ADAMTS13 Gene Mutations in Children with Hemolytic Uremic Syndrome

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We investigated *ADAMTS13* activity as well as the *ADAMTS13* gene mutation in children with hemolytic uremic syndrome (HUS). Eighteen patients, including 6 diarrhea-negative (D-HUS) and 12 diarrhea-associated HUS (D+HUS) patients, were evaluated. The extent of von Willebrand factor (VWF) degradation was assayed by multimer analysis, and all exons of the *ADAMTS13* gene were PCR-amplified using Taq DNA polymerase. The median and range for plasma activity of *ADAMTS13* in 6 D-HUS and 12 D+HUS patients were 71.8% (22.8-94.1%) and 84.9% (37.9-119.9%), respectively, which were not statistically significantly different from the control group (86.4%, 34.2-112.3%) (p>0.05). Five *ADAMTS13* gene mutations, including 2 novel mutations [1584+2T>A, 3941C>T (S1314L)] and 3 polymorphisms (Q448E, P475S, S903L), were found in 2 D-HUS and one D+HUS patients, which were not associated with deficiency of *ADAMTS13* activity. Whether these mutations without reduced *ADAMTS13* activity are innocent bystanders or predisposing factors in HUS remains unanswered.

Key Words: *ADAMTS13*, mutation, hemolytic uremic syndrome, children

Hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) are classified as thrombotic microangiopathy (TMA), which is characterized by occlusive microvascular thrombosis and by following thrombocytopenia, microangiopathic hemolytic anemia, and organ ischemia.[1,2] Acute renal failure is prominent in HUS while neurological impairment and variable degrees of renal abnormalities are found in TTP.[1,2] The clinical distinction between HUS and TTP is sometimes unclear because neurologic symptoms can be found in HUS and significant renal insufficiency can develop in TTP.[1,2]

Deficiency in the von Willebrand factor (VWF)-cleaving protease, also known as *ADAMTS13* (a disintegrin and metalloprotease, with thrombospondin 1-like domains motif 13), is causatively related in 70-80% of TTP,[3,4] either by compound heterozygous or homozygous mutations in the *ADAMTS13* gene in congenital TTP[5,6] or circulating inhibitory antibodies in the acquired form.[4] However, the focus of research in HUS has been on the mechanisms of injury to

the renal endothelium rather than the regulation of VWF.[2] In diarrhea-associated HUS (D+HUS), the bacterial agent Shigatoxin from Escherichia coli and Shigella dysenteriae induces the thrombotic state via direct toxic effects, resulting more in renal injury and clinical HUS.[7] In diarrhea-negative (D-HUS) or atypical HUS, more than 50% of patients have genetic abnormalities in complement regulatory genes, including complement factor H (CFH), factor I (CFI), membrane cofactor protein (MCP), and factor B (CFB) and C3.[8,9] Acquired cases of D-HUS associated with CFH dysfunction due to anti-CFH autoantibodies have also been identified.[8]

Considering the overlapping clinical manifestation of HUS and TTP, we hypothesized that *ADAMTS13* would be an important factor in HUS, and therefore, investigated *ADAMTS13* activity as well as *ADAMTS13* gene mutation in children with HUS.

The samples of 18 patients with HUS were sent to CHA Bundang Medical Center to test for *ADAMTS13* activity and *ADAMTS13* gene mutation from April 2004 to August 2005. The diagnosis was based on the presence or absence of diarrhea, renal dysfunction, microangiopathic hemolytic anemia and thrombocytopenia. Eighteen normal controls were recruited from those who visited the Department of Pediatrics of CHA Bundang Medical Center in the same period. In cases of decreased *ADAMTS13* activity (less than 44%), the presence of autoantibody in the patient's serum and the *ADAMTS13* activity of the parents were tested. The plasma samples were collected from patients and controls with the informed consent, according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the institutional review board of the CHA Bundang Medical Center.

The assay of *ADAMTS13* activity was performed as described previously by Furlan, et al.[3] Briefly, diluted citrated plasma was activated by barium chloride. This activated plasma was added to protease-free VWF (Green Cross, Yongin, Korea). The reaction was stopped by addition of EDTA. The extent of VWF degradation was assayed by multimer analysis using SDS-electrophoresis in 1.4% agarose gels. Following electrophoresis, the proteins were eletrotransferred to nitrocellulose, and VWF was visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG against human VWF (A0082, Dako, Glostrup, Denmark). The activity was calibrated as previously described[10] with 1 : 20 to 1 : 960 dilution of normal human plasma pool.

Inhibition of the *ADAMTS13* was assayed by measuring the remaining protease activity in the mixture of patient's plasma and normal pooled plasma at different dilution. Detection of inhibitory activity was carried out by using a screening test, by 1 : 1 mixing pools of normal plasma and patient plasma with reduced *ADAMTS13* activity (less than 44%) and by incubating for 30 minutes at 37°C. Thereafter, the mixture was diluted to 1 : 10 in Tris/urea, pH 8.0, and processed further to test *ADAMTS13* activity remaining.[11]

Human genomic DNA was isolated from whole blood. All exons of the *ADAMTS13* gene, including the intron-exon boundaries, were PCR-amplified with the primers used by Kokame, et al.[6] and Taq DNA polymerase. Products were sequenced in both directions by using a 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistics were performed using SAS software (version 8.2) and Chi-square test. p value less than 0.05 was considered statistically significant.

In 18 patients tested, 6 were D-HUS, and 12 D+HUS (Table 1). The median and range for plasma activity of *ADAMTS13* in 6 D-HUS and 12 D+HUS patients were 71.8% (22.8-94.1%) and 84.9% (37.9-119.9%), respectively, which were not statistically significantly different from the control group (86.4%, 34.2-112.3%) (p>0.05). No inhibitory activity was detected in patients with *ADAMTS13* activity less than 44%.

Five *ADAMTS13* gene mutations consisting of 2 novel mutations [1584+2T>A, 3941C>T (S1314L)] and 3 polymorphisms [Q448E, P475S, 2710C>T (S903L)] were found in 3 HUS (2 D-HUS and one D+HUS) patients (Fig. 1). All parents of these 3 HUS patients with *ADAMTS13* gene mutations are clinically unaffected. Two novel mutations, 1584 +2T>A, and S1314L, were excluded as common polymorphisms by screening 100 Koreans.

More than 60 mutations of the *ADAMTS13* gene have been found in patients with congenital TTP (The Human Gene Mutation Database at the Institute of Medical Genetics in Cadiff, http://www.hgmd.ac.uk/ac/index.php). However, analysis of *ADAMTS13* gene mutations has generally been restricted to patients already carrying a clear diagnosis of congenital TTP, potentially presenting an ascertainment bias against the identification of mild mutations.[12]

While severe deficiency of *ADAMTS13* (<10%) establishes a diagnosis of TTP unequivocally,[13] not all patients diagnosed with TTP have severe protease deficiency. In addition, *ADAMTS13* deficiency alone may not be sufficient to initiate an episode of clinical TTP.[14] Patients in clinical remission can still demonstrate ultralarge VWF multimers

Table 1. Patients Characteristics

	ID	Sex/Age	*ADAMTS13* (%)	CNS Sx	FHx	Shigatoxin	C3	Recur	Outcome	*ADAMTS13* Mutations
D-HUS	1	F/2 yrs	29.6	-	-	ND	ND	-	Normal	
	2	F/10 yrs	22.8	-	-	-	47	-	MicroHU	
	3*	F/10 months	53.9	-	-	-	112	+	Died	P475S
	4*	F/5 yrs	85.5	+	-	ND	78	+	KidT/NED	1584+2T>A, S903L†
	5	F/3 yrs	94.1	-	-	-	116	-	Normal	
	6	F/1 months	71.8	-	+	ND	ND	-	Normal	
D+HUS	7*	F/17 months	96.9	-	-	E.coli O157 : H7	118	-	Normal	Q448E, S1314L
	8	F/13 yrs	119.9	-	-	ND	ND	-	HTN	
	9	M/10 yrs	55.7	-	-	-	127.7	-	Normal	
	10	F/14 months	76.9	-	-	-	64	-	Normal	
	11	F/4 yrs	76.9	-	-	Klebsiella	112	-	Normal	
	12	M/4 yrs	84.9	-	-	ND	ND	-	Normal	
	13	M/2 yrs	44.5	-	-	-	10.7	-	Normal	
	14	M/3 yrs	95.1	+	-	-	ND	-	Normal	
	15	F/3 yrs	37.9	-	-	-	85	-	Normal	
	16	F/20 months	95.1	-	-	-	ND	-	MicroP/HU	
	17	M/9 yrs	94.0	-	-	-	ND	-	Normal	
	18	F/3 yrs	63.2	-	-	-	80.1	-	Normal	

C3 normal range 77-195 mg/dL.
FHx, family history; ND, not done; MicroHU, microscopic hematuria; KidT, kidney transplantation; HTN, hypertension; MicroP/HU, microscopic proteinuria and microscopic hematuria.
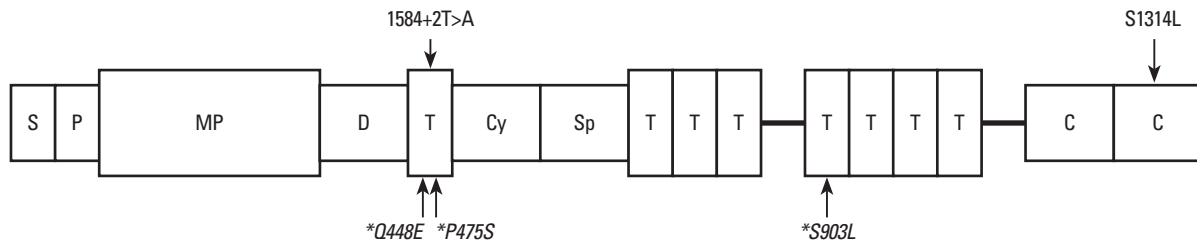*Patients with *ADAMTS13* gene mutations and/or polymorphisms.
†Homozygote.

Fig. 1. *ADAMTS13* gene structure and mutation sites in 3 HUS patients. *Three polymorphisms are indicated by italics. HUS, hemolytic uremic syndrome; S, signal peptide; P, propeptide; MP, metalloprotease domain; D, disintegrin-like domain; T, TSP1 motif; Cys, cysteine-rich domain; Sp, spacer domain; C, CUB domain.

in the plasma and absence of *ADAMTS13* activity *in vitro*.[3] This heterogeneity suggests the presence of genetic modifying factors or environmental triggers other than *ADAMTS13* in TTP.[1]

Complement factor H mutation, known as a causative factor in D-HUS, and Shigatoxin, inducing the thrombotic state in D+HUS, were reported to be risk factors in TTP.[9,15] In addition to the overlapping clinical features between TTP and HUS, these findings suggest possible role of *ADAMTS13* in HUS.

Decreased *ADAMTS13* activity, usually mild to moderate (10 to 40% of normal plasma), has been reported in a wide variety of conditions, including liver cirrhosis, chronic uremia, idiopathic thrombocytopenic purpura, disseminated intravascular coagulation, systemic lupus erythematosus,

leukemia, pregnancy, the postoperative state, the neonatal period, and with advancing age[16] other than congenital TTP. As for HUS, *ADAMTS13* activity is normal or only slightly decreased in typical colitis-associated D+HUS, and severely deficient in a few D-HUS.[17]

In this study, *ADAMTS13* activities were not decreased in 18 HUS patients, which is consistent with previous reports.[18] Although the *ADAMTS13* activities were normal, we found mutations and polymorphisms of *ADAMTS13* gene in 2 D-HUS and one D+HUS patients. We cannot explain the genotype-phenotype dissociation in our 3 HUS patients with *ADAMTS13* gene mutations. Heterozygous mutation, distal location, or interaction with polymorphisms might be possible explanations.

One D-HUS (patient 3) patient had heterozygous P475S

polymorphism and moderate activity (53.9%) of *ADAMTS13* protease. P475S, a well known polymorphism located in the cysteine-rich domain,[6,19] was recombinantly analyzed and was found to be associated with a decline in the proteolytic activity of *ADAMTS13* (5-10% of wild type) despite normal secretion.[20] In Japan, the allele frequency of P475S is about 5%, suggesting that approximately 10% of population are heterozygotes and may possess significantly reduced *ADAMTS13* activity.[19]

The other D-HUS (patient 4) patient had a novel splicing mutation 1584+2T>A in intron 13 (cysteine-rich domain). Also, this patient showed a homozygous S903L mutation in exon 21 (Tsp1-5 domain). Liu, et al.[21] reported a highly suspected congenital TTP patient with significantly reduced *ADAMTS13* activity and compound heterozygote mutation of S903L and R1095W in the *ADAMTS13* gene. Later, S903L was identified as a common polymorphism in Japanese with an allele frequency of 5.5% (7/64).[22] Polymorphisms influence the phenotypic expression of complex disease.[17] Dependent on the sequence context, the same polymorphisms might be either positive or negative modifiers of gene expression.[20] It is unclear whether polymorphism S903L is a positive modifier of *ADAMTS13* expression in the context of 1584+2T>A splicing. The interaction between this splicing mutation and S903L polymorphism might explain normal *ADAMTS13* activity in this patient.

One D+HUS (patient 7) patient had a novel heterozygous mutation of S1314L on exon 28 CUB2 domain and also heterozygous Q448E. It seems that the location S1314L mutation is too distal to have a detrimental effect on the *ADAMTS13* function. However, the cooperative activity between the middle carboxyl-terminal TSP1 repeats and the distal carboxyl-terminal CUB domains is crucial for recognition and cleavage of VWF under flow,[23] and more than 10% of *ADAMTS13* gene mutations associated with congenital TTP are located in the CUB domains.[24] As for Q448E, it is a positive modifier of *ADAMTS13* secretion in the context of P618A and A732V, and a negative modifier enhancing the detrimental effect of the missense mutation in the context of R1336W.[20] Expression tests would be necessary to elucidate the interaction between S1314L and Q448E.

Limitation of this study is the lack of information on complement system except serum C3 levels. In D-HUS, 4 patients tested C3 levels had normal values. One D+HUS patient had low C3 level with unknown significance.

To our best knowledge, this is the first report about *ADAMTS13* gene mutations and polymorphisms in childhood HUS in Korean population. Whether these mutations without reduced *ADAMTS13* activity are innocent bystanders or predisposing factors in HUS remains unanswered. In TTP, relapse is common among patients with *ADAMTS13* deficiency, but rarely occurs in patients without *ADAMTS13* deficiency.[25] In 6 D-HUS patients in this study, only the patient 3 and 4 underwent recurrent attack of HUS, suggesting the possibility that *ADAMTS13* gene mutations may act as a disease modifying or predisposing factor through an unexplained mechanism.

In the future, expression tests are needed to identify the consequences of each *ADAMTS13* gene mutations and polymorphisms found in our HUS patients. Abnormalities in complement system and other genetic or environmental factors, involving TMA, should be investigated. In addition, VWF assay and mutation analysis would be a next assignment in our patients. Long-term follow-up of these HUS patients with *ADAMTS13* gene mutations might also provide an invaluable clue.

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