AGL gene mutation and clinical features in Korean patients with glycogen storage disease type III

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Purpose : Glycogen storage disease type III (GSD-III) is a rare autosomal recessive disorder of glycogen metabolism. The affected enzyme, amylo-1,6-glucosidase, 4-alpha-glucanotransferase (*AGL*, glycogen debranching enzyme), is responsible for the debranching of the glycogen molecule during catabolism. The disease shows clinical and biochemical heterogeneity, reflecting genotype-phenotype heterogeneity among different patients. In this study, we aim at analyzing mutations of the *AGL* gene in three unrelated Korean GSD-III patients, and characterizing their clinical and laboratory findings.

Methods : We characterized the clinical features of three unrelated Korean GSD-III patients by biochemical, histological and imaging studies. The 35 exons and part of exon-intron boundaries of *AGL* were analyzed by direct sequencing using genomic DNA extracted from the peripheral leukocytes of patients.

Results : Diverse clinical features were observed in these patients including hepatomegaly (all patients), seizures (patient 2), growth failure (patients 1 and 2), hyperlipidemia (patients 1 and 3), raised transaminase and creatine kinase concentrations (all patients), and mild cardiomyopathy (patient 2). Liver transplantation was performed in patient 2 due to progressive hepatic fibrosis. Administration of uncooked corn starch maintained normoglycemia and improved biochemical and growth profiles. DNA sequence analysis revealed mutations in 5 out of 6 alleles. Patient 1 was a compound heterozygote of c.1282 G>A (p.R428K) and c.1306delA (p.S603PfsX6), patient 2 had c.1510_1511insT (p.Y504LfsX10), and patient 3 had c.3416 T>C (p.L1139P) and c.1735+1 G>T (p.Y538_R578delfsX4) mutations. Apart from the p.R428K mutation, the 4 other substitutions identified were novel.

Conclusion : GSD-III patients display variable phenotypic characteristics resembling those of GSD-Ia. Molecular defects in the *AGL* gene of Korean GSD-III patients are genetically heterogeneous.

Key Words : Glycogen storage disease typeⅢ, *AGL* gene, Glycogen debranching enzyme, Glycogen storage disease, Metabolic myopathy

INTRODUCTION

Glycogen storage disease type Ⅲ (GSD-Ⅲ) is a rare group of autosomal recessively inherited metabolic disorders that affect glycogen synthesis and degradation. The disease is caused by the deficiency of the glycogen debranching enzyme (*AGL*), resulting in incomplete degradation of glycogen. Glycogen accumulates in the tissues involved, primarily liver, heart and muscle, resulting in progressive damage[1]. The majority of GSD-Ⅲ patients display *AGL* deficiency in both liver and muscle (subtype Ⅲa), while the liver is solely involved in about 15% of the affected subjects (subtype Ⅲb)[2].

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GSD-Ⅲ displays biochemical and clinical heterogeneities. In the most severe form, patients present hepatomegaly, hypoglycemia and growth retardation in infancy and early childhood. Milder cases may present only in adulthood, with asymptomatic hepatomegaly, occult chronic liver disease or myopathy[3]. While fasting hypoglycemia generally improves with age and hepatomegaly regresses, chronic hepatic fibrosis leads to overt cirrhosis and end-stage liver disease in a small proportion of patients with GSD-Ⅲ[4, 5].

AGL is a 165 kDa monomeric protein with two independent catalytic activities, specifically, oligo-1,4-1,4-glucantransferase and amylo-1,6-glucosidase. Complete *AGL* function requires both enzyme activities. The human *AGL* gene, located on chromosome 1p21.2, spans 85kb DNA, and is composed of 35 exons[6, 7]. At least 50 *AGL* mutations have been reported in patients with GSD-Ⅲ. Of these, exon 3 mutations occur exclusively in subtype Ⅲb2). Earlier studies suggest that *AGL* mutations are ethnic-specific. However, in an increasing number of cases, specific mutations are recurrent between different ethnic origins[8-10].

In this report, we analyze mutations of the *AGL* gene in three unrelated Korean GSD-Ⅲ patients, and discuss their clinical and laboratory implications.

MATERIALS AND METHODS

1. Patients

All patients displayed hepatomegaly and elevated aminotransferase levels. Liver biopsy revealed swollen hepatocytes with abundant PAS-positive glycogen material, which led to the diagnosis of glycogen storage disease. *AGL* enzyme deficiency was verified in the liver tissue from the biopsy specimen of one patient. The creatine kinase level in all three patients was elevated at all sequential points over the follow-up period. All patients were clinically diagnosed as GSD-Ⅲ.

2. Clinical data

The following clinical data were analyzed retrospectively from medical records, especially focusing on sex, actual age, age at diagnosis, signs of liver dysfunction, signs and symptoms of skeletal muscle, and cardiac involvement. Muscle involvement was clinically defined as the presence of fixed muscle weakness and/or myalgia and cramping pain induced by exercise. Repeated blood examination, including creatine kinase, aminotransferase and bilirubin levels, prothrombin time, platelet count, lipid profiles, uric acid, and lactic acid levels were performed at 3 to 6-month intervals. All patients were subjected to liver and abdominal ultrasonography, electrocardiography (ECG), and heart echocardiography. Muscle biopsy and electromyogram (EMG) examination were not performed.

3. Molecular analysis

Genomic DNA was extracted from the peripheral leukocytes of patients using a Puregene® blood kit (Gentra systems, Minneapolis, U.S.A.). All the coding exons and exon-intron boundaries of the *AGL* gene were subsequently amplified by polymerase chain reaction (PCR) using the PTC-200 thermocycler (MJ research, Watertown, U.S.A.). PCR products were purified and directly sequenced.

GSD-Ⅲ is indistinguishable from GSD-I during infancy and early childhood. To discriminate between GSD-Ⅲ and GSD-I, the glucose 6 phosphatase gene (*G6PC*) was analyzed in all patients by direct sequencing following amplification, as specified above.

RESULTS

1. Clinical features (Table 1, 2)

All patients were of Korean origin with non-consanguineous parents or no family history of metabolic disease. Median age at diagnosis was 16 months (ranging from 9 months to 6 years). The presenting signs of the disease

were varying between patients. Specifically, patients 1 and 3 were diagnosed due to asymptomatic hepatomegaly with elevated aminotransferase levels. Diagnosis in patient 2 was based on fasting hypoglycemia and hepatomegaly during the evaluation of recurrent seizures.

Hepatomegaly was identified in all cases, and the liver was palpated from 8 cm to 11 cm below the right subcostal margin at diagnosis. Splenomegaly was observed in patients 1 and 2. The spleen was palpated by 8 cm in the physical examination, and moderate portal hypertension was observed by Doppler ultrasonography in patient 2. However, the spleen of patient 1 was not palpated on physical examination, and splenomegaly was only detected by ultrasonography. The kidneys were not enlarged and renal dysfunction was absent. Furthermore, patients showed no evidence of either proximal or distal muscle weakness or wasting. While systolic ejection murmur was not heard in all patients, the ECG findings in patient 2 were consistent with biventricular hypertrophy. Echocardiographic abnormalities, including evidence of cardiac failure, decreased ejection fraction and fractional shortening, were not observed in all patients.

Only patient 1 showed short stature at diagnosis. The height of the patient was below 3rd percentiles (−3.8 height SDS). Patients 2 and 3 were 10th and 50th percentiles in terms of height, respectively. Weight at diagnosis was above 3rd percentiles (>−2 weight SDS) in all patients.

The serum biochemistry profiles of all 3 patients were summarized in Table 2. Elevated liver aminotransferase levels were observed, albeit with varying severity between patients and test time points. The creatine kinase level was elevated in patients 1 and 2, while that in patient 3 was normal at diagnosis and elevated at the follow-up visit. Blood lactate and uric acid concentrations were normal.

The random blood glucose level was normal in all patients. However, following fasting challenges, events of asymptomatic hypoglycemia were observed in patients 1 (30 mg/dL after 7 h fasting) and 2 (55 mg/dL after 10 h fasting). In past medical history, the recurrent seizure might be a manifestation of hypoglycemia, because the electroencephalogram (EEG) and MRI findings were all normal at that time. In patient 3, asymptomatic hypoglycemia (39 mg/dL) was identified in a random blood glucose test at the outpatient clinic. The glucagon stimulation test was not performed in all patients.

Table 1. Clinical and genetic features of GSD-III patients

Patient	Age/Sex	Disease onset	Organomegaly	Cardio-myopathy	Clinical muscle involvement	AGL genotype	Effects
1	7/M	16 mo	HM*, SM**	No	No	c.1282 G>A c.1306delA	p.R428K frameshift
2	18/M	6 yrs	HM*, SM**, PH† -> LULT‡	Mild BVH§	No	c.1510__1511insT unknown	frameshift unknown
3	4/M	9 mo	HM*	No	No	c.3416 T>C c.1735+1 G>T	p.L1139P frameshift

*HM: hepatomegaly, **SM: splenomegaly, †PH: portal hypertension, ‡LULT: living unrelated donor liver transplantation
§BVH: biventricular hypertrophy

Table 2. Serum biochemistry profiles of GSD-III patients

Patient	FBG* (mg/dL)	CK** (IU/L)	AST (IU/L)	ALT (IU/L)	GGT (IU/L)	T-bil† (mg/dL)	Cholesterol (mg/dL)	Triglyceride (mg/dL)
1	30	408-2,584	108-443	27-111	153	0.2-1.5	125-246	123-230
2	55	107-266	190-1277	244-984	37-196	1.4-7.4	86-189	150-275
3	39	129-2,631	143-1073	142-1020	146-162	0.4-1.4	93-258	133-356

*FBG: fasting blood glucose, **CK: creatine kinase, †T-bil: total bilirubin

Liver biopsy findings included swollen hepatocytes with abundant PAS-positive glycogen material, which confirmed the diagnosis of GSD. Periportal hepatic fibrosis was evident in patients 1 and 2. Micronodular cirrhosis and severe chronic inflammation was detected in patient 2, while minimal inflammation was noted in patient 1. No dysplastic cells were present. Tests to establish of the cause of liver disease were all negative, including HBV surface antigen, HCV antibody, autoantibodies, and cooper metabolism. Patient 3 displayed no *AGL* activity in liver tissue.

The fasting tolerance of patient 2 with hypoglycemic seizures improved with age. Moreover, hypoglycemic attacks did not occur after 7 years of age in the absence of corn starch supplementation. The course of hepatic dysfunction was variable between patients. Significant portal hypertension and hematemesis from the esophageal varix developed twice in patient 2. Emergent esophagogastroduodenoscopy revealed active bleeding, and esophageal variceal ligation therapy was performed each time. We observed prothrombin time prolongation (INR 2.4) and progression to end-stage liver disease. Therefore, living unrelated liver transplantation was performed at 16 years of age. Microscopic examination of the liver revealed cirrhosis with fibrosis, but no hepatocellular carcinoma (Fig. 1). After transplantation, the levels of aminotransferase and prothrombin time were normalized by immunosuppressive treatment.

Apart from patient 2, continuous provision of adequate amounts of glucose via uncooked corn starch (1.75-2 g/kg) at 6 h intervals facilitated the maintenance of normoglycemia, normalized growth velocity, and decreased aminotransferase concentrations and liver size.

2. Genetic data from mutation analyses (Fig. 2)

We identified 5 mutant alleles in three unrelated patients with GSD-Ⅲ. Both alleles were detected in two patients. Four of the 5 mutations were novel, and p.R428K was

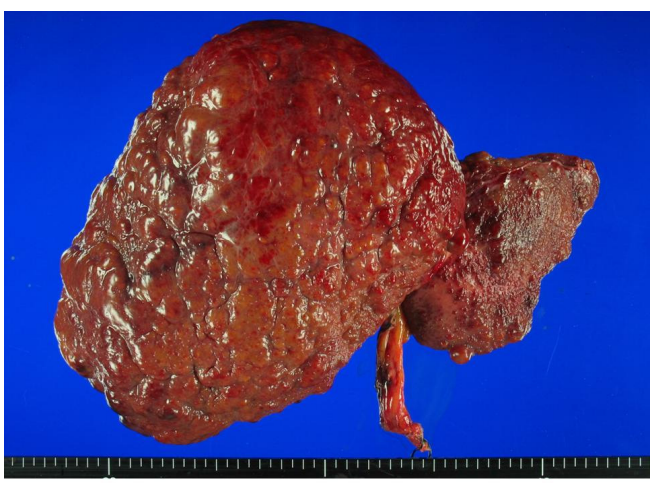

Fig. 1. Explanted liver (23×20×7 cm, 1,215 g) reveals multiple micro and macro cirrhotic nodules on a brownish external surface.

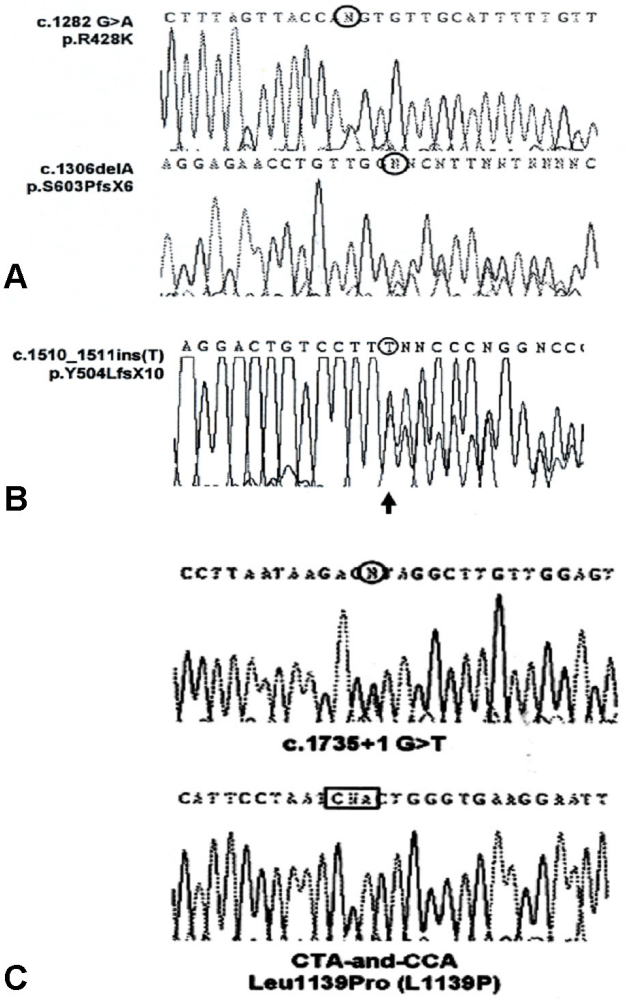

Fig. 2. Sequence analysis of the *AGL* gene. A) c.1282 G>A and c.1306delA patient 1, B) c.1510_1511insT of patient 2, C) c.3416 T>C and c.1735+1 G>T of patient 3.

reported previously[9].

Patient 1 had a compound heterozygote mutation, specifically, a G to A transition at nucleotide 1282 inducing the missense amino acid change from an arginine to a lysine (c.1282 G>A, p.R428K) and a frameshift mutation due to deletion of an adenine at position 1306, altering a serine to a proline and creating a stop codon leading to the premature interruption of translation (c.1306delA, p.S603PfsX6).

In patient 2, only one mutant allele was identified, specifically, insertion of a T between nucleotides 1510 and 1511, altering a tyrosine to a leucine and creating a stop codon resulting in the premature interruption of translation (c.1510_1511insT, p.504LfsX10). Apart from these mutations, two common polymorphisms (c.1160 G>A, p.R387Q and c.3343 G>A, p.G1115R) were identified[11, 12].

Patient 3 carried a compound heterozygote, T to C transition at position 3416, yielding a missense amino acid change from a leucine to a proline (c.3416 T>C, p.L1139P) and G to T transition at nucleotide 1735+1 on the exon-intron boundary, leading to large defects in translation due to the splice-site mutation (c.1735+1 G>T, p.Y538_R578delfsX4).

The *G6PC* gene was also analyzed to differentiate between GSD-I and GSD-Ⅲ. However, no mutation in the *G6PC* gene was found in all patients

DISCUSSION

GSD-Ⅲ is a rare autosomal recessive disorder, also known as Cori's or Forbe's disease. The enzyme affected is *AGL*, which is responsible for debranching of the glycogen molecule during catabolism.

The incidence of GSD-Ⅲ is estimated as 1 in 100,000 live births in North America[13], and higher in Japan. An inordinately high prevalence for this rare disease was observed in the Inuit population, originally of Asian progeny[14]. However, the reported GSD cases in Korea are few and restricted to GSD-I.

The variable phenotype of GSD-Ⅲ patients is explained by differences in tissue expression of the deficient enzyme. In particular, about 80% of GSD-Ⅲ patients with both myopathy and hepatic symptoms are categorized as subgroup Ⅲa. However, in about 15% patients, the disease appears to involve the liver only, which is classified as subgroup Ⅲb2,3). Genetic confirmation of GSD-Ⅲ would require evidence of a mutation in the *AGL* gene encoding the glycogen debranching enzyme. Two substitutions (p.W680X and p.Q6X in exon 3) account for most cases of GSD-Ⅲb. However, the mutations responsible for GSD-Ⅲa are too heterogeneous and ethnic-specific to warrant genetic analysis on a routine basis[10, 11]. Under conditions where GSD-Ⅲ is diagnosed on the basis of clinical features, measurement of *AGL* activity may be useful in liver tissue, blood or skin fibroblast specimens[15].

Our patients were compatible with GSD-Ⅲ with respect to pathologic, clinical and characteristics and further confirmed based on mutation identification of the *AGL* gene, even though the enzyme assay was not undertaken in all cases. Hepatomegaly, fasting hypoglycemia with ketosis, and hyperlipidemia are the common and predominant features during infancy and early childhood in both GSD-I and Ⅲ. Thus, the diseases may be indistinguishable. Hypoglycemia is the primary clinical manifestation of GSD-Ⅲ. In the early stages, hypoglycemia is caused by a defect in glycogenolysis. Due to a deficiency in *AGL* activity in GSD-Ⅲ, only a small proportion of the glucose moieties stored in the liver as glycogen is readily available for homeostasis. As a result, patients may experience hypoglycemia, even after a relatively short fast. However, in contrast to GSD-1, gluconeogenesis is normal in all forms of GSD-Ⅲ. This probably explains why hypoglycemia observed in GSD-Ⅲ is usually less severe than that in GSD-I. Since glucose 6 phosphatase and its transport system are intact in GSD-Ⅲ, the concentrations of phosphorylated glycolytic intermediates are not elevated. Consequently, blood lactate and uric acid levels are usually are within the reference ranges, unlike those of GSD-I. Nonetheless, patients with GSD-Ⅲ can experience sufficiently severe hypoglycemia to induce seizure and brain damage. Recurrent afebrile seizure in the past history of

patient 2 is a manifestation of hypoglycemia associated with GSD-Ⅲ.

Untreated infants and children grow slowly, and puberty is delayed. In this study, only one patient was below 3rd percentiles of height at diagnosis. Muscle weakness is usually minimal and not clinically significant in childhood, but intensified by the third or fourth decade of life[3, 16]. Notably, an adult was diagnosed as a result of mild creatine kinase elevation at 54 years of age with no sign of clinical muscle involvement[17], and an infant was diagnosed by severe hypotonia with no detectable cause other than GSD[14]. In patients with myopathy, varying degrees of muscle weakness is observed, depending on severity and time of onset. The incompletely degraded glycogen molecule, known as limit dextrin, may also accumulate in cardiac muscle, causing hypertrophic cardiomyopathy. Abnormal findings on ECG, as observed for patient 2, are suggestive of subtype Ⅲa. However, overt cardiac dysfunction is rare[18].

Elevated creatine kinase levels were observed in all three patients. This finding was an important factor in the diagnosis of GSD-Ⅲa. However, a normal level of creatine kinase does not rule out muscle enzyme deficiency, and no correlation exists between the levels and extent of myopathy[1].

With the exception of myopathy, symptoms and signs improve with increasing age. While hepatomegaly disappears after puberty, fibrous septa are usually formed in livers of patients with GSD-Ⅲ, and a small proportion of these may progress to severe cirrhosis, as observed for patient 2. Hepatic adenomas are frequent, and hepatocellular carcinoma is reported in patients with end-stage cirrhosis[5, 19].

Frequent or continuous feeding is the mainstream therapy to maintain normoglycemia. Raw corn starch releases glucose slowly, increases growth velocity, and decreases aminotransferase concentrations. A combination with high protein feeding at night may be beneficial for patients with severe myopathy, since gluconeogenesis is intact in GSD-Ⅲ, and protein can be used as a substrate[16, 20]. Alanine applied as a therapeutic is used as a substrate, and thus prevents muscle degradation in gluconeogenesis[21]. However, dietary treatment has little effect in patients with no clinical muscle involvement and those with mild elevation of creatine kinase and aminotransferase levels. Thus, no satisfactory treatment currently exists for progressive myopathy or cardiomyopathy.

Similar to patient 2, subjects who develop end-stage liver failure or hepatocellular carcinoma require surgical intervention, which sometimes includes liver transplantation[22]. The long-term outcomes after liver transplantation are currently unclear.

While the prognosis of GSD-Ⅲ generally varies from patient to patient, the GSD-Ⅲb subtype has better prognosis than the GSD-Ⅲa subtype, which is associated with the possibility of severe myopathy and cardiomyopathy.

Molecular analysis of Korean patients with GSD-Ⅲ reveals heterogeneity of *AGL* gene mutations. In this study, we identified five mutations (four novel and one previously reported) of the *AGL* gene in three GSD-Ⅲ patients. The c.1282 G>A (p.R428K) mutation was formerly identified by Santer in Faroe Islands patients. Insertional (c.1510_1511insT in patient 2), deletional (c.1306delA in patient 1), splice-site (c.1735+1 G>T) and two missense (c.1282 G>A in patient 1 and c.3416 T>C in patient 3) mutations are observed. We could identify only an allelic mutation, c.1510_1511insT, in patient 2. c.1160 G>A (p.R387K) and c.3737 G>A (p.G1115R) mutations, which were found in patient 2, were known polymorphisms[12, 23].

Mutations in exon 3 associated with GSD-Ⅲb were not detected in this study. This DNA-based diagnosis is consistent with the clinical diagnosis of GSD-Ⅲa, as all our patients displayed an elevated creatine kinase level. In contrast to GSD-Ⅲb, GSD-Ⅲa mutations are very diverse, and more than 50 mutations are reported. Most patients are compound heterozygotes, having different combinations of mutations that make genotype-phenotype correlation difficult[11].

In summary, GSD-Ⅲ exhibits variable features with respect to clinical signs and severity. While initial symptoms are mild, these may be accompanied by severe complications, including hypoglycemic brain damage, chronic liver disease and generalized myopathy. The absence of clinical muscle weakness does not exclude GSD-Ⅲ diagnosis. Therefore, early differential diagnosis of GSD-Ⅲ from other GSDs is important. The molecular analysis of the *AGL* gene is useful for the confirmation of GSD-Ⅲ diagnosis unless biochemical assay is available.

한글요약

목 적 : 제3형 당원병은 상염색체 열성으로 유전되는 드문 글리코겐 대사 질환이다. 글리코겐 debranching 효소는 두 가지 효소의 기능을 가지는데, amylo-1,6-glucosidase와 4-alpha-glucanotransferase가 그것이며, 제3형 당원병에서는 글리코겐 debranching 효소의 결핍으로 글리코겐의 불완전한 분해가 초래되며, 다양한 임상 및 생화학적 양상을 보이는 것으로 알려져 있다. 본 연구에서는 3명의 한국인 환자의 임상 및 생화학적 양상을 분석하고, *AGL* 유전자의 돌연변이 형태를 밝히고자 하였다.

방 법 : 서로 혈연 관계가 없는 3명의 한국인 제3형 당원병 환자를 대상으로, 생화학적, 조직학적, 방사선학적 특징을 포함한 임상 양상을 의무 기록을 통하여 조사하였다. 환자의 말초혈액에서 백혈구를 분리하여 추출한 genomic DNA를 사용하여 직접적 염기 서열 분석법으로 *AGL* 유전자의 35개 exon 및 exon과 intron의 경계 부분을 조사하여 돌연변이를 조사하였다.

결 과 : 간비대, 경련, 저신장, 고지혈증, 간효소 수치의 증가, creatine kinase 수치의 증가, 경도의 심근증 등 다양한 임상 양상이 관찰되었고, 한 명의 환자는 진행성 간섬유화로 인하여 간이식 수술을 시행 받았다. 생옥수수 전분가루의 복용은 모든 환자에서 정상 혈당을 유지시키고, 생화학적 검사 소견을 개선시키며 정상적인 성장 속도를 보이게 하였다. *AGL* 유전자 분석 결과 6개의 대립유전자 중 5개에서 돌연변이를 확인할 수 있었으며, 이중에 p.R428K를 제외한 4개의 돌연변이는 이제까지 보고된 적이 없는 새로운 돌연변이(c.1306delA, c.1510-1511insT, c.3416 T>C, c.1735+1 G>T)였다.

결 론 : 제3형 당원병은 임상 증상 및 중등도가 다양한 질환으로, 제1형 당원병의 증상과 유사하여 초기에 감별이 쉽지 않으며, 한국인 환자에서의 *AGL* 유전자의 돌연변이 양상도 매우 이질적이다.

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