Original articles

Frequency of arylsulphatase A pseudodeficiency associated mutations in a healthy population

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Abstract

Arylsulphatase A (ASA, EC 3.1.6.1) is a lysosomal enzyme that catalyses cerebroside sulphate degradation. ASA deficiency is associated with metachromatic leucodystrophy (MLD), a rare autosomal recessive disorder, which is characterised by the storage of cerebroside sulphate. Low ASA activities can be also observed in clinically healthy persons, a condition termed ASA pseudodeficiency. Two mutations responsible for the majority of pseudodeficiency alleles have been defined in the ASA gene. These are both A→G transitions. One causes an asparagine to serine substitution (N350S). The second changes the first polyadenylation signal downstream of the stop codon (1524+95A→G), which causes a severe deficiency of one ASA mRNA species. The incidence of the pseudodeficiency allele is estimated to be high in the general population and can be found in families carrying MLD associated mutations. We report a reliable stratagem for detecting the two PD associated mutations separately, which we have applied to a healthy population. Two homozygotes for the N350S and 1524+95A→G mutations were detected, which gives a population frequency of 2.6%. The overall frequencies of the ASA-PD mutations were shown to be 17.5% for the N350S change and 13.0% for the 1524+95A→G change, estimating each mutation separately. In addition, the frequency of both PD associated mutations occurring together on the same chromosome was found to be 12.3% in our population. The study has also allowed us to establish a new control ASA activity range, which was based on assay of blood from persons who had been shown at the DNA level not to carry ASA PD associated mutations.

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Arylsulphatase A (ASA, EC 3.1.6.1) is a lysosomal enzyme that catalyses cerebroside sulphate degradation. ASA deficiency is associated with metachromatic leucodystrophy (MLD), a rare autosomal recessive disorder, which is characterised by the storage of cerebroside sulphate.¹ The accumulation of this substrate

can be found in many tissues of MLD patients, but affects mainly the nervous system. MLD can be divided into three major clinical forms: late infantile, juvenile, and adult, and the disease incidence is estimated to be around 1:40 000.² The ASA gene maps to the long arm of chromosome 22 and spans 3.2 kb of genomic DNA divided into eight exons.³ The ASA cDNA hybridises to three different mRNA species and would be predicted to code for a protein of 507 amino acids that contains three potential N-glycosylation sites.^{3,4} Two mutations in the ASA gene are responsible for about 50% of the MLD associated mutations in the populations studied to date in northern Europe.^{5,6} One is a G→A change destroying the splice donor site of intron 2 (459+1 G→A) and the other is a C→T transition causing a proline to leucine substitution (P426L) in exon 8.

A much more frequent condition, termed ASA pseudodeficiency (PD), is also characterised by low ASA activities. This phenomenon can be seen in clinically healthy persons and its population incidence is estimated to be between 7.3 and 15%.⁷⁻⁹ Two mutations responsible for the majority of pseudodeficiency alleles have been defined in the ASA gene. These are both A→G transitions at base 1049 and base 1620 (numbering according to cDNA sequence⁴). One causes an asparagine to serine substitution (N350S), which leads to the loss of an N-glycosylation site. The second changes the first polyadenylation signal downstream of the stop codon (1524+95A→G), which causes a severe deficiency of one ASA mRNA species. The loss of this ASA mRNA species accounts for the reduced synthesis of ASA protein and the resultant lower enzyme activity in PD persons.¹⁰

Owing to the high frequency of the ASA PD allele in the general population, it is not surprising that this allele is also found to be segregating in some families carrying MLD associated mutations. This leads to additional difficulty in the establishment of diagnosis of MLD, particularly in prenatal diagnosis. This diagnostic problem necessitated the development of simple and straightforward methods for the detection of the two PD associated mutations in the ASA gene. A PCR based method was recently developed to enable the detection of ASA-PD alleles.¹¹ This method relies on using allele specific oligonucleotides

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Figure 1 Mutations associated with ASA pseudodeficiency. Solid boxes indicate exons and hatched parts untranslated regions. Circles denote potential sites of N-glycosylation. Shaded boxes indicate fragments A and B, which represent genomic DNA amplified for detection of mutations associated with ASA pseudodeficiency. Asterisk represents the site of an introduced mismatch (t) for creation of a DdeI restriction site.

to amplify PCR products from either normal or PD alleles. However, although this assay is useful for identifying persons who carry both PD associated mutations on the same chromosome, it will not detect PD alleles that carry only one of these mutations. PD alleles carrying only one of these mutations have recently been described.¹² We present here a reliable stratagem for detecting the two PD associated mutations separately. The approach combines PCR and restriction endonuclease digestion. We have examined a healthy population by this new method and established the frequency of the two mutations in this population.

Materials and methods

Throughout this manuscript we refer to nucleotide sequence of the ASA cDNA, except when otherwise stated.

DNA PREPARATION

Genomic DNA was extracted from mouthwash samples¹³ from 77 healthy persons.

POLYMERASE CHAIN REACTION AMPLIFICATION

Two fragments of the ASA gene (fig 1) were amplified by PCR using the following oligonucleotide primers designed from the ASA genomic sequence (EMBL X52150).

Fragment A:
ASA 6i-5' (5'-3')
TTGATGGCGAACTGAGTGAC
ASAp 6i-3' (5'-3')
CAGTGCAGGAGGCACTGAGG } 275 bp.

Fragment B:
ASA E-5' (5'-3')
GGTTTGTGCCTGATAAC*TA
ASAp 8i-3' (5'-3')
TTCCTCATTCGTACCACAGG } 114 bp.

(*indicates a mismatch site at position 1524+92).

For the reaction, 500 ng of each pair of exon specific primers was added to approximately 200 ng of template DNA with 2.5 units of *Taq* polymerase (Promega). Genomic DNA was amplified in a total volume of 25 µl containing 0.2 mmol/l dNTPs, 9% dimethylsulphoxide, 10 mmol/l Tris-HCl pH 9, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 170 µg ml⁻¹ bovine serum albumin, and 0.1% Triton X-100. The reaction parameters were 94°C for five minutes, then 30 cycles of 94°C for 30 seconds, followed by 30 seconds at 60°C (fragment A) or 58°C (fragment B), then 72°C for 30 seconds followed by five minutes' extension at 72°C. One fifth of the PCR reaction was analysed on a 2% (w/v) agarose gel.

DETECTION OF THE N350S MUTATION

The N350S mutation creates a *BsrI* site, so population screening for this mutation was carried out by *BsrI* restriction digest of amplified fragment A (fig 1). Approximately 400 ng of PCR product was digested in 40 µl reaction, using 10 U of *BsrI* in 150 mmol/l KCl, 10 mmol/l Tris-HCl, 10 mmol/l MgCl₂, and 20 µg bovine serum albumin. The reaction products were then analysed by electrophoresis on a 2.5% (w/v) agarose gel. In the presence of the mutation the 275 bp fragment A is cleaved into two smaller fragments of 161 bp and 114 bp.

DETECTION OF 1524+95A→G

In this case, a mismatched primer (ASA E-5') was used in the PCR reaction. In the presence of the 1524+95A→G mutation, a *DdeI* site is generated. Hence, *DdeI* cleavage of fragment B was used to screen for this mutation. Approximately 400 ng of PCR product was digested in 40 µl reaction, using 10 U of *DdeI* in 10 mmol/l spermidine, 100 mmol/l NaCl, 50 mmol/l Tris-HCl, 10 mmol/l MgCl₂, and 1 mmol/l dithiothreitol. The reaction products were analysed by electrophoresis on a 10% (w/v) polyacrylamide gel. In the presence of the mutation the full PCR product of 114 bp is cleaved into two smaller fragments of 97 bp and 17 bp. The 17 bp fragment is too small to be resolved on the gels used and so mutation detection depends solely on the generation of a 97 bp fragment.

DNA AMPLIFICATION FOR DETECTION OF THE N350S AND 1524+95A→G MUTATIONS ON THE SAME CHROMOSOME

Fragments 1 and 2 (fig 1) were amplified according to a method described previously by Gieselmann.¹¹ This method uses two pairs of primers for amplification of the region between the two mutations (fragment 2). One pair of primers is homologous to the normal ASA sequence and allows amplification of fragment 2 when neither mutation is present. The other pair of primers is homologous to the mutant sequence and a product is generated only when both mutations are present on the same chromosome. Fragment 1, an internal positive con-

Figure 2 Detection of N350S and 1524+95A→G mutations. (A) Detection of N350S mutation: fragment A was amplified by PCR, products were digested with BsrI and fractionated in a 2.5% (w/v) agarose gel. Lane 1 is homozygous for normal sequence; lane 2 is homozygous for mutant sequence; lanes 3 and 4 are heterozygotes. (B) Detection of 1524+95A→G mutation: fragment B was amplified by PCR, products were digested with DdeI and fractionated in a 10% polyacrylamide gel. Lane 1 is homozygous for normal sequence; lane 2 is homozygous for mutant sequence; lanes 3 and 4 are heterozygotes.

trol for the assay, is amplified from all ASA genes. Primer sequences for this assay are as described previously.¹¹

ASA ENZYME ASSAY IN LEUCOCYTES
Leucocytes (WBC) were isolated from a 10 ml heparinised blood sample by the dextran sedimentation method¹⁴ and stored at -30°C. Each WBC pellet was resuspended in 500 µl of deionised water and disrupted by sonication. Total protein concentration was determined by the method of Lowry *et al.*¹⁵ ASA activity was determined in sonicated WBC pellets using 4-nitrocatechol sulphate as the substrate, as described by Lee-Vaupel and Conzelmann,¹⁶ with an incubation time of 18 hours at 0°C and protein content of 100–150 µg/assay.

DIRECT SEQUENCE ANALYSIS OF PCR PRODUCTS
PCR amplified DNA was purified using Gene-clean II (Bio101) and sequenced by standard methods with Sequenase (USB) and appropriate primers.¹⁷

Results

VERIFICATION OF THE N350S ASSAY
Fig 2A shows the results obtained for the detection of the N350S mutation. The presence of the 275 bp full length fragment A alone indicates a subject who is homozygous for the normal sequence (fig 2A, lane 1). In persons who are homozygous for the N350S mutation, the amplified fragment A is cleaved and two smaller fragments of 161 bp and 114 bp are seen (fig 2A, lane 2). Persons who are heterozygous for the N350S mutation show three fragments after BsrI digestion of fragment A, the 275 bp product from the normal allele, and the 161 bp and 114 bp fragments from the allele carrying the N350S mutation (fig 2A, lanes 3 and 4). In each case, the mutation was confirmed by direct sequence analysis.

VERIFICATION OF THE 1524+95A→G ASSAY
Results from the assay for the detection of the 1524+95A→G mutation are shown in fig 2B. The 5' primer used in the amplification of fragment B contains a mismatched base (relative to the normal genomic DNA sequence)

Summary of genotypes found

Genotypes		No of subjects
N350S	1524+95A→G	
+/+	+/+	2
+/+	-/-	1
+/-	+/-	16
+/-	-/-	5
-/-	-/-	53

-/- represents homozygous for normal sequence.
+/- represents heterozygous.
+/+ represents homozygous for mutant sequence.

that generates a restriction site for DdeI in the presence of the 1524+95A→G mutation. After DdeI digestion of fragment B, a subject who is homozygous for the normal sequence shows a single full length fragment B of 114 bp (fig 2B, lane 1). A person who is homozygous for 1524+95A→G shows a single smaller fragment of 97 bp (fig 2B, lane 2) (the 17 bp product is not resolved on the gels used here). In a subject who is heterozygous for the 1524+95A→G mutation, two fragments of 114 bp and 97 bp are observed (fig 2B, lanes 3 and 4). Results were confirmed by sequence analysis.

POPULATION FREQUENCIES OF THE N350S AND 1524+95A→G MUTATIONS
A summary of N350S and 1524+95A→G mutation genotypes found in our population is shown in the table. We have detected mutations in 24 out of 77 persons screened. Two were homozygous for both mutations, 16 were heterozygous for both of them, and five were heterozygous for the N350S mutation alone. One was homozygous for the N350S mutation alone.

The 16 persons who were shown to be heterozygous for both mutations were re-screened using the method described by Gieselmann¹¹ in order to establish whether both mutations were located on the same chromosome. The results obtained using this method showed that 15 of these 16 persons carried the N350S and 1524+95A→G mutations on the same chromosome. However, one showed no amplification of the mutant or normal fragments, despite amplification of the internal control fragment, suggesting that the two mutations were located on different chromosomes.

Figure 3 Relationship between ASA activity and genotype. -/- represents homozygous for normal sequence; +/- represents heterozygous; +/+ represents homozygous for mutant sequence; solid bar denotes MLD range; hatched bar indicates previous control range.

CORRELATION OF ASA PD MUTATION AND ENZYME ACTIVITY

ASA activity was measured in leucocytes of a number of persons with each combination of ASA PD alleles, as shown in fig 3. Two were homozygous for the N350S and 1524+95A→G mutations and one was homozygous for the N350S mutation alone. Nine persons were heterozygous for both the N350S and 1524+95A→G mutations while two were heterozygous for the N350S mutation alone. Nine persons who were homozygous for the normal allele were also included in this screening. Also included in the figure is an ASA activity range observed in MLD patients (solid bar) as well as a control range (hatched bar) which was established before this study in healthy subjects who were not tested for ASA PD associated mutations (unpublished data). Although the numbers are small, it appears that subjects who are homozygous for both ASA PD associated mutations have the lowest residual ASA activity. Further, the N350S mutation alone does not seem to affect ASA activity levels as has been described previously.¹⁰

Discussion

The molecular basis of arylsulphatase A pseudodeficiency was first defined as an allele carrying two linked lesions in the ASA gene, the N350S and the 1524+95A→G mutations.¹⁰ However, recent data have shown

that these two mutations do not always occur together and that at least the N350S mutation may be found alone.^{9,12} To date the 1524+95A→G mutation has not been reported in isolation.

We have developed a new approach to detect the ASA PD associated mutations separately instead of using the combined PCR based method described by Gieselmann.¹¹ This approach allowed us to establish the frequency of the different genotypic groups within our normal population. Subjects from each group were reassessed in order to establish the relationship between ASA PD genotype and ASA enzyme activity.

We have detected two homozygotes for the N350S and 1524+95A→G mutations, which gives a population frequency of 2.6% (four of 154 alleles). The ASA enzyme activities of these two persons were found to be just above the MLD range (fig 3), with values of 0.7 and 1.0 nmol/h/mg protein, while the control range was established to be from 6.0 to 11.6 nmol/h/mg protein. Sixteen persons were found, by our method of detection, to be heterozygous for both mutations. When these 16 persons were then rescreened by the method described by Gieselmann,¹¹ which gives positive results only when both mutations are located on the same chromosome, 15 subjects appeared to carry the ASA PD allele. Hence, one person presumably carries the two mutations on different chromosomes. ASA activities were also measured in eight persons who are heterozygous for both mutations and carry them on the same chromosome (fig 3) and from the person who presumably carries the two mutations on different chromosomes. All these persons were found to have lower ASA activities than those who are homozygotes for the normal sequence. However, their activities fall within the ASA activity range established previously for normal ASA alleles, at the lower end of the range. This result suggests that the normal ASA activity range established previously on a control group drawn from the same population probably included samples from persons carrying the PD allele.

The one subject who was found to be homozygous for the N350S mutation had an ASA activity (fig 3) of 3.9 nmol/h/mg protein, while the control range was determined to be 6.0 to 11.6 nmol/h/mg protein. This shows that the homozygote for the N350S mutation in this study has an ASA activity below the normal range, but similar to those of heterozygotes for both mutations. Five persons were found to be heterozygotes for the N350S mutation alone and the two of these who were available for measurement of ASA activity had levels within the normal range. These results indicate that the N350S mutation alone does not seem to be responsible for a major reduction of ASA activity in our population. Although the numbers studied by us are still small, the observations confirm the data of Gieselmann *et al.*¹⁰

The overall frequencies of the ASA PD mutations have been shown here to be 17.5% (27 of 154 alleles) for the N350S change and 13.0%

(20 of 154 alleles) for the 1524+95A→G change. These frequencies have been estimated for each mutation separately. In addition, the frequency of both PD associated mutations occurring together on the same chromosome was estimated to be 12.3% (19 of 154 alleles) in our population. The frequency of the PD allele obtained here is higher than the data obtained in the Australian (9.6%)⁹ and German (7.3%)⁸ populations. This variation may be because of different assays used in each study. The method used here enables the establishment of the frequency of the ASA PD associated mutations alone as well as the frequency of the ASA PD allele carrying both mutations. The study has also allowed us to establish a new control ASA activity range, which was calculated using measurements on persons who had been shown at the DNA level not to carry ASA PD associated mutations. The data obtained in this study will be useful in precise diagnosis of mutation status in families segregating PD associated as well as MLD associated mutations.

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