Isolated Mild Intellectual Disability Expands the Aminoacylase 1 Phenotype Spectrum

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Abstract Aminoacylase 1 (ACY1) deficiency is a rare inborn error of metabolism presenting with heterogeneous neurological symptoms such as psychomotor delay, seizures, intellectual disability and it is characterized by increased urinary excretion of N-acetylated amino acids. We report on a new patient who presented ACY1 deficiency in association with isolated mild intellectual disability, but neither neurological symptoms nor autistic features. The child showed a compound heterozygous mutation (p.Glu233Asp) and a novel p.Ser192Arg fs*64, predicting an unstable transcript and resulting in very low protein levels.

This new ACY1 deficient child was identified through regular screening for inborn error of metabolism adopted in our department in all cases of intellectual disability. This report supports a recommendation to perform metabolic investigations in patients with isolated mild intellectual disability.

Introduction

Aminoacylase 1 (ACY1, EC 3.5.1.14) deficiency (MIM 609924) is a rare inborn error of metabolism (IEM) characterized by increased urinary excretion of N-acetylated amino acids with the exception of N-acetylaspatic acid (NAA). Since the first report by Van Coster et al. (2005) less than 20 additional patients have been described, most of them presenting with heterogeneous neurological symptoms such as psychomotor delay, seizures, intellectual disability (Sass et al. 2007; Ferri et al. 2013). ACY1 deficiency has also been reported associated with autistic feature in one patient (Tylki-Szymanska et al. 2010). The diagnosis of ACY1 deficiency is based on urinary analysis of organic acid by gas chromatography-mass spectrometry (GC/MS) (Gerlo et al. 2006) or NMR spectroscopy (Engelke et al. 2008), and confirmatory molecular genetic and enzyme analyses (Sass et al. 2006).

Aminoacylase 1 is a soluble homodimeric zinc binding enzyme that catalyzes the formation of free aliphatic amino acids and acetic acid from N-acetylated precursors. Compared to other tissues, the kidney contains the highest levels of ACY1 mRNA, protein and enzyme activity, with a localization throughout the renal tubular epithelium (Lindner et al. 2008). This seems to be in line with a possible functional role of ACY 1 in recycling of N-acetylated amino acids in the kidney and can explain the high concentrations of N-acetylated amino acids in the urine of patients with the biochemical defect (Sass et al. 2006). *ACY1* is also highly expressed in brain and liver, and to a lesser extent in almost all the other tissues (Cook et al. 1993; Sass et al. 2006), suggesting a pivotal role of the enzyme in amino acid metabolism of these organs.

ACY1 is located on the short arm of chromosome 3 (3p21.1) (Naylor et al. 1979, 1982; Miller et al. 1990; Cook

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et al. 1993), and the protein is highly conserved, supporting an evolutionary role in physiology. Until now ten different mutations have been reported (Van Coster et al. 2005; Sass et al. 2006, 2007; Tylki-Szymanska et al. 2010; Ferri et al. 2013), with no clear-cut phenotype–genotype correlation.

We report a new deficient patient presenting with mild intellectual disability harboring compound heterozygous mutations in *ACY1*.

Patient

A 10-year-old girl was admitted in our department to evaluate her language and learning disabilities. She was born at term after an uneventful pregnancy as the first child of unrelated healthy parents. At birth she presented a mild respiratory distress (APGAR score after 1 and 5 min was 7–10); birth weight was 3,500 g, length 52 cm, OFC 34.5 cm.

With the exception of multiple abortions reported by the mother there was no family history of epilepsy, other neurologic or psychiatric disorders. The child presented a normal motor development with language delay and mild difficulties in relationship with her peers. The parents also reported several episodes of asthmatic bronchitis since the first year of life.

The phenotypic features of the child were normal; height and weight were in the 75th percentile and head circumference between the 50th and 75th percentile.

At the neurological examination the girl showed only a mild clumsiness; her behavior was characterized by impulsivity, attention deficit, and provocative attitude.

Diagnostic Work-Up

Blood and urine samples from the proband and her parents were obtained after informed consent for metabolic and genetic analyses.

Organic acids determination was performed as part of the metabolic work-up, including amino acids, creatine, thyroid hormones evaluation, and karyotype analysis (550 band level), as recommended by the diagnostic flowchart in our department in all cases of non-syndromic intellectual disability.

Moreover brain Magnetic Resonance Imaging (MRI) with proton spectroscopy, awake and sleep EEG, and fundus oculi evaluation were performed.

For organic acids analysis urine was extracted with ethylacetate (Tanaka et al. 1980) and trimethylsilylated (TMS) by TMCS + BSTFA at 60°C for 1 h prior to the analysis with an Agilent 6890 N GC equipped with an HP5MS capillary column (0.25 mm × 30 m, film thickness 0.25 µm) and an Agilent mass spectrometer 5973 N. Reagents were purchased from Sigma-Aldrich (Italy).

Mutation Analysis

Genomic DNA was purified from peripheral blood using standard procedures. PCR primers were designed by *Primer 3* software (<http://frodo.wi.mit.edu/primer3>) to cover the 14 exons and part of the flanking intronic regions of the *ACY1* gene (primer sequences are available upon request). Fragments were directly sequenced using the BigDye 3.1 chemistry (Applied Biosystems, Foster City, CA) on an ABI3130xl Genetic Analyzer (Applied Biosystems).

A punch skin biopsy from the anterior area of the left forearm of the patient was obtained for fibroblast culture. Cells were grown in regular DMEM supplemented with 10% fetal bovine serum following standard procedures.

Total RNA was isolated from cultured skin fibroblasts using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Germany) according to the manufacturer's protocol. Both integrity and concentration were assessed on 1% agarose gel. Identical amounts of RNA were retrotranscribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and the cDNA was PCR-amplified using primers 5'-3'ACY1-F CTAAGTCCAGGCCACAGTCA and 5'-3'ACY1-M13R CAGGAAACAGCTATGACCGGGGGAAGAG-GAGGTCCTT.

About 40 µg of cell proteins were separated by 15% denaturing SDS-PAGE as previously described (Nogueira et al. 2013) and subjected to western blotting using a monoclonal anti-mouse antibody specific for the human Aminoacylase 1 protein (dilution 1:100; Novus Biologicals, USA). Quantitative blot analysis was performed using ImageJ software (<http://rsbweb.nih.gov/ij/>). The same ACY1 antibody was used for immunocytochemical analysis, following procedures already described elsewhere (Fiorillo et al. 2012).

Enzyme Assay

Enzymatic activity of ACY1 was measured in the supernatant of homogenized fibroblasts, according to Sass et al. (2006) with minor modifications. Methionine concentrations produced after incubation of the cells with *N*-acetylmethionine (NAM) were determined by a JLC-500/V amino acids analyzer (Jeol, Japan). Proteins were quantitated by the Lowry method (Lowry et al. 1951). ACY1 activity in the patient and in seven controls cells was analyzed in duplicate.

Neuropsychological Phenotype

The assessment of neuropsychological profile was performed according to the protocols of our department: the WISC IV (Wechsler 2003) for the cognitive level, a short

Fig. 1 Section of the total-ion chromatogram of organic acid from urine of the patient after extraction with ethylacetate and derivatization with BSTFA + TMCS (1% v/v) at 60°C for 60 min. In the figure only the TMS derivatives of the N-acetylated amino acids are highlighted with capital letters. The suffixes 1 or 2 indicate the number of

trimethylsilyl groups, according to Gerlo et al (2006): (a) *N*-acetylalanine-1; (b) *N*-acetyl-alanine-2; (c) *N*-acetylglycine-1; (d) *N*-acetylglycine-2; (e) *N*-acetylvaline-1; (f) *N*-acetylvaline-2; (g) *N*-acetylleucine-1; (h) *N*-acetylserine-2; (i) *N*-acetylthreonine-2; (j) *N*-acetylmethionine-2; (k) *N*-acetylmethionine-1; (l) *N*-acetylglutamate-3

version of a pictured multiple-choice comprehension grammar test for children (TCGB) (Chilosi and Cipriani 1996) for the sentence comprehension, the Peabody Picture Vocabulary Test (PPVT) (Dunn and Dunn 1997; Italian standardization, Stella et al. 2000) for the receptive vocabulary, and a naming test (Gugliotta et al. 2009) for the productive vocabulary.

Results

Diagnostic Work-Up

Amino acids and creatine levels in plasma and urine of the patient were normal, as well as karyotype and standard blood and urine examinations. Brain MRI with proton spectroscopy, EEG awake and sleep, and fundus oculi evaluation were also normal.

Organic acids analysis in three different urine samples disclosed the characteristic pattern of ACY1 deficiency, with the presence of numerous peaks corresponding to N-acetylated amino acids. The most abundant were methio-

nine and glutamate (mono- and di-) TMS derivatives, but alanine, glycine, valine, leucine, serine, and threonine were easily identified (Fig. 1).

All the usual organic acids, including *N*-acetylaspartic acid, both in urine and blood, were within the normal range. Organic acids in the urine of the parents of the patient were normal.

Molecular and Structural Findings

Sequencing of the coding region of *ACY1* revealed that the patient was compound heterozygous for the missense mutation c. 699A>C (p.Glu233Asp) in exon 10 and the c.574_575insG insertion in exon 8. The latter mutation predicts a frameshift and the synthesis of a prematurely truncated protein (p.Ser192Arg fs*64) with a carboxy-tail containing 64 new amino acids. Segregation was confirmed in the parents (Fig. 2).

The expression of Acy-1 protein, evaluated by Western blotting and immunocytochemistry in patient cultured skin fibroblasts, was nearly absent if compared to normal controls (Fig. 3).

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Fig. 3 ACY1 protein analyses on cultured skin fibroblasts, showing a significant reduction of the expression in the patient compared to controls both by Western blot (**a**) and by immunofluorescence (**b**). For immunoblot quantification, ACY1 content was normalized using a

monoclonal anti-porin antibody (Mitosciences, USA); DAPI was used for nuclei counterstaining in immunocytochemistry. Representative experiments are shown. *Ct* controls, *Pt* patient

adopted in our department for intellectual disability, whereas a complete metabolic screening is commonly performed only in patients in whom an IEM is suspected.

The mild developmental impairment observed in the child suggests that ACY1 deficiency could be often overlooked for various reasons, such as the clinical phenotype or some technical problems in the diagnostic laboratories. The identification of N-acetylated amino acids, the biochemical hallmark for the ACY1 deficiency, is fairly easy for many laboratories through the organic acids analysis. Unfortunately the extraction and derivatization of these metabolites present a great variability depending on the conditions adopted for the analysis. As reported by Gerlo et al. (2006) the major problems in identifying the N-acetylated amino acids are the lack of reference spectra in most commercial mass spectral libraries, the presence of coeluting compounds and multiple derivatives in the chromatograms. On this respect, the results of qualitative organic acids scheme of the European Research Network for evaluation and improvement of screening for Diagnosis and treatment of Inherited Disorders of Metabolism (ERNDIM) in 2012, where a sample of urine from a patient with ACY1 deficiency was included, showed that only 73% of the participants ($n = 86$) correctly identified the disease.

To date, ten mutations in *ACY1* have been described, the majority in isolated cases, including small deletions, insertions, and missense mutations. The clinical aspects of the patients previously reported are extremely heterogeneous

and comprise symptoms as psychomotor retardation, brain and spinal malformations, hypotonia, developmental retardation, seizures (Van Coster et al. 2005; Sass et al. 2006, 2007; Engelke et al. 2008; Tylki-Szymanska et al. 2010).

Mutation analysis of *ACY1* in the proband revealed the presence of two heterozygous mutations, one of which (p.Glu233Asp) has been already reported by Sass et al. (2006) in a heteroallelic child who was evaluated because of a slight psychomotor delay. The second mutation (c.574_575insG/p.Ser192Arg fs*64) is novel and resulted in very low levels of ACY1 protein, as shown by immunoblot and immunocytochemistry. This finding is supported by expression studies on transfected cells with Glu233Asp mutation, in which the enzymatic activity was completely lost (Sommer et al. 2011). Both mutations identified in our patient involve evolutionary conserved amino acids and are localized in the protein dimerization domain. In particular, they are localized towards the second monomer and may contribute to the stabilization of the homodimeric structure (Sommer et al. 2011). Because no phenotypic association has been found so far for different types of mutations, it is likely that additional factors modify the ACY1 phenotype in affected patients. The question of the pathogenicity of ACY1 deficiency requires the identification of additional subjects with this condition; similarly the role of the N-acetylated amino acids in the central nervous system should be better understood. Decreased expression of ACY1 has been found in murine models of Huntington's Disease and Fragile-X syndrome (Zabel et al.

2006), suggesting a role of this enzyme in regulating apoptosis and complex metabolic pathways through protein–protein interactions (Zabel et al. 2006; Maceyka et al. 2004). Among the N-acetylated amino acids, ACY1 preferentially hydrolyzes *N*-acetyl-leucine, NAM, *N*-acetyl-cysteine, and to a lesser extent *N*-acetylglutamate (NAG) (Lindner et al. 2008). NAG is the principal excitatory neurotransmitter in the brain, involved in many brain functions including development, cognition, memory and learning, but also in acute or chronic neurodegenerative processes; chronic alterations of its levels can affect these functions. The presence of NAM in the human brain has prompted Smith et al. (2011) to hypothesize that it could be a key player to understanding why individuals with the ACY1 deficiency have neurological or psychiatric defects. They proposed NAM as a secondary source of acetate, similarly to the *N*-acetylaspargate which provides acetate for the myelin lipids synthesis. In addition, NAM could serve as a storage pool of methionine, which has a prominent role in the brain; in this respect it is interesting to note that NAM is the major N-acetylated amino acid excreted in urine of patients harboring pathogenic mutations in *ACY1*. Moreover methionine can be converted to cysteine, the rate-limiting factor for the synthesis of glutathione that is the most important cellular antioxidant; accordingly, the depletion of methionine and cysteine, due to the impairment of ACY1 activity, could lead to an increased susceptibility to oxidative stress, which affects metabolism, signaling, and functioning of the cells. Finally it must be noted the role of methionine as precursor of *S*-adenosylmethionine, which in turn provides methyl groups for transmethylation reactions or can be decarboxylated for polyamine synthesis; these metabolites are involved in various physiological processes and have a critical role on the central nervous system function.

In conclusion, the patient here reported expands the phenotypic spectrum of the ACY1 deficiency and the number of mutations in the responsible gene. Our findings underscore the recommendation to perform a metabolic work-up even in patients with isolated intellectual disability of unknown origin.

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Concise 1 Sentence Take-Home Message

The isolated mild intellectual disability in a child harboring two mutations in ACY1 recommends thorough metabolic investigations in patients affected by intellectual disability of unknown origin.

Compliance with Ethics Guidelines

Conflict of Interest

Maria G Alessandri, Manuela Casarano, Ilaria Pezzini, Stefano Doccini, Claudia Nesti, Giovanni Cioni, and Roberta Battini declare that they have no conflict of interest.

Informed Consent

All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with Helsinki Declaration of 1975, as revised in 2000. Parents of the proband gave their informed consent to participate in the study.

Contributions of Individual Authors

Maria G Alessandri: biochemical analyses, concept and drafting of manuscript. Manuela Casarano, Roberta Battini: clinical data and drafting of manuscript. Ilaria Pezzini: performed mutation analysis. Stefano Doccini: performed protein and cellular studies. Claudia Nesti: directed molecular genetic studies. Giovanni Cioni: design and revision of manuscript.

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