, speak or walk.

The human ASPA gene maps to 17pter-p13 and covers a span of ~30 kb of genomic DNA. The cDNA is 1435 bp in length and contains 6 exons encoding a protein of 313 amino acids (Kaul et al., 1993). CD is especially prevalent among individuals of Ashkenazi Jewish ancestry in which the carrier frequency approximates 1 in 37-57 (Feigenbaum et al., 2004). Nearly all (98%) of the mutant alleles in this population are due to two founder mutations, E285A and Y231X. A third mutation, A305E, accounts

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for approximately 40 percent of mutant alleles in non-Jewish patients. Most other mutations appear to be private, confined to single families or to small geographic areas.

A total of 53 mutations of the ASPA gene are listed in the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/search/231014.html). Our laboratory first reported 14 of these mutations (Zeng et al., 2002), and has recently discovered another 10 novel mutations, all in non-Jewish families. These mutations were found in the course of our study of 38 non-Jewish families with CD. This report reviews the ASPA mutations we have observed in these families and illustrates the importance of molecular genotyping for the prenatal diagnosis of CD.

2. METHODS

Blood, and in some cases cultured skin fibroblasts were obtained from patients following informed written consent. In one case, only fetal skin and cartilage were available. In this particular case, and in many others, blood was also obtained from the parents. The diagnosis of CD was based on typical clinical, neuroradiologic and biochemical criteria. The clinical manifestations of the patients are summarized in Table 1. Their ethnic origins are included in tables 2 and 3.

Table 1. Clinical manifestations 38 non-Jewish CD patients

Manifestations		**n**
Age of onset	**<2m**	**14**
	3-4m	**20**
	>6m	**4**
Poor head control		**38**
Macrocephaly		**38**
Visual failure		**38**
White-matter disease		**38**
Seizures		**13**
Alive	**no**	**2**
	yes	**31 (one is >17y)**
	unknown	**5**

Procedures for cell culture, isolation of peripheral blood lymphocytes and extraction of total RNA and genomic DNA are described in Zeng et al. (2002). Genomic DNA was amplified using primers previously described (Kaul et al. 1993, 1994, 1996; Zeng et al., 2002). The procedure of Zeng et al. (2002) was also followed for the synthesis and amplification of cDNA. DNA and cDNA PCR products were either directly sequenced or

cloned in pGEM-T Easy-Vector according to the manufacturer's suggested protocols and then sequenced. Automated sequencing was used.

In vitro mutagenesis, ASPA cDNA expression and assay of ASPA activity were performed as described by Zeng et al. (2002).

3. RESULTS AND DISCUSSION

Our general strategy involved, first, a search for the three common mutations (E285A, Y231X, A305E) using site-specific restriction endonuclease digestion of PCR products from genomic DNA. Then PCR products from both cDNA and genomic DNA were sequenced. To confirm the alterations found, specific restriction endonuclease digestion analysis of genomic DNA was performed. When possible, the carrier status of the parents was also confirmed. *In vitro* mutagenesis and expression of mutant cDNA's was performed for many of the mutations reported in our earlier series (Zeng et al., 2002) but have not yet been done for the mutations identified more recently.

3.1. Mutations in 39 Non-Jewish Patients with Canavan Disease

The mutations found are shown in Tables 2 and 3. Novel mutations included 12 missense mutations, 2 nonsense mutations, 5 deletions, 1 insertion mutation, 1 case of two variations in a single allele, 1 elimination of a stop codon and 2 splice accepter site mutations. Table 4 list all mutations found by class.

The A305E mutation is known to be present primarily in patients of European origin and that was the case in our cohort; 16/17 alleles with this mutation were of European ancestry. The smaller percentage of our cases with the A305E mutation (21.7% 17/78) than in other case series (39.5-60%; Elpeleg & Shaag, 1999, Sistermans et al., 2000) may reflect the large number of non-European patients in our series.

3.2. Characteristics of 24 Novel Mutations

From 39 patients, we identified 24 novel mutations (tables 2-4). Within human ASPA are esterase-like sequences including amino acid motifs GGTHGNE, DCTV and VNEAAYY. Two of the mutations identified, G18R and E24G, produce substitutions of invariable amino acid residues within the first esterase catalytic domain consensus sequence GGTHGNE in the first and last residues. No ASPA activity was detected in COS-7 cells transfected with mutant cDNA containing the E24G mutation whereas the activity was markedly increased after transfection with normal ASPA cDNA. This suggested that the E24G mutation caused malfunction of ASPA.

Of the two splice accepter site mutations found, one (IVS 1-2A→T) caused retention of 40 nucleotides of intron 1 on the upstream side of exon 2 while the other (IVS 4-1G→C) resulted in skipping of exons 5 and 6. In the case of the IVS 1-2A→T, intron retention occurred due to the presence of a cryptic splice acceptor sequence within the intron introducing new amino acids (Zeng et al., 2002).

Table 2. Mutations in 22 non-Jewish CD Patients*

Patient	Mutation 1	Mutation 2	Ethnic origin
1.	E24G	P181T	German
2	D68A	D249V	British
3	923delT	245insA	Jamaican
4	D249V	unidentified	British
5	Q184X	A305E	German/British
6	C152W	C152W	Yemenite
7	E285A	33del13	German/Italian
8	G27R	H244R	Italian
9	A305E	E214X	German/British/Italian
10	A287T	10T→GG	German
11	698insC	698insC	Yemenite
12	A305E	244delA	African American
13	IVS1-2A→T	not identified	German
14	X314W	X314W	Guam
15	A305E	not identified	European/Greek/Irish
16	G27R	not identified	Italian
17	P181T	not identified	German/French
18	A305E	A305E	British
19	A305E	876del4	German/American Indian
20	A305E	I16T	Italian
21	D114E	D114E	Turkish
22	A305E	G123E	European

***Zeng et al, 2002; Novel mutations are underlined.**

Within exon 2, a run of seven adenines occurs at positions 238-244 that is prone to slipped mispairing at the replication fork. The seventh adenine at nucleotide 244 was deleted in one of our patients and in another, $244\text{-}245_{AT}$ was deleted. In two other patients, we encountered insertion of an adenine at position 245, previously described by others. Hence, this site tends to be associated with both insertion and deletion, leading in both cases to a truncated non-functional gene product.

We identified a normal mutation in the cystine residue at position 152 (C152W) which resulted in complete deficiency of ASPA activity on *in vitro* mutagenesis and expression in COS-7 cells. As noted (Zeng et al., 2002) the C152 residue participates in the creation of a strong β-sheet structure and may be required for maintaining ASPA in a conformationally active state (Kaul et al., 1995). The existence of two other mutations in this same residue, i.e. C152R (Kaul, et al., 1995) and C152Y (Kaul, et al., 1996), highlights the importance of this site for the structural integrity of ASPA.

Several missense mutations resulted in a change in electron charge of the substituted amino acid or conversion of a polar amino acid to a hydrophilic one. These included D68A (aspartate →alanine), N121I (asparagine→isoleucine) T166I (threonine→isoleucine), and D249V (aspartate→valine). In the case of D68A expression of the mutant

cDNA did not produce any ASPA activity in COS-7 cells. All 12 novel missense mutations are in conserved regions of the ASPA gene.

Homozygosity for these rare novel mutations was found in the cases of G18R C152W, H244R, 244 del AT, X314W and 698 ins C. Consanguinity was present only for the 698 ins C mutation (Yemenite). While an ethnic predilection for each of these mutations is a possibility, only for two of the mutations, H244R and D249V, were they present in more than one patient of the same ethnic origin.

Table 3. Mutations in non-Jewish CD Patients*

Patient	Mutation 1	Mutation 2	Ethnic origin
1	I143F	IVS4-1G→C	Guatemala
2	I143F	IVS4-1G→C	Guatemala
3	A305E	N121I	German/Polish
4	A305E	A305E	German
5	A305E	P181L	Dutch/German
6	H244R	H244R	Italian
7	G18R	G18R	Persian/Iran
8	A305E	L272P	Italian
9(Fetus)	E285A	IVS4+1G→T	Jewish/Turkish
10	A305E	A305E	German
11	R168C	382delC	Italian
12	244delAT	244delAT	Venezuelan
13	245insA	T166I	Mexican
14	P181T	V14G	German/Polish-Dutch
15	A305E	unidentified	German/British
16	A287T	unidentified	German/European
17	H244R	unidentified	Italian

***Mutations identified subsequent to Zeng et al, 2002; Novel mutations are underlined.**

3.3. Genotype: Phenotype Correlations

While all patients presented with severe psychomotor delay, poor head control and truncal hypotonia and developed progressive spasticity and macrocephaly, the clinical onset and time appearance of seizures varied. Clinical manifestations were noted at birth in two youngsters of British ancestry, both of who possessed one allele containing the D249V substitution. Both died early. Another infant with an onset at birth carried the E214X mutation which produces a stop codon.

Several other novel mutations were associated with an onset before two or three months and early seizures. These include V14G, C152W, P181L, 244 del AT, X314W, 698 inc C and 923 del T. In several of these examples, either a stop codon is introduced or eliminated or a frameshift occurs. In the case of the C152W mutation, a disulphide bond critical for the molecular conformation of ASPA is disrupted. Therefore, clinical

variations in Canavan disease may result from differing effects exerted on the expression of ASPA activity by specific mutations.

Table 4. Results of Mutation Analysis in 39 Non-Jewish CD Patients*

Summary	n	Mutations
Missense mutations	**21**	**V14G, I16T, G18R, E24G, G27R, D68A, D114E, N121I, G123E, I143F, C152W, T166I, R168C, P181T, P181L, H244R, D249V, L272P, E285A, A287T, A305E.**
Nonsense mutations	**2**	**Q184X, E214X**
Deletions	**6**	**33del13, 876del4, 244delA, 244delAT, 382delC, 923delT.**
Insertion mutations	**2**	**245insA, 698insC.**
Two variations in one allele	**1**	**10TindelGG (10T→G and 11insG)**
Elimination of the stop code	**1**	**X314W (941A→G, TAG→TGG)**
Splice acceptor site mutation	**2**	**IVS1-2A→T, IVS4-1G →C.**

***Novel mutations are underlined.**

3.4. Prenatal Diagnosis of Canavan disease

Aspartoacylase activity is low to undetectable in chorionic villus and amniocytes. Therefore, a prenatal diagnosis of Caravan disease cannot be made reliably by enzyme activity. As an alternative, the concentration of NAA in amniotic fluid can be determined using a stable-isotype dilution technique (Kelley, 1993). This method was used to detect Canavan disease in a pregnancy originally thought to be at low risk.

The mother was one-quarter Jewish and a carrier for the E285A mutation. Her husband, who was non-Jewish and of Turkish, Persian and Indian ancestry, tested negative for the three common Canavan disease mutations. Nevertheless, the couple elected to have the amniotic fluid analyzed at 17 weeks of pregnancy. The amniotic fluid NAA was reported as elevated and was again elevated when repeated at 21 weeks of gestation (Dr. Richard Kelley, Kennedy-Krieger Institute, Baltimore, Maryland). The fetus was aborted. The father's genomic DNA was sequenced and a mutation at IVS4+1G→T (Rady et al., 2002) was found. Both this mutation and the mother's E285A mutation were confirmed in the fetal tissue.

Mutation analysis of fetal cells is the "gold standard" for prenatal diagnosis of Canavan disease but may not always be possible. In another family with an affected child, we were able to positively identify a fetus as unaffected using polymorphisms within the ASPA gene. The mother carried the A305E mutation but it was not possible to identify the father's mutated allele since neither the father nor the affected child produced mRNA from this allele. The 693C→T polymorphism could not be used because the father appeared to be homozygous whereas the affected sibling was heterozygous for this polymorphism. Sequencing of the father's genomic DNA revealed that he also carried a new variation at IVS2-284A→T on one of his ASPA alleles. His wife also carried this variation on one of her alleles and the affected sibling was homozygous for the new polymorphism. However, the fetus was heterozygous for the novel variation. With cDNA, the affected child had a one allele pattern, whereas the fetal cDNA contained two alleles confirming the diagnosis of an unaffected fetus.

Consequently, when one mutation remains unidentified in either parent, the search for polymorphisms may be a useful alternative to determine the genotype of an at risk fetus.

4. CONCLUSIONS

Whereas two mutations in the ASPA gene account for more than 98% of all mutant alleles causing Canavan disease in the Ashkenazi Jewish population, many different mutations can be found in non-Jewish individuals with Canavan disease. In our investigation of 40 non-Jewish patients with Canavan disease, we have found 24 novel mutations and one new polymorphism in the ASPA gene.

On the basis of this experience, it is concluded that the diagnosis of non-Jewish persons with Canavan disease requires sequencing of all exons and their splice sites as well as a search for insertions and deletions. When mRNA cannot be found so that cDNA is unavailable for sequencing, it may be possible to use polymorphisms in place of the actual mutations for prenatal diagnosis.

5. ACKNOWLEDGEMENTS

These studies were supported by grants from the Morris and Alma Shapiro Foundation, the Margaret Enoch Fund, the Freddy Teft Memorial Fund, and the Genzyme Corporation.

6. QUESTION AND ANSWER SESSION

SESSION CO-CHAIR MATALON: Any comments? We fixed the microphone. You can talk now. Let's give it a try. Good.

PARTICIPANT: I'm fairly new to the Canavan's field. I'm a spectroscopist. Do the carriers have any manifestations? And have the carriers been evaluated with spectroscopy or imaging?

DR. KOLODNY: Perhaps others in the audience can answer this question. We haven't imaged any of our carriers. It is said that if you do psychological testing that carriers

might have visual, spatial, or other maybe executive function abnormalities. I haven't really seen that in our patient cohort.

DR. ROSS: In response to the question that occasionally parents will hop into the magnet when their children aren't around. We haven't ever seen any hint, but, of course, we haven't usually known which parent was the carrier; double-double blind study.

DR. KOLODNY: Looks better.

PARTICIPANT: The reason why I ask, we have discovered -- it's SLC6AA -- a creatine transporter deficiency. And the parents, the female carriers, the grandmother, mother, and aunt all have psychological deficits; and I did spectroscopy. And they did have relatively normal creatine levels, but what was interesting was we imaged a nine-day-old carrier who had a deficiency of creatine.

Now, with her mother and her aunt and her grandmother, as I just said, it normalizes with the functioning allele. So that's why I was wondering if anyone had looked to see if there were any kind of myelin disruptions or maybe a reduced -- or, yes, an NAA that was somewhere between a normal and the Canavan's might be interesting.

DR. KOLODNY: Carriers don't normally have elevations in NAA.

PARTICIPANT: Hello. Yes. In CSF analysis, can that be more informative in an early diagnosis or if possible to avoid genotyping for that matter in a Canavan's disease patient suspected of hydrocephalus?

DR. KOLODNY: You're asking whether CSF NAA can be used for the early diagnosis of Canavan disease?

PARTICIPANT: We had a child with hydrocephalus, and we were suspecting CD. So CSF analysis, can that be more informative? Or directly going on to the genotyping that according to the early detection can --

DR. KOLODNY: Using CSF rather than urine analysis of organic acids? I can't answer that because we haven't done CSF exams. Has anyone? Reuben? CSF, Canavan children?

DR. LEONE: Yes, we did it.

DR. BURLINA: Of course, we are also concerned because, you know, you have to make a lumbar puncture in a small child. But the response is very high. And in the ten cases we had until now, eight also had a very high level of NAAG. It was very high because it went from 100 micromoles to 250 micromoles per liter in the CSF.

DR. KOLODNY: It is interesting that for some of our patients, we have had normal organic acids. Then after making the diagnosis of Canavan, we have asked the lab to repeat it, and it has been elevated. I think you have to be very careful when you review laboratory results for urine NAA levels.

DR. BURLINA: There is a question for that one because usually NAA was always considered an organic acid, not an acetylated amino acid. So you went through the analysis with the organic acid with the usual analysis for organic acid, but you risked overestimating with the organic acid. And you lose the higher peak.

So you risk that you don't have a real number. So this is the reason that sometimes you have to reject it and use a different system. So we use capillary electrophoresis for that reason.

DR. KOLODNY: So that's an important caution.

Dr. Tsipis, you had a question?

DR. TSIPIS: Just a quick question. With genotype/phenotype, is there anything in there that is interesting?

DR. KOLODNY: There are three children that we reported in our paper in 2002 where seizures and irritability and failure to thrive really began immediately after birth, very soon, before two months.

But that isn't the story in most patients. And in others who have the same genotype, there was a more typical clinical picture. So it's very hard to make a story, even when you have termination stop codons early in the gene so that protein isn't made. Still we have children surviving and doing quite well.

DR. NAMBOODIRI: Can you comment on any kidney complications in your patients?

DR. KOLODNY: Well, with regard to that, I can only answer that we really stress the kidney by creating a strong acidosis with Diamox and calcium acetate driving the bicarb level way down, as low as 12 milliequivalents. And families know enough that when the child starts hyperventilating with a respiratory acidosis, that they will back off on the drug.

So I think that both the lungs and the kidneys seem to be working very well, in spite of the metabolic stress that we put on them with these two drugs.

SESSION CO-CHAIR MATALON: Maybe just before the break, I'll throw something. You can think about it when we come back. Among all of the patients I have seen, which is quite a few, and that I have heard of, there was one patient who had nephritic syndrome. So I don't really know if this has any relation.

Thank you.

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