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GENOTYPE AND NATURAL HISTORY IN UNRELATED INDIVIDUALS WITH PHENYLKETONURIA AND AUTISTIC BEHAVIOR

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ABSTRACT - We describe three unrelated individuals, two males (ages 35 and 9) and a female (age 8) presenting with late diagnosed phenylketonuria (PKU) and autistic behavior, all showing poor adherence to the dietary treatment resulting in high plasmatic phenylalanine levels, particularly in the oldest subject. Clinical findings included hair hypopigmentation, microcephaly, severe mental retardation with absent development of verbal language and autistic symptoms in all three patients, whereas variable neurological signs such as seizures, spasticity, ataxia, aggressivity, and hyperactivity were individually found. Homozygosity for the IVS10nt11g/a (IVS10nt546) was found in all. This is the first report of molecular findings in subjects with PKU also presenting with autistic features. The authors discuss if this mutation is particularly involved in the association of autistic symptoms in untreated PKU individuals.

KEY WORDS: autism, natural history, pervasive developmental disorders, phenylalanine hydroxylase, phenylketonuria.

Genótipo e história natural em indivíduos não aparentados com fenilcetonúria e comportamento autístico

RESUMO - Descrevemos três indivíduos não aparentados, dois do sexo masculino (com idades de 35 e nove anos) e um do sexo feminino (com idade de oito anos) apresentando fenilcetonúria diagnosticada tardiamente e comportamento autístico, todos com adesão limitada ao tratamento dietético resultando em altos níveis plasmáticos de fenilalanina, especialmente no indivíduo mais velho. Os achados clínicos incluem hipopigmentação de cabelos, retardo mental grave com ausência de desenvolvimento da linguagem verbal e sintomas autísticos nos três pacientes, enquanto outros achados neurológicos como convulsões, espasticidade, ataxia, agressividade e hiperatividade são descritos em um indivíduo, cada. Homozigose para a mutação IVS10nt11g/a (IVS10nt546) foi encontrada em todos. Este é o primeiro relato de achados moleculares em indivíduos com fenilcetonúria que desenvolveram características autísticas. Discute-se se essa mutação estaria particularmente envolvida no desenvolvimento de sintomas autísticos em indivíduos com fenilcetonúria não tratada.

PALAVRAS-CHAVE: autismo, história natural, distúrbios invasivos do desenvolvimento, hidroxilase de fenilalanina, fenilcetonúria.

Autism is a behavioral syndrome with still poorly understood etiologies. In most cases, it is probably caused by multifactorial inheritance that means the association of genetic factors to environmental factors. On the other hand, in 10 to 20% of all cases a specific cause can be determined, including chromosomal abnormalities¹, metabolic disorders², monogenic conditions like tuberous sclerosis, fragile X syn-

drome or other entities, as well as "pure" environmental factors³.

Concerning the metabolic disorders, phenylketonuria (PKU) was frequently associated with autistic symptoms. This association has almost vanished since the introduction of early detection and treatment for phenylalanine hydroxylase (PAH) deficiency. However, even in countries where newborn screen-

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ing is available, one may miss the diagnosis for several reasons, including births outside hospitals, too early screening, samples mismatches or false negative results⁴. Thus, phenylketonuric individuals presenting with neurological impairments including autistic behavior still occur.

To date, no information on the molecular basis has been reported in individuals with autistic symptoms due to PKU. The present study describes clinical and molecular findings in three unrelated patients with this association.

METHOD

Three unrelated individuals were evaluated. The first patient was referred with a previous diagnosis of PKU while autistic symptoms were diagnosed during clinical assessment. The remaining two patients were enrolled in a more comprehensive study on 84 individuals with pervasive developmental disorders⁵; PKU was previously known in one subject and was diagnosed by laboratory tests in the other.

Informed consent for the clinical and molecular study was obtained from parents in all three cases after approval of the Ethics Review Board, FCM/Unicamp.

Individuals and close relatives underwent molecular analysis of the PAH gene in the following protocol: DNA was isolated from leukocytes of each subject by standard methods⁶ and analyzed for mutations by PCR amplification of the 13 segments comprising the entire coding sequence, splicing functions and the promoter region of the PAH gene, followed by DGGE⁷ and sequencing⁸. When DGGE analysis of exon 11 of the PAH gene revealed an abnormal pattern for the patients suggesting the IVS10nt-11g/a (IVS10nt546) mutation, gene sequencing was also performed to confirm this finding.

Clinical reports

Patient 1 – Male, 35 years old, 10th child of consanguineous parents (first-cousins) of Portuguese origin and proceeding from the State of Paraná in South Brazil. An oldest sister died at the age of two years by a “mental disorder”. The family history is otherwise unremarkable. Pregnancy was uncomplicated. Delivery was at home, with no medical assistance. At the time of his birth, in the 1960's, there was no neonatal metabolic screening in Brazil. His neuromotor development was globally delayed and at the age of four he was diagnosed as having PKU by Guthrie bacterial inhibition assays in urinary samples. The family was then oriented to follow dietary treatment with low protein intake, which was performed for three years. Since there was no improvement of the neurological impairment, the diet was interrupted. There was psychomotor delay with no language development and he could never be toilet trained. At the age of 12 he started to present auto-aggressivity. There was no history of seizures.

In his first clinical assessment at the age of 35, his stature was 168 cm (25th centile), weight 58 kg (25th centile), and OFC 53 cm (3rd centile). He was not collaborative for a

detailed evaluation but there were no relevant dysmorphic features, except for an extensive pilous nevus on the dorsum and left arm. His hair and beard were red, different from the dark haired familial pattern. There were multiple scars over the scalp caused by repetitive head hitting against the wall and callosities over the dorsum of the hands from self-biting. He did not collaborate with neurological examination but he was noted to present spasticity in the upper members. He avoided eye contact and did not use verbal or gesture language, most of the time presenting body-shaking and worm-like movements of the fingers, as well as a frequent unintelligible mumbling or “singing”. There was a significant impairment in social interaction with other family members and strangers manifested as failure in developing peer relationships and lack of emotional reciprocity, becoming auto and hetero-aggressive every time someone approached him. There was also adherence to alimentary routines and inflexibility in dealing with environmental changes. Thus, he fulfilled DSM-IV criteria for autism.

A first plasmatic phenylalanine measure resulted 48.5 mg% for a reference value of up to 4 mg%. Dietary treatment was reintroduced with the assistance of a nutritionist. The family reported some improvement in behavior, aggressivity and sleep. Unfortunately the patient manifested high resistance to this diet resulting in a weight lost of 3 kg in a few weeks. The plasmatic phenylalanine levels never fall below 17 mg% during this time. Less than one year after reintroducing treatment, the family decided to definitely interrupt it.

Chromosomal analysis in lymphocytes with G-banding technique and a resolution of 400 bands resulted in a 46,XY normal male constitution. A brain single photon emission computed tomography (brain SPECT) with Tc^{99m}, which revealed heterogeneous distribution of the radiopharmaceutical in the whole cerebral cortex, thus suggesting diffuse brain damage.

Molecular analysis of the PAH gene revealed homozygosity for the IVS10nt-11g/a mutation. The father and one sister were heterozygous for the same mutation and two unaffected sisters were heterozygous for the L385L substitution. The mother was deceased and no molecular test could be performed but most probably she was a double heterozygous for the IVS10nt-11g/a mutation and the L385L polymorphism.

Patient 2 – Male, nine years old, the first child of a couple of Portuguese and Amerindian ancestry proceeding from the State of Paraíba in Northeastern Brazil. The parents denied consanguinity but they were both born in a small village with 10,000 inhabitants according to the Brazilian populational census in 2000. The family history is otherwise unremarkable. Pregnancy and delivery were uncomplicated and he was born at term, weighting 2.8 kg and measuring 47 cm. Neonatal screening was not performed. His development was globally delayed and he never acquired verbal language. At the age of five, he started to present tonic-clonic seizures with good response to phenobarbital. Besides severe impairment of verbal and non-verbal skills, he avoided physical contact with strangers,

had no adequate peer relationships, used to treat persons as objects, and had abnormal interest on bicycle wheels, water and puzzles small pieces. He also presented hand and head shaking stereotyped movements. At the age of six, he was sent to a school for autistic children.

When the youngest sister was born, neonatal screening was performed and a diagnosis of hyperphenylalaninemia was established. She was adequately managed since then and presents normal somatic and neurological developments. Because of her neonatal screening, a diagnosis of PKU was investigated and confirmed in patient 2 and dietary treatment was introduced at the age of five with some behavioral improvement. Despite that, the family was unable to keep a rigid diet and at the age of nine he showed high phenylalanine plasmatic levels, with values around 18 mg% in several consecutive measurements.

At the first clinical evaluation, he presented normal weight and stature with an OFC of 50 cm (3rd centile). There were no relevant dysmorphic features. The patient presented brown hair, contrasting with the dark haired family pattern. Neurological assessment revealed intermittent eye contact, inadequate answers to simple questions, hyperactivity and stereotyped movements like hand flapping and head shaking. There was no abnormal muscular tonus or other neurological signs. He fulfilled DSM-IV criteria for typical autistic disorder.

Other complementary tests included chromosomal analysis in lymphocytes with G-banding technique and a resolution of 400 bands (46,XY), molecular analysis for the FRAXA, FRAXE and FRAXF mutations (negative) and a brain computed tomography which revealed widening of the lateral ventricles, suggesting mild *ex vacuo* hydrocephaly.

Molecular analysis of the PAH gene revealed homozygosity for the IVS10nt-11g/a mutation in the patient and the younger sister. Parents were heterozygous for the same mutation and the unaffected sister showed homozygosity for the normal allele.

Patient 3 – Female, eight years old, the second child of a consanguineous couple (first cousins) of Portuguese origin proceeding from the State of São Paulo in Southeastern Brazil. Pregnancy was uncomplicated. Delivery was at term, weighting 3.3 kg and measuring 49 cm. Apgar scores were 9 and 10. Neonatal screening was not performed. Development was globally delayed and she never acquired verbal language. At the age of 15 months, she presented myoclonic seizures with spontaneous resolution. She attends a school for autistic children since the second year of life.

At the first clinical assessment, she presented normal weight and stature with an OFC of 49 cm (3rd centile). Dysmorphicologic evaluation revealed thin habitus, prominent upper central incisors and mild cubitus valgus, which were present in the mother and considered familial features without other clinical significance. She also presented brown hair, different from the dark haired family pattern. The neurological evaluation revealed hyperactivity, ataxic gait, dysmetria, intention tremors of the upper extremities and flapping. She did not interact with other children and presented rigid routines and abnormal interest for rotatory

objects such as wheels or blenders. She fulfilled DSM-IV criteria for typical autistic disorder.

During the laboratory investigation, an amino acid chromatography revealed increased phenylalanine in urine samples. A plasmatic phenylalanine measure at this age resulted 19.6 mg%. Dietary treatment was introduced with some behavioral improvement after a few months.

Other complementary tests included chromosomal analysis in lymphocytes with G-banding technique and a resolution of 400 bands (46,XX), molecular analysis for the FRAXA, FRAXE and FRAXF mutations (negative), EEG (diffuse epileptic activity in both cerebral hemispheres) and a brain SPECT (normal). Molecular analysis of the PAH gene revealed homozygosity for the IVS10nt-11g/a mutation.

DISCUSSION

Since the description of PKU in 1934 by Fölling⁹, several authors, mainly from the earlier medical literature, reported individuals with autism and PKU¹⁰⁻¹⁷. This issue has been previously reviewed^{10,18}. Most authors did not specify diagnostic criteria for autism and to date no information on the PAH mutation(s) has been provided in such cases. In this report, the IVS10nt11g/a mutation was found in three unrelated individuals. It consists in a single base substitution that creates a novel splice site in intron 10, causing aberrant splicing and the addition of nine nucleotides corresponding to three extra amino acids (Gly-Leu-Gln) between the normal sentences of intron 10 and intron 11. This change is incompatible with the structural maintenance of architecture of the PAH protein which can be detected in normal amounts but shows no activity. The resulting phenotype is of severe phenylalanine intolerance and classical PKU¹⁹.

The IVS10nt11g/a is the most common molecular defect of the PAH gene causing PKU in Mediterranean populations. It seems to be originated in the Turkey-Israel area and introduced in Italy and in the Iberian Peninsula due to gene flow²⁰. The migratory events from the 16th century introduced the mutation in Brazil²¹, as well as in Chile, Argentina, and Mexico²². The L385L polymorphism, detected in family 1, is also common in the Portuguese population²³. In Brazil, gene frequencies for the PAH mutations varies in each State and region, also as a consequence of the migratory flow. For this reason, the IVS10nt 11g/a is rare (2.4% of the allele frequency) in Southern Brazil²¹, where a significant part of the population descends from immigrants of Central European countries. On the other hand, this mutation is the most prevalent (17.4%) in the State of São Paulo⁸, where the influence of Portuguese, Spanish, Greek, Lebanese, Turkish, and Jewish immigrants is great.

An allele frequency of up to 20% for this mutation was also found in the Northeastern Brazilian population²⁴, where Portuguese and Afro-Brazilians represent the most frequent ethnic background of its population.

It is interesting to note that many individuals with untreated PKU who developed autism came from the Mediterranean area and countries that received immigrants from this region: Italy^{14,17}, Tunisia¹⁵, and Venezuela¹⁶, although molecular findings were not reported by these authors. This raises the possibility that a few or even one specific mutation could be associated with autism in individuals with PKU. Our results suggest that the IVS10nt-11g/a could be this mutation or at least one of them.

A confinable percentage of patients with PKU having autistic symptomatology is difficult to determine, specially after introduction of treatment. In 1975, Knobloch and Pasamanick²⁵ found 14 (21.8%) phenylketonuric individuals among 64 patients with autism diagnosed by Kanner criteria, which may be an overestimation. More recently, we found two subjects with PKU (present patients 2 and 3) in a sample of 84 individuals with pervasive developmental disorders diagnosed by DSM-IV criteria, giving a frequency of 2.3% in the total sample comprising all PDDs or 2.7% if Asperger syndrome was excluded and only autism, either typical or atypical, was considered⁵. On the other way, Baieli et al.¹⁷ reported 35 individuals with classic PKU late diagnosed, all with mental retardation and two (5.7%) presenting autism based on the Autistic Diagnostic Interview Revised (ADI-R) and the Childhood Autism Rating Scale (CARS) criteria.

In conclusion, the present study reports three unrelated individuals presenting with PKU and autistic features diagnosed by DSM-IV criteria. It is the first work to describe the molecular findings in such cases. All three individuals were homozygous for the IVS10nt-11g/a raising the question whether this mutation could be associated with the development of autistic symptoms in untreated individuals.

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