Magnetic resonance spectroscopy and molecular studies in ornithine transcarbamylase deficiency novel mutation c.802A>G in exon 8 (p.Met268Val)

Spektroskopia rezonansu magnetycznego i badania molekularne w nowej mutacji powodującej niedobór transkarbamylazy ornitynowej c.802A>G w egzonie 8 (p.Met268Val)

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Abstract

Ornithine transcarbamylase (OTC) deficiency, an X-linked, semidominant disorder, is the most common inherited defect in ureagenesis, resulting in hyperammonaemia type II. The *OTC* gene, localised on chromosome X, has been mapped to band Xp21.1, proximate to the Duchenne muscular dystrophy (*DMD*) gene. More than 350 different mutations, including missense, nonsense, splice-site changes, small deletions or insertions and gross deletions, have been described so far. Almost all mutations in consensus splicing sites confer a neonatal phenotype. Most mutations in the *OTC* gene are 'private' and are distributed throughout the gene with a paucity of mutation in the sequence encoding the leader peptide (exon 1 and beginning of exon 2) and in exon 7. They have familial origin or occur *de novo*. Even with sequencing of the entire reading frame and exon/intron boundaries, only about 80% of the mutations are detected in patients with proven OTC deficiency. The remainder probably occur within the introns or in regulatory domains. The authors present a 4-year-old boy with the unreported missense mutation c.802A>G. The nucleotide transition leads to amino acid substitution Met to Val at codon 268 of the OTC protein.

Key words: novel mutation c.802A>G in exon 8, ornithine transcarbamylase deficiency, brain spectroscopy, children.

Streszczenie

Niedobór transkarbamylazy ornitynowej (OTC), dziedziczony w sposób sprzężony z chromosomem X, to najczęstsza jednostka chorobowa ureagenezy, stanowiąca hiperamonemię typu II. Gen *OTC* zlokalizowany jest w obrębie chromosomu X, zmapowany w rejonie Xp21.1, proksymalnie do genu dystrofii mięśniowej Duchenne'a (gen *DMD*). Do chwili obecnej opisano ponad 350 różnych mutacji typu zmiany sensu, braku sensu, zmiany ramki odczytu czy małych delecji lub insercji albo dużych delecji. Prawie wszystkie mutacje związane ze zmianą ramki odczytu wywołują fenotyp noworodkowy. Większość mutacji w genie *OTC* to mutacje prywatne z dystrybucją w obrębie całego genu oraz z brakiem mutacji w sekwencji kodującej główne białko (egzon 1 lub początek egzonu 2) i w egzonie 7. Mają one pochodzenie rodzinne lub pojawiają się *de novo*. Sekwencjonowanie całej ramki odczytu oraz granic egzon/intron pozwala na wykrycie 80% mutacji u pacjentów z rozpoznanym *OTC*. Pozostałe mutacje prawdopodobnie występują w obrębie intronów lub domen regulatorowych. Autorzy prezentują przypadek 4-letniego chłopca z nieopisaną mutacją typu zmiany sensu c.802A>G. Przemieszczenie nukleotydów prowadzi do zamiany metioniny na walinę w kodonie 268 białka OTC.

Słowa kluczowe: nowa mutacja c.802A>G w egzonie 8, deficyt transkarbamylazy ornitynowej, spektroskopia mózgu, dzieci.

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Introduction

Ornithine transcarbamylase (OTC) deficiency, an X-linked, semidominant disorder, is the most common inherited defect in ureagenesis, resulting in hyperammonaemia type II. Incidence of this metabolic disorder is estimated as 1 : 80 000 live births in the Japanese population [1,2]. The *OTC* gene, localised on chromosome X, has been mapped to band Xp21.1, proximate to the Duchenne muscular dystrophy (*DMD*) gene. More than 350 different mutations have been described so far [3]. Almost all mutations in consensus splicing sites confer a neonatal phenotype. Most mutations in the *OTC* gene are 'private' and are distributed throughout the gene with a paucity of mutation in the sequence encoding the leader peptide (exon 1 and beginning of exon 2) and in exon 7 [1,3-5]. They have familial origin or occur *de novo*. Even with sequencing of the entire reading frame and exon/intron boundaries, only about 80% of the mutations are detected in patients with proven OTC deficiency. The remainder probably occur within the introns or in regulatory domains [1,3].

The *OTC* gene is expressed in the liver and in the mucosa of the small intestine [1]. A deficit of this enzyme results in disturbed incorporation of ammonia into the cycle of transformations, leading to urea synthesis, which, consequently, results in hyperammonaemia. A part of the cycle of transformations takes place in the mitochondrial matrix; thus, any disorders of mitochondrial functions may reduce urea production.

Clinical symptoms of hyperammonaemia include, regardless of its cause, a lack of appetite, irritability, respiratory disorders (heavy or rapid breathing), vomiting, apathy, coma, somnolence, and cerebral oedema. The following symptoms are seen in the neonatal period with the onset usually on the second day of life: aversion to sucking, intermittent agitation and apathy, vomiting, tachypnoea, respiratory alkalosis, apnoea, convulsions and hypothermia, frequent intracranial haemorrhages, death of neonates without diagnosis in the medical history of the family [1]. The clinical picture of the patients is extremely variable. Male hemizygotes are usually severely affected during early childhood, while in heterozygotes the course of the disease may be milder or asymptomatic. About 15% of heterozygous females have life-threatening hyperammonaemic comas. Both symptomatic and asymptomatic carriers show increased orotic acid extraction, especially under the protein loading test [6].

The diagnosis of OTC deficit is based on identification of hyperammonaemia, in chromatographic partition of amino acids, hypocitrullinaemia and increased levels of glutamine, alanine and lysine, in the course of acute disorders, and increased excretion of orotic acid in urine is a characteristic feature [1,3]. The diagnosis is confirmed by measuring the enzyme activity in biopsy of the liver or the intestine coupled with molecular diagnostics.

In the differentiation process, the following factors should, among others, be taken into account: other primary hyperammonaemias and secondary ones – organic acidurias, disorders of fatty acid oxidation and mitochondrial cytopathies.

In this report we describe a 4-year-old patient with clinical manifestations of hyperammonaemia type II. The diagnosis was proved by biochemical and molecular tests that revealed a novel mutation of the *OTC* gene in the patient and his mother.

Case report

The case concerns a 4-year-old boy, a son of young, healthy and unrelated parents, a child from the first pregnancy, born on time by spontaneous delivery, with a birth weight of 2800 g, and Apgar score of 10 points. The family history includes death of an infant in the mother's family. The psychomotor development was normal. From the tenth month of life symptoms of decompensation reoccurred, with vomiting and behavioural disorders (with intermittent apathy or agitation), most often preceded by an infection of the upper respiratory tract with fever.

In additional examinations, performed in periods of metabolic decompensation, elevated activities of aminotransferases and creatinine kinase (CK) were found: AspAT (aspartate transaminase) 400 U/l; ALAT (alanine transaminase) 300 U/l; CK 600 U/l. In the tenth month of life, hepatitis was suspected but infection with HBV (hepatitis B virus), HCV (hepatitis C virus), CMV (cytomegalovirus) or *Toxoplasma gondii* was excluded.

At the age of 18 months, the patient was referred to diagnostics with suspected muscular dystrophy because of periodically occurring weakness of lower limbs and frequent falls. In physical examination no abnormalities were found, while neurological examination revealed muscular hypotonia with preserved tendon reflexes, and hypertrophy of calves. Developmental quotient (DQ) = 82 (assessed according to the Brunet-Lezine scale). In additional studies: CK 1830 U/l, serum lactate 3.5 mmol/l. Organic acids in urine by the GC/MS (gas chromatography mass spectrometry) method showed dicarboxy-

lic aciduria without ketosis. The results of other studies (blood cell counts, aminotransferases, glycaemia, ammonia, urea, creatinine, orotic acid in urine, serum aminogram, the profile of acylcarnitines) were normal.

Magnetic resonance imaging (MRI) of the head did not reveal abnormalities. Electromyography and ultrasonography of abdominal organs were normal.

In the 22nd month of life, in the course of an infection of the upper respiratory tract with fever, the child's condition rapidly deteriorated: vomiting was observed, with general weakness, and growing quantitative consciousness disturbances. In physical examination, an infection of the upper respiratory tract was confirmed. Neurological examination revealed generalised muscular hypotonia with maintained tendon reflexes. Additional examinations showed: ALAT 2046 U/l, AspAT 3789 U/l, CK 1271 U/l, lactic acid 4.2 mmol/l, ammonia in serum 146 mmol/l, serum aminogram – increased concentrations of alanine, asparagine and glutamate, citrulline concentration below detectability level, orotic acid in urine – 229 μg/μmol creatinine (control value < 3 μg/μmol of creatinine), organic acids in urine by the GC/MS method – orotic aciduria was found, associated with hyperammonaemia. In computed tomography (CT) of the head, calcifications were identified in subcortical nuclei. Electromyography was abnormal, with features of a myopathic pattern.

On the basis of the clinical picture and the results of performed studies, a suspicion of urea cycle disorders – hyperammonaemia type 2 – was postulated. The diagnostics were then extended towards OTC deficiency – a protein load test and a test with allopurinol, performed in the mother (for identification of heterozygotes), resulted in normal results. Subsequent episodes of general condition breakdown – metabolic crises, induced by infection of the upper respiratory tract – took place at the age of 2.5 and 3.5 years.

Magnetic resonance spectroscopy measurements

At the age of 4 years, nuclear magnetic resonance measurements were done using the whole-body 2T magnetic resonance imaging/magnetic resonance spectroscopy (MRI/MRS) system operating at a proton resonance frequency of 81.3 MHz. Four voxels of 1.5 × 1.5 × 1.5 cm^3 were localized using the PRESS (point resolved *in vivo* spectroscopy) sequence (TR 1500 ms, TE 35 and 136 ms and 100 acquisitions) on bilateral subcortical nuclei and bilateral dorsolateral prefrontal white matter regions. Water suppression was achieved with the help of CHESS (chemical shift selective) pulses. The spectra were normalized to 1 within the range 0.0-4.2 ppm. ^{1}H MRS (proton magnetic resonance spectroscopy) was done in a stable condition, when the child was treated with a protein restriction diet. During the last metabolic crisis the child was in a bad condition and anaesthesia was contraindicated.

The metabolite ratios were obtained using the user-independent method of spectra analysis [7,8]. N-acetylaspartate (NAA), choline (Cho), creatine and phosphocreatine (tCr), myo-inositol (mI), glucose (Glc), glutamine and glutamate (Glx), and lipids and lactate (LacLip) integral intensities were calculated.

Magnetic resonance spectroscopy showed a dec - reased value of the NAA/tCr ratio in all areas, and the Cho/NAA ratio was increased, which confirmed reduced NAA (a neuronal marker) (Fig. 1A). In all the spectra, there was an intensive strip, derived from lipids, lactate and macromolecules, which is reflected in the high values of the LacLip/tCr ratio (Fig. 1B).

The Cho/tCr values in the subcortical nuclei were normal; in the frontal white matter, however, the tCr signal was reduced, which implies artificially increased Cho/tCr ratios. The mI/tCr values were markedly reduced in the subcortical nuclei, whereas the corresponding values measured in the frontal lobes were normal, but when taking into account the lower integral intensity of the tCr signal in this area, the mI/tCr values seem to be overestimated (Figs. 1B and 2).

The Glx/tCr ratios exceeded the normal values in both the frontal lobes (Fig. 2); however, a reduction of the tCr peak may be of importance.

Another remarkable finding was the increase of the broad peak intensities at 0.8-1.5 ppm in the frontal lobes spectra, which can be assigned to the lipid/lactate complex. Although it is difficult to differentiate lipid with lactate clearly in the short echo-time proton MR spectra, most of the broad peak intensities at 0.8-1.5 ppm may be contributed from increased mobile lipids within the investigated regions.

Molecular analysis

To confirm the clinical diagnosis of hyperammonaemia type II, molecular analysis of the *OTC* gene was performed in the patient and his mother. Genomic DNA was isolated from peripheral blood samples and 10 coding exons of the *OTC* gene were screened for mutation using the PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) and sequencing analysis.

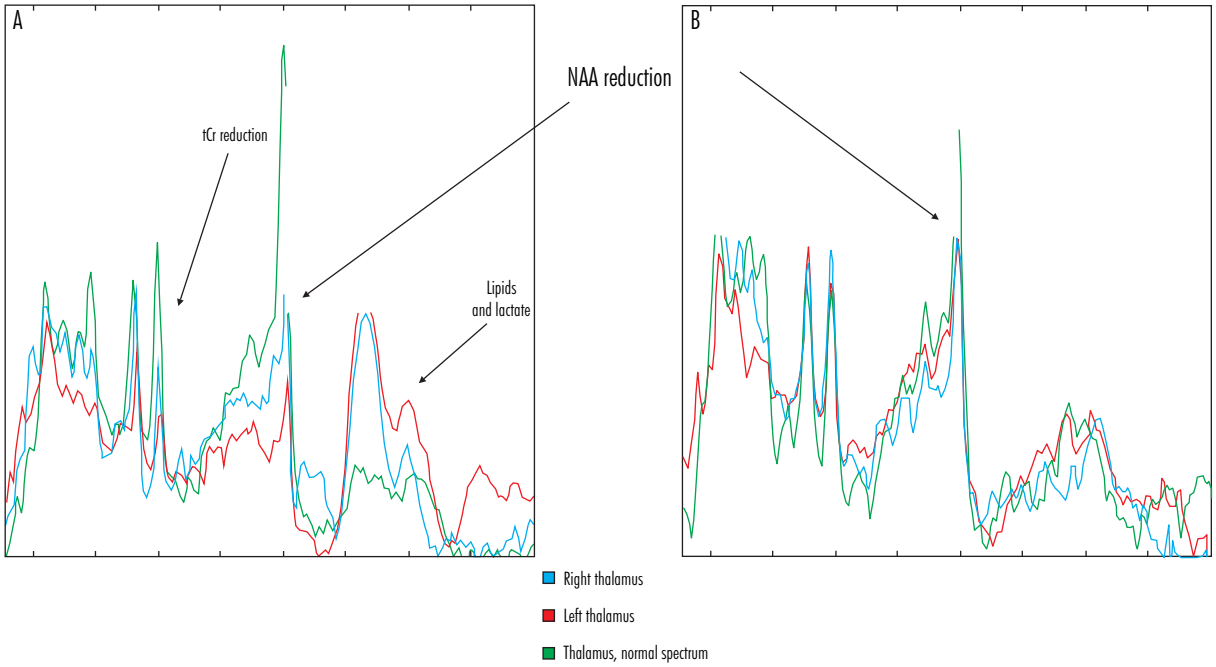

Fig. 1. Comparison of the ^{1}H MRS (TE = 35 ms) spectra acquired from the patient's frontal lobes and thalamus, and the spectra obtained from the respective localizations in the healthy patient at a similar age

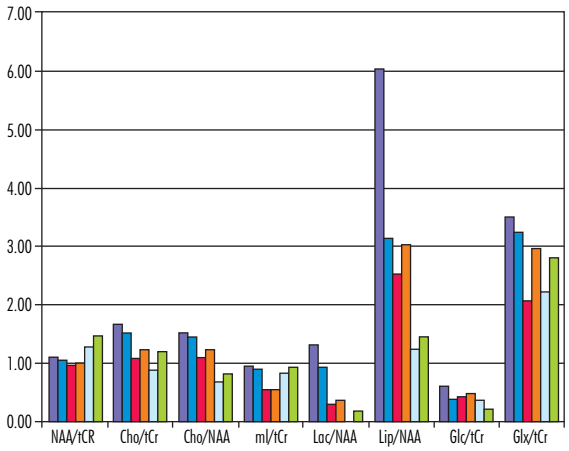

Fig. 2. The Glx/tCr ratios exceeded the normal values in both the frontal lobes. According to order of appearance: left frontal lobe, right frontal lobe; left thalamus, right thalamus, frontal lobes (normal), thalamus (normal)

The sequence of the shifted fragments (exon 8) was compared with the *OTC* cDNA sequence (Gene Bank Ref-Seq: NM_000531) and OTC protein sequence (Gene Bank NP_000522).

The patient and his mother were shown to carry a heretofore unreported missense mutation, c.802A>G. This nucleotide transition leads to amino acid substitution Met to Val at codon 268 of the OTC protein (Fig. 3). Based on the protein sequence alignment, Met268 appears to be the most conserved amino acid located in protein region SMG, having 100% homology in all studied organisms, and is thought to be critical for enzyme activity [8]. The c.802A>G mutation was not detected in 40 control DNA samples, indicating that this allele is not a common polymorphism (data not shown).

Observation of muscular hypotonia and a high CK value prompted us to perform molecular analysis of the *DMD* gene. No deletion in any fragment of the gene was found. However, presence of a point mutation was not excluded.

Discussion

Ornithine transcarbamylase deficiency is one of the most frequent causes of genetically determined liver encephalopathy. It is assumed that in liver encephalopathy, the blood-brain barrier is injured, increasing its permeability not only to ammonia, but also to aromatic amino acids and to γ-aminobutyric acid (GABA) [9,10]. The cause of increased permeability has not yet been completely explained; it is assumed that it may be associated with impaired transporting mechanisms of capil-

lary vessels, especially those acting in pinocytosis of astrocytes.

Ammonia is the main substance with neurotoxic activity; however, methionine, mercaptans, β-hydroxyl derivatives of biogenic amines and short-chain fatty acids may also take part in the pathogenesis of hepatic encephalopathy [9]. It is supposed that these substances, by disturbing the activity of membrane ATPase (adenosine triphosphate), damage the membrane transport of neurons and their energetic metabolism. Ammonia disturbs mainly the metabolism of glutaminic acid by suppressing the activity of glutamine synthetase. Both these processes, combined with the abnormal metabolism of glucose, lead to energetic disorders in the neurons.

The increased permeability of the blood-brain barrier to aromatic amino acids, caused by hyperammonaemia, changes their proportion in the CNS to the amino acids with branched chains, causing an intensification of the synthesis of the so-called false neurotransmitters (tyramine, octopamine and phenylethanolamine) [10].

Another significant mechanism considered in the pathogenesis of liver encephalopathy is the blockade of the benzodiazepine receptor by γ-aminobutyric acid, which disturbs the balance between the systems of GABAergic and glutaminergic transmitters [9,10].

The results of liver tests and ammonia concentration do not always correspond with the degree of liver encephalopathy. MRI imaging, performed in the initial stages of the disease, may not show any abnormalities in brain structures. In such a case MRS, as a sensitive diagnostic tool providing insight into the biochemistry of the brain, may be especially useful. In MRS examinations of patients with liver encephalopathy a triad of symptoms is usually found: increased concentration of glutamine/glutaminic acid, decreased level of myo-inositol and choline and, initially, normal concentrations of NAA. In the studied case only one of the mentioned typical spectral characteristics is present – the reduced mI/tCr ratio. Additional findings are reduced NAA and the increased lipid/lactate complex at 0.8-1.5 ppm deriving from increased mobile lipids within the investigated regions. It seems unlikely that this latter finding is caused by fat contamination from the scalp or bone marrow because it was observed even in the spectra obtained from the subcortical nuclei, where the volume of interest was far from the scalp or bone marrow fat. We speculate that the increase of mobile lipids may be caused by neuroglial cell membrane damage or disintegration due to severe metabolic stress, and may be a sign foretelling brain damage.

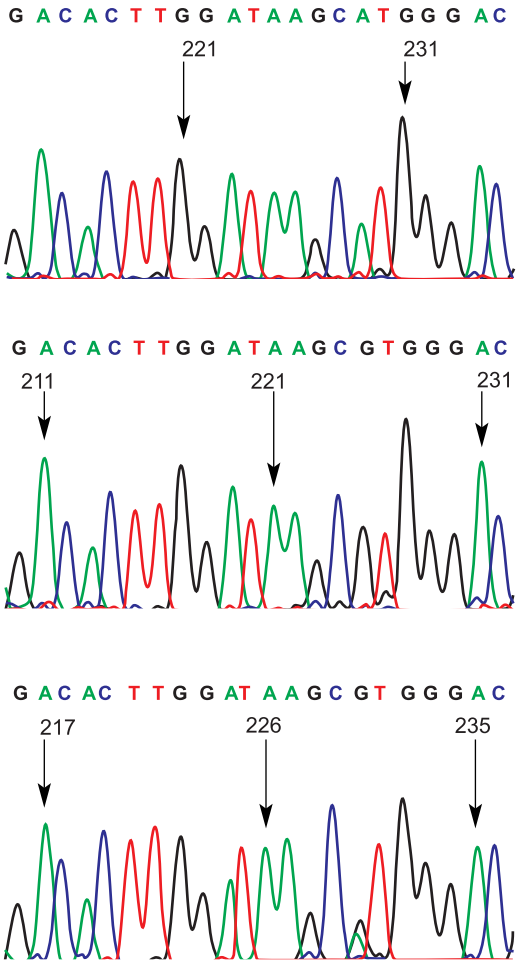

Fig. 3. Sequencing of the *OTC* gene showing the c.802A>G (p.Met268Val) mutation in exon 8 in the patient and his mother

A reduction of the cerebral NAA concentration is commonly understood as a reduction of neuroaxonal cellular density and/or dysfunction. In this study NAA loss is especially marked in the frontal lobes, indicating neuronal/axonal damage. The observed total creatine pool loss seen in the frontal lobe white matter is another indicator of cellular disintegration. Total creatine is often used as an internal standard assuming that its level is constant in the brain. However, the creatine levels can vary according to the nature of clinical diseases, and the lowering of the tCr signal may represent a cerebral energy deficit [1].

Under the effect of increased ammonia concentration in the brain, the synthesis of glutamine in astrocytes is usually enhanced as well as glutamine storage [9]. These are the only cells in the central nervous system (CNS) with glutamine synthesis ability, this reaction

being the chief means of ammonia elimination from the nervous system. One may speculate that the increased glutamine accumulation triggers a compensatory decrease in myo-inositol since both these compounds exert an osmoregulatory function. Such an effect has been described for cultured astrocytes [10].

However, the Glx levels seem to be normal in this study (only an artificial increase due to the total creatine loss is observed within the frontal lobes). Since myo-inositol is considered to be an astrocyte marker, a dec - reased myo-inositol level may alternatively indicate a lower number of astrocytes rather than a compensation for glutamine increase [11]. In the case of a drastic reduction of the number of astrocytes vs. neurons, a significant decrease in creatine should be expected and, in fact, its signal at 3.03 ppm is drastically low in the frontal white matter MRS spectra of the studied subject. No correlation was found between the level of myo-inositol and the degree of hepatic encephalopathy; thus it is suggested that other, osmotically active substances, such as taurine and betaine, may play some role in the process as well. Changes in the myo-inositol and choline content and often normal level of NAA in patients with liver encephalopathy indicate that this pathology is, first of all, a result of oedema and disturbed function of astrocytes without any damage to neurons.

The outcomes of MRS studies confirm the theories that, in liver encephalopathy, there are disorders in neurotransmission, especially at the level of the junction of glial cells and neurons. The observed changes in choline concentration have not yet been explained. It is suggested that they may result from disorders of transport, metabolism or absorption from the gastrointestinal tract. The increased perfusion of basal ganglia makes them more susceptible to the effects of metabolic disorders. In neuroimaging studies, hyperintensity, especially of the globus pallidus, was observed.

In liver encephalopathy, the cerebral metabolism of glucose is significantly disturbed, together with increased lactate concentration, which results from decreased oxidation of pyruvate and enhanced synthesis from glucose. The increased concentration of lactate in the CNS causes an increase in intracranial pressure.

One of the hypotheses assumes that liver encephalopathy may be induced by disturbed function of astrocytes, resulting from changes of expression of genes encoding for proteins, the changes caused by hyperammonaemia. It further results in transport disorders and elimination of glutaminic acid, glycine, glucose and monoamines.

The most important issue in this case report is that the patient escaped from the diagnostics for such a long time. Despite the fact that previous episodes suggested metabolic dysfunction of the CNS, the first abnormal ammonia level was confirmed when the child was almost two years old. Probably the former measurements were within the normal range because during the crises the child received intravenous replacement of fluids in the local hospitals. After the recommendation of taking a blood sample before the intravenous treatment, hyperammonaemia was observed.

The identified substitution c.802A>G in exon 8 (p.Met268Val) is a novel mutation, being one of over 350 different mutations in the *OTC* gene reported to date. The mutation results in replacement of methionine 268 by a valine residue. According to Tuchman *et al*. [8] exon 8 contains many conserved nucleotides (particularly in codons 263, 267 and 268), which code the amino acids forming the enzyme active site. The mutation identified in the Polish family is therefore highly likely to be disease causing. The pathogenic, not polymorphic character of the mutations is firmly supported by the absence of p.Met268Val substitution in any of 80 families with OTC deficiency and in 20 control females.

The substitution p.Met268Val, like the described mutation Met268Thr in the same amino acid position, produces a mild phenotype of OTC deficiency termed 'late-onset' hyperammonaemia. Morizono *et al.* [12] analysed some patients with mild disease phenotype and the mutation Arg277Trp, located in exon 8 in a 9 amino acid residue loop remote from the active site. Compared to the wild type, Arg277Trp mutant protein had nearly 70-fold lower affinity for L-ornithine, one of two substrates of OTC enzyme, and characterized by reduced thermal stability. It may be predicted that mutated protein with Met268Val substitution will also present reduced affinity for L-ornithine and lower activity of the OTC enzyme.

Molecular analysis in the asymptomatic proband's mother revealed the presence of the same nucleotide substitution c.802A>G on one *OTC* gene allele. Despite this, an allopurinol loading test showed no elevated orotic acid level and resulted in no identification of the disease-carrier status of the woman. Our observation of the normal orotic acid level in the allopurinol loading test in the patient's mother, along with the identified *OTC* gene mutation, supported the probability of undefined or false negative results reported previously for heterozygous females [13-17]. Direct mutation analysis of the *OTC* gene is therefore important for providing accura-

te carrier detection and prenatal diagnosis in OTC deficiency families.

Disclosure

Authors report no conflict of interest.

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