X-LINKED ADRENOLEUKODISTROPHY: PROFILES OF VERY LONG CHAIN FATTY ACIDS IN PLASMA AND FIBROBLASTS IN EIGHT SERBIAN PATIENTS

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ABSTRACT

X-linked adrenoleukodystrophy is a severe neurodegenerative disorder with impaired very long chain fatty acid metabolism. The disease associated ABCD1 gene encodes a peroxisomal membrane protein which belongs to the superfamily of ATP-binding cassette transporters. We investigated eight male X-ALD patients diagnosed among 142 suspected patients referred for investigation. Plasma levels of very long chain fatty acids were measured at our laboratory using capillary gas chromatography. Eight cases of childhood X-ALD were diagnosed. This is the first published series of Serbian patients with X-ALD. In addition, diagnosis identifies carriers, which could be benefit for genetic counselling and prenatal diagnosis.

KEY WORDS

X-linked adrenoleukodystrophy, peroxisome disorders, very long chain fatty acids, capillary gas chromatography

INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD; McKusick 300100) is the most frequent peroxisomal disorder, with an estimated frequency of 1:20,000 in males (1,2). It should be differentiated clinically and biochemically from neonatal ALD, an autosomal recessive disorder of peroxisome biogenesis in which the function of at least five peroxisomal enzymes is impaired.

The accumulation of VLCFA's in tissues of X-ALD patients results from their impaired capacity to degrade these substances. This reaction normally take place in a peroxisome as a part of the cell. The plasma concentration of very long chain fatty acids (VLCFA) is elevated in more than 99 % of males with X-ALD of all ages regardless of the presence or absence of symptoms. This assay is extremely specialized and therefore is performed only in a few laboratories worldwide.

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Patients with X-ALD lack one of the proteins required for this degradation. The protein that is missing or defective is called ALDP (X-ALD protein). X-ALD is due to mutations or defects in the gene that codes for ALDP. This gene is located on the X-chromosome (ABCD1 gene). Molecular analysis of the ABCD1 gene is available clinically (3). It is used primarily for genetic counseling for determination of carrier status in at risk female relatives and for prenatal diagnosis (4). Here we report the results of our study on the VLCFA profiles in specimens from controls and patients affected by X-ALD.

MATERIALS AND METHODS

Chemicals : Organic solvents such as chloroform, methanol, n-hexane, toluene and diethylether were the highest available grade including the standards of fatty acids were obtained from Sigma-Aldrich.

Biological samples : Plasma or serum: venous blood (with or without anticoagulant) 2-5 ml, is centrifugated for 10 min at 800 g, preferably within 1 h of collection to avoid hemolysis, and the plasma or serum is separated. The sample is stored at -4 °C or lower temperature until analysis can be performed.

Cultured fibroblasts: human skin fibroblasts were cultured in

HAM F-10 medium (Gibco, Invitrogen), supplemented with 10 % fetal calf serum, penicillin (100 IU/ml), streptomycin (100 IU/ml) and 25 mM Hepes buffer with L-glutamine (1 mM) in a humidified atmosphere of 5 % CO₂. Unless otherwise stated cells were cultured at 37°C. Cells were used between passage numbers 6 and 18. For fatty acid analysis, cells were harvested either by trypsinization or by 'scraping'. The culture media is removed after centrifugation for 15 min at 800 g and the fibroblast pellet is washed three times with phosphate buffered saline (PBS). After the last wash, the liquid is drained and the pellet is stored at -20°C until analysis (5,6).

Sample preparation

To 200 ml of plasma is added 30 ml of heptacosanoic acid (C27:0) solution as internal standard and 1.25 ml of chloroform-methanol 1:2 (v/v), cap and vortex vigorously for 5 min. Centrifuge the extracted plasma at 1000 g for 10 min and transfer the lower phase to another tube and dry under nitrogen stream. Dissolve the dried lipid extract in 100 ml of chloroform and apply 150 ml of the solution in a 0.5 cm band on a precoated TLC plate, a reference mixture and four samples can be run simultaneously. The plate is developed 10 min in n-hexan:diethylether:chloroform:acetic acid 52.5:15:7.5:0.75 (v/v).

After air drying for 30 min, the sample bands were stained by 2',7'-dichlorofluorescein, removed by scrapping on the weighing paper, transferred to another tube and transesterified with 500 ml of methanol and 80 ml of acetylchloride and heat the tubes for 45 min at 85°C in a metal block thermostat.

After cooling to room temperature add 700 ml of n-hexan, vortex 1 min. Transfer the upper n-hexan phase into another tube and dry under nitrogen stream. The dried residue is finally taken up in 60 ml n-hexan and 3 ml subjected to GC for the analysis of VLCFA.

For VLCFA analysis of fibroblasts, suspend the fibroblast pellet (containing at least 300-500 mg of protein) in 400 ml of water and disrupt the cells by sonication to form a homogeneous suspension. Take two aliquots of this suspension for duplicate protein analysis and transfer 200 ml of the remaining suspension to a glass tube with teflon-lined screwcap. Add 30 ml of the heptacosanoic acid solution and proceed as described above the plasma (7,8).

Capillary gas chromatography : For the assay of VLCFA in our laboratory we use a HP5 capillary column (30 m x 0.32 mm I.D., 0.25 mm film thickness; crosslinked 5 % phenylmethyl

Table 1. Chromatographic parameters of derivatized standards of very long chain fatty acids (VLCFA) on HP5 column.

Elution order	VLCFA	Abbreviation	RRT	RRF	CV (%) n=6
1	Docosanoic	C22:0	1.157	0.428	2.0
2	Tetracosanoic	C24:0	1.086	0.498	1.68
3	Hexacosanoic	C26:0	1.029	0.434	2.66
4	Heptacosanoic (IS)	C27:0	1.0	1.0	1.12

Table 2. Mean values $\pm$ SD of very long chain fatty acids concentrations in plasma and fibroblasts of controls and X-linked adrenoleukodystrophy (X-ALD) patients.

Plasma n	Controls 50	X-ALD 8
C26:0/C22:0 ratio	0.017 $\pm$ 0.007 (0.010-0.035)	0.404 $\pm$ 0.031 (0.17-1.16)
C24:0/C22:0 ratio	0.896 $\pm$ 0.042 (0.30-0.90)	1.457 $\pm$ 0.265 (0.97-2.10)
C26:0 mg/L	0.378 $\pm$ 0.248 (0.010-0.80)	0.995 $\pm$ 0.204 (0.85-1.46)
C24:0 mg/L	16.880 $\pm$ 3.028 (8.0-35.7)	15.887 $\pm$ 4.118 (6.5-21.1)
C22:0 mg/L	22.010 $\pm$ 4.514 (9.5-50.0)	12.400 $\pm$ 1.233 (11.0-29.5)
Fibroblasts n	Controls 20	X-ALD 8
C26:0/C22:0 ratio	0.058 $\pm$ 0.040 (0.08-0.20)	0.740 $\pm$ 0.153 (0.48-1.71)
C24:0/C22:0 ratio	1.890 $\pm$ 0.082 (1.70-2.00)	2.759 $\pm$ 0.140 (2.50-3.1)
C26:0 mg/g protein	0.162 $\pm$ 0.021 (0.15-0.35)	0.789 $\pm$ 0.092 (0.60-1.90)
C24:0 mg/ g protein	2.709 $\pm$ 0.291 (1.0-4.65)	2.849 $\pm$ 0.338 (1.40-7.0)
C22:0 mg/ g protein	1.527 $\pm$ 0.508 (0.86-3.0)	0.716 $\pm$ 0.091 (0.55-2.61)

siloxane, Hewlett Packard, USA). Nitrogen is used as the carrier gas, at a flow rate of 1.5 ml/min. The flame ionization detector is 280°C and the on-column injector temperature 60°C. The oven temperature is programmed as follows: the initial temperature of 60°C is immediately increased to 300°C at 6°C/min and held there for 5 min. Instrument was used HP6890 gas chromatograph with a flame ionization detector (Hewlett Packard, USA).

Statistical analysis : Data were compared by using the Student's t-test procedure (9).

Fig.1: GC profile of an standarde mixture of very long chain fatty acids analyzed on the HP5 column. A 1µl aliquot was injected onto the column in the splitless mode (1:10).

The labeled peaks represent a following acids: C22:0, docosanoic acid; C24:0, tetracosanoic acid; C26:0, hexacosanoic acid; C27:0, heptacosanoic acid added as an internal standard.

Fig.2: Typical GC profile of very long chain fatty acids in plasma from a patient with X-ALD (A) and an control (B).

The labeled peaks represent a following acids:

C22:0, docosanoic acid; C24:0, tetracosanoic acid; C26:0, hexacosanoic acid; C27:0, heptacosanoic acid added as an internal standard.

Fig.3: Typical GC profile of very long chain fatty acids in fibroblasts from a patient with X-ALD (A) and an control (B). Note that in fibroblasts from a control hexacosanoic acid is not detectable.

The labeled peaks represent a following acids: C22:0, docosanoic acid; C24:0, tetracosanoic acid; C26:0, hexacosanoic acid; C27:0, heptacosanoic acid added as an internal standard.

RESULTS AND DISCUSSION

Peroxisomal disorders are an extremely heterogeneous group of genetic disorders without strict correlation between clinical picture and biochemical abnormalities. The analysis of VLCFA in plasma and fibroblasts is necessary as the first screening test for diagnosis of X-ALD. 142 samples of ill male children with suspicion diagnosis of X-ALD were analyzed. Eight of them have abnormal VLCFA profile in plasma and fibroblasts (5.6 %).

Retention times (RT) and response factors (RF) of the

individual VLCFA on the HP5 column were determined with respect to heptacosanoic acid (C27:0) as internal standard. The overall reproducibility of the procedure, expressed as percent variation from the mean, was determined from six independent analyses of the standards (Table 1). Analytical recoveries exceeding 90 % of a very long chain fatty acids. As shown in chromatogram in Fig. 1, VLCFA listed in Table 1 were separated on HP5 column on conditions described in Material and Methods. A normal and pathological plasma and fibroblast profiles of VLCFA is shown in Fig. 2 and Fig. 3. Identification of the analytes was based on comparison of the retention times with those of standard solutions. The lower

detection limits for docosanoic acid were 9.5 mg/L, tetracosanoic acid 8.0 mg/L and for hexacosanoic acid 0.01 mg/L. Mean values and range of VLCFA concentrations and ratios C26/C22, C24/C22 in plasma and fibroblasts from controls and X-ALD patients are given in Table 2. In controls, the range of plasma C26:0 fatty acid concentration was 0.010-0.035 mg/L (mean 0.0378). In X-ALD patients the plasma concentrations of C26:0 and C26/C22 and C24/C22 ratios were significantly increased ($p < 0.01$). The range for the plasma concentration of hexacosanoic acid (C26:0) was 0.85-1.46 mg/L (mean 0.995). Also, in fibroblasts of X-ALD patients, the C26:0 concentration and C26/C22 and C24/C22 ratios were significantly increased ($p < 0.01$).

Our results obtained with X-ALD show that in virtually all affected patients the three parameters of C26:0 concentration as well as C26/C22 and C24/C22 ratios are significantly elevated in both plasma and fibroblasts. Although there is a great variability among individuals. False negative results in plasma from X-ALD patients appear also to be possible, as reported by Wanders et al. (10). In one case of clinically suspected patient the plasma VLCFA level were found normal, whereas they were clearly elevated in cultured fibroblasts. Whenever there is clinical evidence for X-ALD and plasma VLCFA concentrations are difficult to interpret or normal, analysis of fibroblasts is recommended.

Detection of index cases in families is important for detection of further X-ALD cases, treatment of asymptomatic or barely symptomatic cases to avoid or delay symptom appearance, detection of heterozygotes and providing genetic counselling and prenatal diagnosis for at risk subjects. Although VLCFA analysis is still an important tool for diagnosis of patients and for the treatment follow up.

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