한국 von Willebrand병 환자의 von Willebrand 인자 유전자 돌연변이 조사

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Investigation of von Willebrand Factor Gene Mutations in Korean von Willebrand Disease Patients

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Background : We intended to find the mutations of von Willebrand factor (VWF) gene as the most important contributing factor of von Willebrand disease (VWD) in Korean patients.

Methods : In 40 known vWD patients mutations of vWF gene were sought by direct sequencing of PCR products targeting exons 18, 19, 20, 26, 28 and 52 frequently implicated as the locations of mutation. For factors other than VWF gene contributing to VWD phenotype, we tested ABO blood group and measured ADAMTS13 activity in VWD patients.

Results : Twenty-seven cases (67.5%) were type 1 vWD, 3 cases (7.5%) type 3, and 5 cases (12.5 %) type 2A. Three cases were type 2A or 2B (7.5%) and 2 cases were suspected to be type 2N (5.0 %). Among them six candidate missense mutations were found: V1279I, R1306W, R1308C, and V1316M were previously reported in type 2B and type 1 vWD, and C858W and T1477I were novel findings. All patients were heterozygotes. Blood group O was overly represented in VWD patients, while ADAMTS13 activity of the patients was not significantly different from that of normal control.

Conclusions : Mutation of VWF gene detected by genetic studies can significantly improve the diagnostic accuracy, especially in subtype assignment of VWD. Two novel mutations, C858W and T1477I associated with VWD were found and expected to contribute to the elucidation of its pathophysiology. *(Korean J Lab Med 2007;27:169-76)*

Key Words : *von Willebrand disease, von Willebrand gene, ADAMTS13*

INTRODUCTION

Von Willebrand disease (VWD) is the most common bleeding disorder resulting from quantitative and qualita- tive abnormality of von Willebrand factor (VWF). VWF is synthesized from 8.8 kb transcript in endothelial cells and megakaryocytes and stored in Weibel-Palade bodies and α-granules. Its precursor protein of 2813 amino acids contains 22 aa signal protein, 741 aa pre-peptide, and 2050 aa mature peptide and composed of four repeated domains, each of which possesses distinct functions in its own syn- thesis and hemostasis[1]. Many genetic defects were iden- tified recently and vast amount is enlisted in database dedicated to this bleeding disorder (http://mmg2.im.med. umich.edu)[2]. Most of them were identified in type 2 VWD and their association with each subtype of type 2

VWD is so specific as to endow the genetic study with diagnostic value. Furthermore the location of the mutations in each domain and corresponding derangement of the specific function of the domain is so well correlated that speculation of pathogenic mechanism of individual cases is not difficult in the majority of cases. On the contrary, genetic studies in type 1 VWD were always completed with low yield of mutation detection and a large part of its pathogenesis still remains uncovered[3-8]. Significant overlap of VWF level from type 1 VWD patients and general population obscures clear distinction between VWD and normal phenotype[9]. This may explain the mysterious genetic aspect of type 1 VWD to some extent. In Korea, despite the widespread appreciation of high prevalence of VWD, there has rarely been an attempt of genetic study of this disease. Only one case of VWD with novel candidate mutation of VWF gene has been reported till now[10]. We supposed that overall the mutation associated with VWD in Korea would not be greatly different from those enlisted in database collected worldwide, although there may be an occasional novelty. So, we screened the mutation of VWF gene in 40 patients with known VWD by PCR and direct sequencing method. Additionally, to evaluate ABO blood group and ADAMTS13, VWF cleaving plasma metalloproteinase as the contributing factors other than VWF gene in the pathogenesis of VWD, we tested ABO blood group and measured ADAMTS13 activity in VWD patients.

MATERIALS AND METHODS

1. Patients

Forty patients were included who were previously diagnosed with VWD in our and other institutions and referred then for related clinical tests to the department of laboratory medicine of our institution. At the time of the visit, whole blood collected in EDTA tube were obtained, aliquoted, and frozen at -80℃ until the genetic studies were done. Twenty seven (67.5%) of them were previously classified as type 1 VWD, 5 (12.5%) as type 2A, 3 as type 2A or 2B (7.5%; absence of large multimers without the result of ristocetin induced platelet aggregation test), and 3 (7.5%) as type 3 according to the findings from VWF multimer study (Table 1). Two of them were

suspected to have VWD type 2N (decreased factor VIII: C with normal VWF:Ag and VWF activity in female patient or family member). Specimens for family study were available in five of them, in one of which was found a highly suspected candidate mutation of VWF gene associated with VWD.

2. Measurement of VWF:Ag and VWF activity

We retested the VWF:Ag and VWF activity for every possible case for confirmatory purpose. VWF:Ag and VWF activity were measured in citrate plasma drawn from patients by enzyme-linked immunosorbent assay (ELISA) methods using a VWF:Ag kit (Corgenix, Westminster, CO, USA) and VWF activity kit (Axis-Shield Diagnostic, Dundee, UK) according to the procedure provided by the provider. VWF activity assay detects a functional epitope of VWF and can be used in replacement of the conventional ristocetin cofactor activity assay using fixed platelet and aggregometer. For multimer analysis, non-reduced plasma samples were analyzed by sodium dodecyl sulfate (SDS)-agarose electrophoresis (0.7% and 1.2%) and detected after blotting using anti-VWF antibody, biotinylated secondary antibody, avidin horse radish peroxidase and bromophenol-blue. The factor VIII:C was measured by STA deficient VIII (Diagnostica STAGO, Paris, France). Ristocetin induced platelet aggregation (RIPA) was measured in platelet rich plasma (PRP) using Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA) with ristocetin concentration of 1.0, 0.6 and 0.3 mg/mL.

3. Mutation screening

The genomic DNA was extracted from EDTA whole blood after thawing using QIAamp DNA blood Mini kit

Table 1. Number of each subtype among the VWD patients included and positive results of the mutation screening

Classification	Subtype	N of cases (%)	N of mutations detected (% of cases)
Type 1		27 (67.5)	3 (11.1)
Type 2	2A	5 (12.5)	0 (0.0)
	2A or 2B	3 (7.5)	3 (100.0)
	2N	2 (5.0)	0 (0.0)
Type 3		3 (7.5)	0 (0.0)
Total		40	6

(Qiagen, Valencia, CA, USA). The size (coding sequence of 8,439 bp) and complexity of VWF gene caused great difficulty in searching for candidate mutation. To save the time and resources consumed in searching for the mutations of VWF gene, we gave the investigational priority to the exons 12, 14, 16, 18, 19, 20, 24, 26, 27, 28 and 52, because they frequently appear in database as the locations of known mutations. The regions spanning each exon and exon-intron boundary was PCR amplified and direct sequencing was done on the purified PCR products. The presence of VWF pseudogene, which is located on chromosome 22 long arm and shows about 97% homology with exons 23 to 34 of VWF gene[11], further hampered the study necessitating the use of allele specific oligomers for PCR (For some of them the sequences were kindly presented by Dr. Baronciani of University of Milan, Italy and the others were designed separately). The sequences of primers can be given upon request. DNA sequence analysis was performed by the fluorescent dideoxy terminator method. In this study VWF cDNA was numbered from the first nucleotide in the ATG initiation codon.

4. Measurement of ADAMTS13 activity

Plasma ADAMTS13 activity was measured using fluorogenic substrate of ADAMTS13, FRETS-VWF73 (Peptide International, Louisville, KY, USA)[12]. Briefly, diluted substrate was mixed with citrate plasma obtained from normal healthy individuals as standard and each patient diluted in assay buffer (5 mmol/L Bis-Tris, 25 mmol/L $CaCl_2$, 0.005% Tween-20, pH 6.0). Fluorescence was measured by FLUOstar OPTIMA microplate reader (BMG LABTECH, Offenburg, Germany) and the enzyme activity was calculated by comparing the development of fluorescence versus time in patient sample with those of standards.

RESULTS

1. Mutation screening results

Forty patients with known von Willebrand disease were screened for mutation of von Willebrand factor gene. Overall, 6 nucleotide substitutions with consequent amino acid substitutions were found in type 1 and type 2 VWD patients. Small to large deletions or insertions known to be associated with type 1 or type 3 VWD were not found. Maybe PCR direct sequencing method we relied on was not appropriate for the detection of gross deletion or insertion occasionally associated with type 3 VWD. The results of screening are summarized in Table 2 with other laboratory data. All the repeated measurements showed almost the same results as tested when they were diagnosed. Three of the 6 variations correspond to R1306W (3916 C>T), R1308C (3922 C>T) and V1316M (3946 G>A) were located in domain A1 and all previously reported in association with type 2B VWD[13-18]. Another was V1279I (3835 G>A) reported in type 1 VWD[3]. All the nucleic acid substitutions in our study were found in heterozygous state. Association of each of these mutations with VWD subtypes in the literature was reaffirmed in our cases. In these cases the association of each mutation with VWD seems clear, although thorough family studies were not available due to incompliance or follow up loss.

2. C858W and T1477I found in two unrelated VWD patients

The other two variations, resulting in C858W and T1477I amino acid substitutions have not been previously reported and we examined their prevalence in general population by studying 50 individuals (100 alleles) without known history of bleeding diathesis. Both C858W and T1-

Table 2. Laboratory data of the patients for whom candidate implicated mutations of VWF gene were found and past reports of each mutation

| Patient No.	vWF:Ag (%)	vWF activity (%)	FVIII:C (%)	Activity/Ag ratio	Multimer analysis	Amino acid substitution (nucleotide substitution)	Domain	Past reports
2	16	13	19	0.8	Type 1	V1279I (3835 G>A)	Domain A1	Eikenboom et al. (1993)
4	30	11	27	0.4	Type 2A or 2B	R1306W (3916 C>T)	Domain A1	Cooney et al. (1991)
20	42	10		0.1	Type 2A or 2B	R1308C (3922 C>T)	Domain A1	Cooney et al. (1991)
35	74	17	-	0.2	Type 2A or 2B	V1316M (3946 G>A)	Domain A1	Cooney et al. (1991)
36	42	37	-	0.9	-	T1477I (4430 C>T)	Domain A1	-
37	35	22	5	0.7	Type 1	C858W (2574 C>G)	Domain D'	-
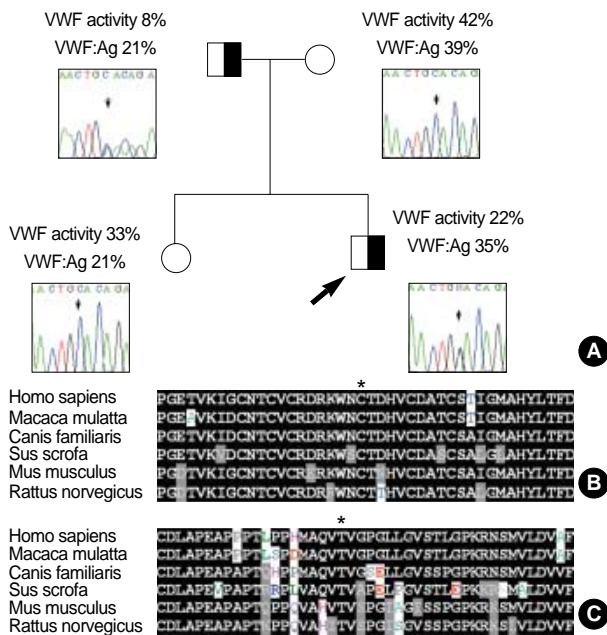
Fig. 1. The probable role of the novel findings C858W and T1477I in causing VWD. The C858W segregated with decreased VWF activity within the family of the patient 37, while mildly decreased VWF:Ag was observed in all members of the family (A). The residues 858C and 1477T (indicated by asterisk) within VWF are more strictly preserved throughout several different mammalian species than other immediate neighboring residues, implying its essentiality for wholeness of VWF in regard to its synthesis, stability or function (B, C).
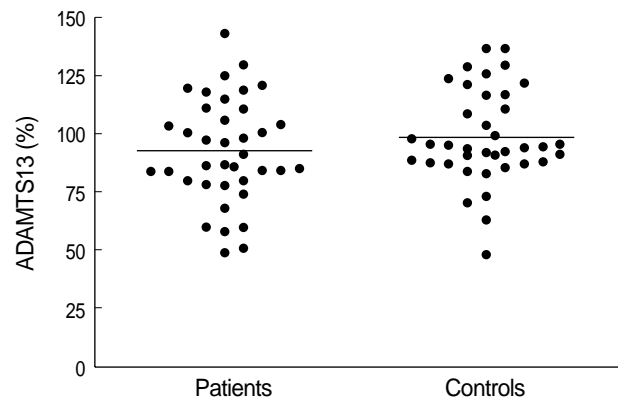

Fig. 2. ADAMTS13 activity in VWD patients compared with normal controls. There was no significant difference between ADAMTS13 activity between VWD patients (n=38) and normal healthy control (n=38).

tial for proper function or synthesis of VWF (Fig. 1C).

3. Factors other than VWF gene

Because mutation of VWF gene was found in only a small number of patients from a substantial size of patient group studied, we suspected that certain factors other than VWF itself has affected VWF activity. First, we reexamined the sequencing data for the presence of polymorphism Y1584C, which was recently reported to be prevalent in type 1 VWD implicating the susceptibility to AD-AMTS13 as an important factor influencing the development of VWD[19, 20]. But there was not a case with C1584 allele among the forty patients. We examined the effect of blood group, especially blood group O, which is known to be associated with low VWF level[9, 21]. Among the VWD patients who were blood typed, the prevalence of blood group O was significantly higher than it is known in general population (67% vs. 28%)[22]. This implies that along with other unknown factors, increased clearance of VWF in blood group O can reduce its level to the extent of VWD[21]. Next we also tested another factor related to VWF clearance, ADAMTS13. We measured ADAMTS13 activity with recently described fluorogenic synthetic substance[12]. ADAMTS13 activity in VWD patients was not significantly different from that measured in normal healthy individuals ($P=0.238$; Fig. 2). Nor conld there be observed an apparent correlation between ADAMTS13 activity and VWF activity in plasma (data not presented).

477I substitution were not found. A family study including the parents and sister was done for the patient with C858W substitution. Factor VIII:C activity of the patient was markedly decreased to 4%. The patient's father also carried the same nucleotide substitution and his VWF:Ag and VWF activity were 21% and 8%, respectively, (35% and 22% for the patient), while those of his mother and sister who did not carry the substitution were 39%, 42% and 21%, 33%, respectively (Fig. 1A). His family members included in this study had no noticeable past medical histories and did not complain of bleeding tendency. Amino acid sequences of VWF from other species (Macaca mulatta, Sus scrofa, Canis familiaris, Mus musculus, Rattus norvegicus) were aligned with that of human for comparison. Cysteine at the 858th amino acid of VWF is well conserved throughout the species while neighboring amino acids showed some degree of interspecies variation (Fig. 1B). This implies the importance of Cys858 for proper function of VWF, although the exact role of this residue cannot be unraveled to the structure level at this point. T1477I is also strictly conserved and expected to be essen-

DISCUSSION

V1316M (3946 G>A), R1306W (3916 C>T), and R1-308C (3922 C>T) substitutions found in three sporadic cases in our study have been repeatedly described in association with type 2B VWD[13-18]. Increased binding of VWF to platelet with subsequent consumptive clearance is the pathogenic mechanism with these mutations. Large multimers were absent in multimer study of patients 4 and 20 possessing R1306W and R1308C alleles. But due to unavailability of RIPA test in them, only the putative diagnosis of type 2A or 2B has been made. So, these are the cases where genetic tests have the determining role in subtype assignment. V1279I (3835 G>A) substitution has been reported as a genetic defect in type 1 VWD with recessive inheritance pattern[3]. Many cases of type 1 VWD showing recessive inheritance are known to be compound heterozygote (but the other genetic defect of patient 2 has not been found yet). Additional study targeting exons omitted in this study may reveal the other mutation. The amino acid substitution C858F is well known for its association with type 2N VWD[23-26]. Considering its location in D' domain of VWF, which is involved in interaction with factor VIII, the association is quite expectable. Substitutions at other residues in the domain are also associated with type 2N subtype, among which R854Q was reported to be the most common, comprising about 73% and the next R816F, about 10%. The association of C858W with decreased VWF activity per se is an unexpected finding in this context with restricted structural information of the domain available at now. Confirmation with additional studies including factor VIII binding assay not included in this study and in vitro expression study should be followed and would be of help in elucidating the association with functional aberration. Interestingly the patient's family members who did not carry the C858W showed slightly decreased VWF:Ag levels. But VWF:Ag of these levels is known to be uncommonly associated with bleeding symptom and shows low heritability[27, 28]. Substitution of Cys858 with tryptophan might have influenced the protein structure to the level where the delicately balanced function of VWF is affected. The amino acid substitution T1477I has not been previously reported either in association with type 1 VWD or as a neutral polymorphism. Linkage with VWD could not be fully evaluated because the family study was not available. But the absence in general population and high degree of conservation throughout different species imply an important functional role in synthesis or stability of VWF. H1472 in proximity to T1477, where SNP (H14-72D) has been reported shows great interspecies diversity as shown in Fig. 1C. The activity of the variant VWF does not seem to be significantly impaired considering the similarity between VWF activity/VWF:Ag ratios over 0.6. It can also be anticipated from the fact that the residue T1477 is located outside and between the functional domains D3 and A1. There exist very few reports of amino acid substitution related to type 1 VWD in contrast to its high prevalence. The majority of them are also located outside the functional domains. Some of them are substitutions of cysteine residue (C1130F, C1149R) involved in disulfide bond formation, resulting in gross structural alterations[5]. Another is in close proximity to those cysteine residues (T1156M)[7]. The other two amino acid substitutions V1229G and N1231T were reported in type 1 VWD showing apparent autosomal recessive pattern of inheritance[6]. In vitro expression study has been done in few cases and the exact mechanism of aberrant synthesis is not yet fully described. Such an approach can be tried for the further investigation of our case with limited opportunity of thorough linkage study.

ADAMTS13 catalyzes the proteolysis of VWF between Y1584 and M1585 and consequently results in the clearance of large VWF multimers. Recently, it was reported that Y1584C substitution renders VWF susceptible to the action of ADAMTS13 and consequently results in increased clearance of VWF from plasma[19]. Actually the corresponding allele was found to be overly represented in type 1 VWD patients and their families by 25% and 27% respectively, compared with about 1% in general population[20]. The frequency of C1584 allele in east Asians is nearly zero (Entrez SNP database) and accordingly the substitution Y1584C was not found among VWD patients included in our study. Blood group O VWF is by far more susceptible to the cleavage by ADAMTS13 than other blood groups[29]. It can explain the well known phenomenon, a decreased plasma VWF level in blood group O individuals, which is also found in this study. Both the findings imply the significant role of ADAMTS13 activity in determining plasma VWF level. It is a plausible hypothesis that increased ADAMTS13 activity facilitates the clearance of plasma VWF and consequently lowers

VWF level. Thus we further examined if ADAMTS13 activity is increased in VWD patients compared with normal healthy individuals. It has long been suggested without definite evidence that factors other than VWF itself can be related with VWD[30, 31]. Probably that may explain the paucity of reports of type 1 VWD related VWF gene mutation. But in this study there was no significant difference in ADAMTS13 activity between the patient and control groups. There was no correlation between ADAMTS13 activity and VWF activity, either. This is a quite different finding from that reported for type 3 VWD where ADAMTS13 activity was significantly increased in the patient group[32]. Despite our results contradicting the essential role of ADAMTS13 in determining VWF level, the possibility of partial contribution of ADAMTS13 to the development of VWD cannot be entirely excluded. In regard to the unimpressive results of mutation screening in VWD, especially type 1, one should take into account the effect of variations in untranslated region of the VWF gene[33]. Enhancer and silencer sequences as well as cell type-specific promoter region on VWF gene are being continuously revealed[34, 35]. But, the information implicating these regions with VWD is still insufficient. Examination targeting these regions can possibly reveal a new aspect of the pathophysiology of VWD. In conclusion, mutation of VWF gene detected by genetic study can significantly improve the diagnostic accuracy especially in subtype assignment of VWD, as well as providing contributory information for elucidating pathophysiology of the disease.

요 약

배경 : 한국인 폰 빌레브란트 병 환자를 대상으로 폰 빌레브란트 병의 가장 중요한 발병인자인 폰 빌레브란트 인자 유전자의 돌연변이를 조사하였다.

방법 : 폰 빌레브란트 병으로 진단된 40명의 환자를 대상으로 유전체 DNA를 추출하여, 중합효소연쇄반응 및 염기 서열 분석 방법을 통해 폰 빌레브란트 인자 유전자의 돌연변이를 조사하였다. 돌연변이가 주로 발견되는 것으로 알려진 엑손 18, 19, 20, 26, 28 및 52를 대상으로 하였다. 또한 유전자 이상 외에 발병 요인으로 작용할 수 있는 ABO 혈액형 및 ADAMTS13 활성을 측정하였다.

결과 : 27명(67.5%)의 1형 폰 빌레브란트 병이었고, 3명(7.5%)의 3형, 5명(12.5%)의 2A형을 대상으로 하였다. 그리고, 3명 (7.5%)은 2A 또는 2B 그리고, 2명(5.0%)은 2N형의 의심되었다. 6개의 과오돌연변이를 발견하였으며 모두 이형접합체였다. V1-279I, R1306W, R1308C, V1316M은 2B형 및 1형 폰 빌레브란트 병에서 이미 보고되었으며, 1형에서 발견된 C858W와 T1477I는 아직 보고된 바가 없다. 결손이나 삽입은 발견되지 않았다. O 혈액형군이 환자군에서 일반인에 비해 매우 높은 빈도로 발견되었으며, ADAMTS13 활성은 정상인 대조군과 비교하여 의미 있는 차이를 보이지 않았다.

결론 : 폰 빌레브란트 유전자의 돌연변이 검사는 폰 빌레브란트 병 아형 분류의 정확성을 향상시키는데 기여할 것으로 사료된다. 또한 C858W와 T1477I는 아직 보고된 바가 없으며 폰 빌레브란트 병의 병리 기전 규명에 기여할 것으로 생각된다.

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