대한 유전성 대사질환 : 제 2 권 제 1 호, pp72~76, 2002

Late-onset Ornithine Transcarbamylase Deficiency in Male Patients : Spectrum of Clinical Presentations, Molecular Pathology and Molecular Epidemiology

Makoto Yoshino, M.D.

Department of Pediatrics & Child Health, Kurume University School of Medicine

Introduction

Ornithine transcarbamylase(OTC) is the enzyme that catalyses citrulline synthesis from carbamylphosphate and ornithine in the sequential reactions of ureagenesis, known as the urea cycle or Krebs-Henseleit cycle that exists exclusively in the liver. This enzyme is located in the mitochondrial matrix. Congenital deficiency of this enzyme(OTCD) causes hyperammonemia and increased pyrimidine synthesis, as indicated by increase in excretion of orotic acid and uracil.

Early studies on families with this disease suggested that this disease was inherited in an X-linked dominant manner(Scott et al., 1972; Short et al., 1973, Campbell et al., 1973).

Later on, Lindgren et al.(1984) mapped the locus of this enzyme to Xq21.1 by a cytogenetic technique. Thus, it was established that this disease is an X-linked trait.

The structure of cDNA(Horwich et al, 1984) and genomic arrangement(Hata et al, 1988) of human OTC gene are now determined. The human OTC gene consists of ten exons and nine introns, encoding for a 36 kDa precursor protein, or pOTC.

The first 32 amino acid residues from the N terminus(signal peptide) of pOTC is cleaved to form mature OTC(mOTC) after it is transported across the inner mitochondrial membrane with the aid of some molecular chaperons. The mOTC associates to form homotrimer and the trimer exhibits full enzyme activity.

There is a significant difference in severity of this disease and age at onset between male and female patients, and among female patients. In hemizygous male patients, the severity of the disease is determined almost exclusively by the mutational nature of the mutant allele. While in female individuals carrying a mutant OTC allele, it is also affected by the pattern of X-inactivation. The OTC gene, located in Xp21.1, is subjected to X-inactivation. Consequently, the liver of a female individual carrying a mutant OTC gene consists two heterogeneous groups of hepatocytes, one expressing the wild type allele and the other, expressing the mutant allele. Thus, severity of the disease can be highly variable in female individuals carrying a mutant OTC gene, depending on the proportion of the two different populations of hepatocytes, in addition to the nature of the mutant gene.

Thus, it was a common description that those male OTCD patients almost invariably developed hyperammonemic crisis in the neonatal period or early infancy and died. We now designate such clinical phenotype "classical" presentation.

In 1990, we reported three male patients with OTCD whose first episode of hyperammonemia

was delayed until late adolescence or adulthood (Yoshino et al, 1990). We then proposed that these patients constituted a clinical entity separate from male patients with classical presentation, or so-called "late-onset" presentation in which the onset of the disease was in preschool-age(Finkelstein et al, 1990).

Clinical Phenotype

Here we describe an index patient - patient 1. This 58-year old man was admitted to Kurume University Hospital because of progressive disturbance of consciousness. Past history was noncontributory except that he ate bean curd("tofu") every day. He had been well until 56 years of age, when he began to have episodes of nausea and malaise. Seven days prior to admission, he developed abnormal speech, unintentional strolling and agitation. One day before admission he developed sudden depression in consciousness level.

Laboratory studies on admission revealed massive hyperammonemia(2,377 μM), slightly increased glutamine concentration(1,045 μM) and decreased citrulline concentration(8 μM) in plasma and marked orotic aciduria(663 mmol/mol creatinine, normal control range, <1.5). These results suggested a defect in citrullinogenesis.

Biochemical Characteristics of the Mutant OTC

The patient died two days after admission. Activity of OTC in liver tissue obtained by autopsy decreased to 1-2% of the normal control mean value, while carbamylphosphate synthetase-I (CPS-I) activity was not reduced. Analysis of OTC protein by immunoblot revealed a faintly stained band with a molecular weight that was indistinguishable from that of wild OTC.

We then had an opportunity to examine the enzymological characteristics of OTC of another patient, patient 4, who was later proved to carry R40H mutation. The Vmax was reduced to less than 5% of control mean value. The Km values for L-ornithine and carbamylphosphate in this patient were both very similar to those of wild-type control. pH optimum was between 7.8-7.9, which was again indistinguishable from control values. We were thus unable to determine whether or not these patients had inherited OTCD.

Tracking Heterozygotes in Families

To answer whether or not the OTCD in these patients was inherited, we employed protein loading test to identify possible heterozygotes and hemizygotes in family members of the patients, because technology of human gene analysis was not yet available at this time.

Family members were fed 1 g/kg of protein and followed for plasma ammonia level and orotic acid concentration in urine. In the family of patient 1, a female sibling of the propositus disclosed a mild but significant increase in orotic acid in urine, indicating heterozygosity. An elder brother of the propositus had died at the age 48 years from a disease what was reported to be "fulminant hepatitis". It was later found that the son of a daughter of this male sibling was found to have OTCD. These observations indicated that the OTCD in the propositus was inherited in a manner consistent with an X-linked dominant mode, and the mutant gene could be transmitted through both maternal and paternal side. This was the first to show that mutant OTC gene can be transmitted from male patient to his daughter. Similar results were obtained in other families of patients 2 and 3.

In the mean time, along with development of molecular techniques, analysis of human OTC gene became available. We then analyzed OTC

gene in the first three patients. In patient 1, a G to C transversion at nucleotide position 119, altering arginine to histidine in the codon 40, or R40H mutation, was found(Nishiyori et al, 1997). In the second patient, a G to A transition at position 166 was found. This transition caused tyrosine to asparagine in codon 55, termed Y55D mutation (Nishiyori et al, 1998).

We then tested by in vitro expression experiments whether or not these mutations identified in these patients were of etiological significance. Wild type OTC cDNA or cDNA harboring either R40H or Y55D mutations was ligated into an expression vector, pCAGGS, driven by chicken beta action promoter. The plasmid was cotransfected with a plasmid bearing bacterial beta galactosidase gene into Cos I cells by lipofection. The cells were harvested after 72 hours of incubation.

The RNA blot analysis of the transfected Cos I cells with wild-type and the mutant human OTC cDNA's indicated that levels of mRNA of both R40H and Y55D were very similar to that of wild-type, indicating that these mutant mRNA's were as stable as the wild-type mRNA.

During this experiment, a liver specimen from a patient carrying R40H mutation obtained by biopsy was referred to us. We then examined mRNA level in this tissue, as well as control tissue obtained by autopsy. The mRNA level in the patient was again indistinguishable from that of control.

The enzyme activity of wild-type OTC expressed in Cos I cells transfected by the wild-type cDNA was 30 fold that of mock transfected cells, when normalized to beta galactosidase activity. The activity levels of R40H and Y55D OTC's, as normalized for the beta galactosidase activity, were 33% and 28% of the wild type OTC activity, respectively. The levels of R40H and Y55D OTC's in the transfected cells, when determined by immunoblot analysis, were both decreased. These results, along with the RNA blot data, indicate that R40H and Y55D OTC genes were transcribed and spliced at an efficiency similar for the wild-type OTC, and that the mutant R40H mRNA was as stable as wild-type in vivo.

The wild-type and the two mutant OTC's expressed in Cos I cells were subjected to 5 cycles of freezing and thawing to examine physical stability of the enzymes. The wild-type OTC was barely affected by this treatment, whereas R40H OTC was deceased to 6% level of pre-treatment activity, indicating physical instability of R40H OTC. While activity of Y55D OTC was not affected by this treatment, and mechanism of deficiency of the mutant OTC remained unsolved.

Later in 2001, Mavinakere and his colleagues showed that R40H pOTC tends to be degraded in the cytosol before the preprotein is imported into mitochondria. Our previous data, taking their results together, show that transcription, splicing and translation of R40H gene is comparable to that of the wild-type gene, but the mutant pOTC was more prone to be degraded before it was transported into mitochondria. The R40H OTC was still capable of forming homotrimer, a form that exhibited full activity of the enzyme, once it was transported into mitochondria.

Molecular Family Tracking

While we were processing in vitro studies, a specimen from a 6 year-old boy who developed hyperammonemia was referred to us for genetic diagnosis. Scrutiny of family tree of this boy revealed that this boy was related with patient 1. This boy was a son of a daughter born to a male sibling of patient 1. This male sibling died at the age of 48 years from what was reported to be fulminant hepatitis. Thus, we had a rare opportunity to perform gene tracking in family of pa-

tient 1. R40H mutation creates a novel *Nla*III site. By the use of this RFLP, we determined that the boy carried R40H mutation. Thus, transmission of a mutant OTC gene through not only maternal side but also through paternal side was demonstrated. This is the first demonstration that a mutant OTC can be transmitted paternally.

Variability of Clinical Presentation

We identified nine patients carrying R40H mutation to date, each of them is not related, with the exception of patient 1 and the related 6 year-old boy. This table summarizes clinical characteristics of the patients. There is significant variation in clinical features among the patients. The age at onset of the disease has been variable, ranging from 6 years to 58 years. The reasons for this variation have remained unanswered.

Variation of protein intake can be a candidate for factors that affect the clinical phenotype. Matsuda and his colleague found a general tendency in male individuals who carried R40H mutation that the earlier was the date of birth of patient, the younger was the age at onset of the disease. Statistical survey showed that average amount of protein intake has been increasing yearly since 1950th in Japan. This could have partly attributed to the onset of the disease at a younger age in patients in the later generation. Further search for additional factors that would affect the phenotype is necessary.

Molecular Epidemiology

The R40H mutation observed in our independent families may be the product of recurrent mutation, because the nucleotide resides in a CpG island, a mutational hot spot. Indeed, this mutation has been reported in other ethnic groups. However, it is possible that the mutant allele tends to be retained in a limited geographic area because it can be transmitted through not only maternal side but through paternal side.

The residential locations of patients carrying R40H mutation were scattered in an area with a radius of 100 km in the northern part of Kyushu Island. This observation suggests that the mutant allele has accumulated as a consequence of founder effect in this area.

The prognosis of patients carrying the R40H mutation has been generally poor, and they have often succumbed to the disease in the first episode of hyperammonemic crisis. However, it is not known whether or not an early intervention improves prognosis. Here we describe two patients with the R40H mutation. Patient 7 had been well until 32 years of age, when he first developed hyperammonemia and died without specific therapy. Postmortem liver tissue was referred to our laboratory for enzymatic and genetic examination and diagnosis of OTCD due to the R40H mutation was made. Four years after the death of patient 7, a 23 year old man, a maternal cousin of patient 7, developed hyperammonemic crisis. This man, patient 9, was referred to our medical center four days after the onset of disease.

Because of the family history, therapy with intravenous infusion of sodium benzoate was initiated immediately after admission. Consciousness became alert within 48 hours after the initiation of the therapy, and he was discharged without any sequelae. This instance implies that with adequate early intervention, patients can survive the first attack and therefore early recognition of the R40H mutation is meaningful.

Brain edema has been often a fatal complication of hyperammonemic crisis. We tried to identify a biochemical parameter that would predict brain edema and found that the concentration of glutamine in plasma could serve an indicator of brain edema. However, it is not known whether or not

the ratio is useful as a predictor of brain edema. Development of effective strategy for prevention of brain edema seems to improve prognosis of life.

Conclusion

OTCD should be considered as a diagnostic possibility in adult male patients with hyperammonemia.

Most of the male patients with late-onset disease in our district carry R40H mutation.

The R40H OTC has enzymatic properties that are indistinguishable from that of wild-type enzyme, but is less stable when subjected to physical stress.

The mutation can be transmitted not only maternally but also paternally, and hence can make the mutation retained in a population.

Prognosis of patients carrying R40H mutation, once developed hyperammonemic crisis, has been generally poor, but early recognition and intervention seems to improve prognosis.

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