

## Original Article

# Classification of exon 18 linked variants of VWF gene in von Willebrand disease

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**Abstract:** Defects in von Willebrand factor, a crucial protein in haemostasis, lead to the most common inherited coagulopathy in man, von Willebrand disease. To date, over 350 mutations and 170 single nucleotide polymorphisms of VWF gene have been reported. In the present study, the distribution of two linked VWF gene variants, rs1063856 and rs1063857 have been assessed. The proportional frequency of rs1063856 (2365A/G) and rs1063857 (2385T/C) in healthy individuals were 0.70/0.30. Frequency of polymorphisms was in agreement with predicted geographical distribution. von Willebrand disease was more common in subjects with 2365A and 2385T alleles (odds ratio=1.35), although the difference was not statistically significant (p-values>0.05). The perfect correlation between these two single nucleotide polymorphisms supports their joint contribution in von Willebrand factor biology.

**Keywords:** von Willebrand disease, VWF gene, genetic polymorphisms, allele frequency

## Introduction

von Willebrand Factor (VWF) as a pro-coagulant protein, plays an important role in haemostasis. In addition to make a bridge between exposed subendothelium and platelets, it also carries the coagulation factor VIII in the circulation [1]. VWF abnormalities cause the von Willebrand disease (VWD) in three main types. The quantitative defects in VWF lead to either type 1 or type 3 phenotype. Type 3 VWD is the most severe form of the disease with serious mucocutaneous bleeding. In type 1 VWD, the partial depletion of the factor, causes mild bleeding symptoms. Type 2 VWD is composed of 4 subtypes (2A, 2B, 2M, and 2N) and characterized by a qualitative defect in VWF function [2, 3].

The VWF gene spans ~178kb on short arm of chromosome 12 and includes 52 exons. The first 17 exons encode the signal peptide and propeptide in the subsequent protein. The mature VWF subunit corresponds to exons 18 to 52, which finally undergoes multimerization.

Exon 18 encodes the first 51 amino acids of VWF D<sup>+</sup> domain [4-6]. This domain is involved in binding of VWF to FVIII [7-9]. According to the ISTH-SSC VWF Online Database (<http://www.ragtimedesign.com/VWF/mutation>), exon 18 has the highest frequency of mutation per nucleotide among the 52 exons of the VWF gene (**Figure 1**).

In addition to these mutations, there are two linked single nucleotide polymorphisms (SNPs) (e.g. rs1063856 and rs1063857) on exon 18. rs1063856 is a nonsynonymous Threonine to Alanine 789 alteration and rs1063857 is a synonymous Tyrosine 795 variant. These SNPs correspond to 2365A/G and 2385T/C when nucleotides are numbered from the first adenine in the initiator ATG codon on exon 2 or 2615A/G and 2635T/C when nucleotides are numbered from the first adenine of untranslated exon 1, respectively.

Allele frequencies of the polymorphisms are studied in several populations but this is the first report regarding Iranian VWD patients and

rs1063856 and rs1063857 SNPs of VWF gene

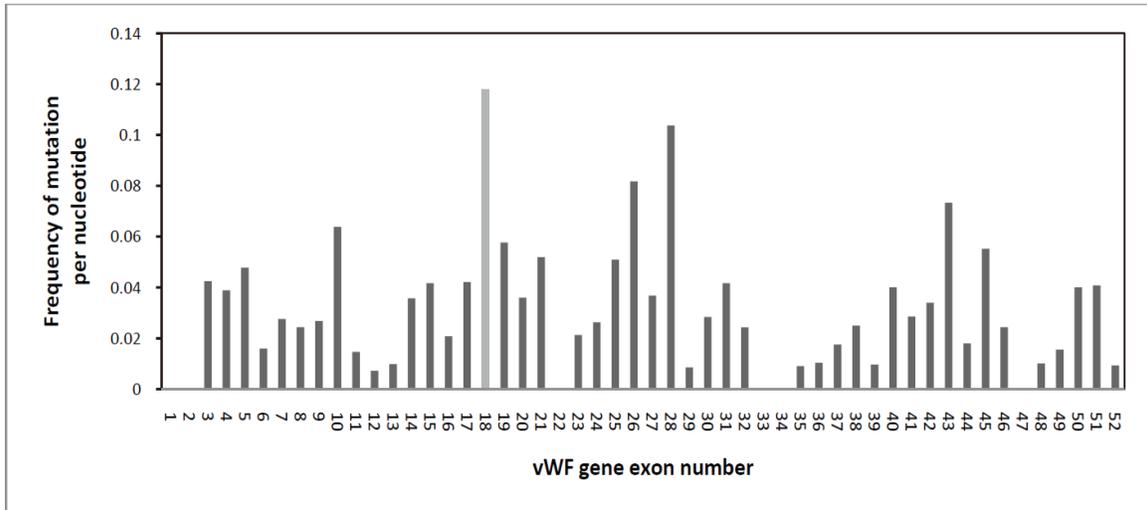


Figure 1. The frequency of mutation per nucleotide in VWF exons.

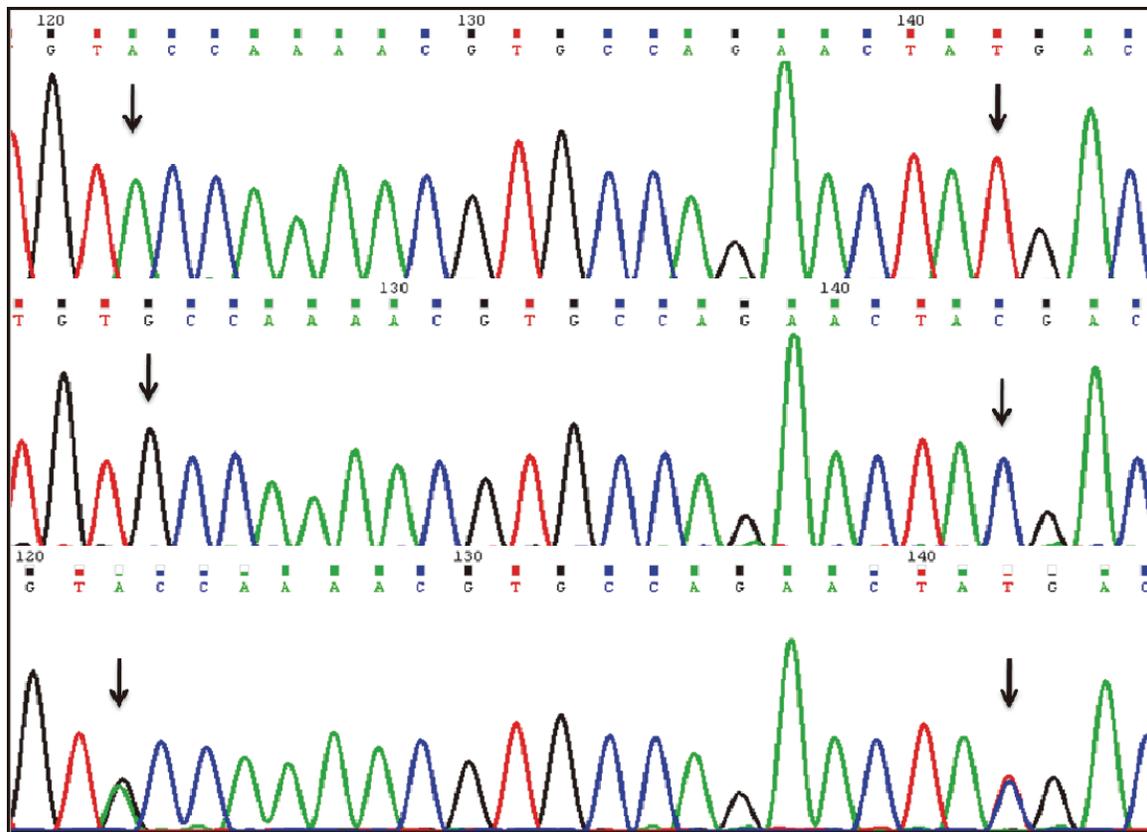


Figure 2. DNA sequencing chromatograms: homozygote 2365A and homozygote 2385T (above), homozygote 2365G and homozygote 2385C (middle), heterozygote 2365A/G and heterozygote 2385T/C (below)

normal population. Recent studies have linked either rs1063856 or rs1063857 polymorphisms to VWF plasma levels. In this study, we have evaluated the distribution and genotype correlation of these SNPs.

## Materials and methods

### Subjects

Thirty six VWD patients including 23 type 3, 8 type 2 and 5 type1 participated in this study. To estimate the frequency of the two SNPs in Iranian population, 104 healthy individuals were randomly selected from different ethnic groups. Lack of abnormal bleeding history was the main criteria for the selection of healthy individuals. To verify this, we used the standardized MCMDM-1 questionnaire in which negative scores indicate no VWD [10]. All patients and healthy individuals were notified of participating in a genetic study by obtaining written informed consent. Medical examination and coagulation analysis were performed based on standard protocols as previously described [11]. VWF:RCo and VWF:Ag ratio less than 0.7 was considered as type 2 whilst ratio more than 0.7 rated as type1.

### DNA extraction and polymerase chain reaction

5ml of peripheral blood from each patient or healthy individual was collected in EDTA anticoagulant containing tubes. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, ZistBaran, Iran) as recommended by the manufacturer's instruction.

Specific primers were designed to amplify exon 18 of VWF gene. The SNPs located on the amplicon were checked on the latest version of the single nucleotide polymorphism (SNPs) database (dbSNP 129; <http://www.ncbi.nlm.nih.gov/projects/SNP/>) along with the SNPs available on the ISTH SSC VWF Database. The primers specificity for their target sequences was evaluated on BLAST website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). PCR reactions were set up using 0.2 mM of each primers and 100 ng of genomic DNA in 2X PCR master mix (GenetBio, AryaToos, Iran) containing 1 unit of Prime Taq DNA Polymerase, Tris-HCl (pH 9.0), PCR enhancer, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 mM MgCl<sub>2</sub>, enzyme stabilizer, sediment loading dye and

2.0 mM dNTPs mixture. The PCR amplification carried out according to the following program; an initial denaturation step for 5 min at 94°C and 30 cycles of [94°C for 30 s, 57°C for 30 s and 72°C for 1 min], followed by a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 2% agarose gel.

### DNA sequencing

All PCR products were purified and sequenced on ABI sequencer. The results were analyzed using ChromasPro V.1.5 software and verified on the BLAST website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). For polymorphic amino acid residues, designations were given before the codon position number, whereas for polymorphic nucleotide, designations were given following the nucleotide position number (<http://www.VWF.group.shef.ac.uk/nomenclature.html>) (Figure 2).

### Statistical analysis

The collected data were analyzed using statistical analysis software SPSS V.16. The analyzed data were reported as odds ratio along with 95% confidence intervals (CIs). The p-values less than 0.05 were considered to be statistically significant. Hardy-Weinberg principle was calculated manually.

## Results

### Linked genotypes of rs1063856 and rs1063857 polymorphisms

The rs1063857 is located 20 nucleotides downstream to the rs1063856 SNP. In this study, the genotype pattern of the two SNPs was exactly the same in all samples. This is due to the perfect linkage of 2365A and 2365G alleles to 2385T and 2385C alleles, respectively (<http://pga.gs.washington.edu/>).

### Allele frequency and correlation study

Both gene variants were characterized in each sample. The proportional frequency of G/C alleles was 0.30 in healthy individuals and 0.23 in VWD patients. Weighted allele frequency was correlated to disease but not statistically significant (Odds ratio=1.357, CI = 0.725-2.541, P-values> 0.05).

## rs1063856 and rs1063857 SNPs of VWF gene

Distribution of both SNPs in normal population was in agreement with Hardy-Weinberg equilibrium. Most of the patients were type3 and descendent of consanguineous marriage, therefore, the data were not appropriate for the genotype and phenotype correlation study.

### *von Willebrand factor plasma levels*

The mean value of vWF-antigen level was calculated for each genotype in control subjects. There was no significant difference between vWF-antigen levels among the genotype groups (Table 1).

### Discussion

Single nucleotide polymorphisms are genetic varieties that make the diverse spectrum of susceptibility and predisposition to disease. The VWF gene has numerous known polymorphisms distributed throughout the gene. Among them rs1063856 (2365A/G, T/A789) and rs1063857 (2385T/C, Y/Y795) located on exon 18 are getting noticed in recent publications.

2365G and 2385C are conserved in the genome of all six non-human primates according to Ensemble website data base ([http://www.ensembl.org/Homo\\_sapiens/Gene/Compara\\_Alignments?g=ENSG00000110799;r=12:6058040-6233936](http://www.ensembl.org/Homo_sapiens/Gene/Compara_Alignments?g=ENSG00000110799;r=12:6058040-6233936)). As shown in Table 2, these two nucleotides were mainly converted to 2365A and 2385T in human. Another alternative nucleotide replacement (2365A/C) has been reported for 2365A/G SNP. This substitution was found as a disease-causing mutation in an English and an Iranian VWD family [12]. Our data support the mutational nature of this variant, because we observed no A→C substitution in healthy Iranian population.

The distribution of rs1063856 (2365A/G) has been studied in different human populations. The highest G allele frequency has been reported in Black North American (0.54) and the lowest in Chinese population (0.06) [13, 14]. The VWF ISTH database indicates the more frequency of 2385C allele in African compared to Asian population (0.70 in Nigerian vs. 0.04 in Chinese and 0.06 in Japanese population)

**Table 1.** Genotype frequency and VWF antigen of controls and VWD subtypes.

		2365A / 2385T 2365A / 2385T	2365A / 2385T 2365G / 2385C	2365G / 2385C 2365G / 2385C
Controls	n	52	42	10
	Mean±SD	91±8%	102±13%	96±10.5%
Type3	n	16	3	4
	Mean±SD	≤5%	≤5%	≤5%
Type2	n	5	2	1
	Mean±SD	80±14%	77±5.5%	91%
Type1	n	4	0	1
	Mean±SD	18±10.5%	-	23%

**Table 2.** The evolutionary evidence of rs1063856 and rs1063857 genetic linkage.

Homo sapiens	CTCGAGTGTACCAAAACGTGCCAGAACTATGACCTGGAG
Pan troglodytes	CTCGAGTGTGCCAAAACGTGCCAGAACTACGACCTGGAG
Pongo abelii	CTCGAGTGTGCCAAAACGTGCCAGAACTACGACCTGGAG
Macaca mulatta	CTCGAGTGTGCCAAGACGTGCCAGAACTACGACCTGGAG
Gorilla gorilla	CTCGAGTGTGCCAAAACGTGCCAGAATTACGACCTGGAG
Callithrix jacchus	CTCGAGTGTGCCAAGACGTGCCAGAACTACGACCTGGAG
Nomascus leucogenys	CTGGAGTGTGCCAAAACGTGCCAGAACTACGACCTGGAG

(<http://www.ragtimedesign.com/VWF/polymorphism/polytableresults.php>).

In this study, the frequency of 2365G allele in healthy population (0.30) was in agreement with its predicted geographical distribution. This value is between the allele frequency in European (0.35-0.45) and Indian (0.20) populations. Based on the available data, there is the same justification for the frequency of 2385C allele. Our statistical data lend support to the view that the frequency of G allele decreases from Africa to the Far East which is compatible with the out-of-Africa theory.

The 2365G homozygote genotype has been previously reported only in type 3 and type 1 VWD but not in healthy controls or type 2 patients [15]. In the present study, the homozygote 2365G genotype and therefore its counterpart, homozygote 2385C, were detected in type 2 and even in healthy individuals. Our results also revealed an association of A/T alleles to VWD.

VWF circulates in the plasma at the concentration of 5-10 µg/ml [16, 17]. The plasma level of the factor is under the influence of various inter or intragenic elements such as ABO blood groups and VWF gene polymorphisms [18-20]. Several studies have attempted to assess the significance of rs1063856 and rs1063857 SNPs in modifying plasma VWF antigen levels.

The rs1063856 showed a significant correlation with VWF concentration and the higher occurrence of coronary heart disease (CHD) in type I diabetes [21]. A similar study reported no association between this SNP and the risk of CHD in patient with type II diabetes although increased plasma VWF levels were observed in the patients [22].

In a comprehensive study published by the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium, rs1063857 variant was attributed in elevation of VWF antigen levels [23]. In a similar study, van Schie and colleagues confirmed this results and an association between rs1063857 and the risk of CHD was reported as well [24]. Using the Human CVD BeadChip in healthy individuals, Zabaneh et al, have concluded that rs1063856 affects VWF plasma concentration

[25]. The contribution of rs1063856 in plasma VWF and venous thrombosis was demonstrated in a population-based case control study [26]. Recently, the ARIC (Atherosclerosis Risk in Communities) cohort has identified rs1063857, but not rs1063856, involved in changing VWF antigen plasma levels [27]. Our study showed no significant differences between the VWF plasma levels in normal controls. It may be due to the few GG/CC genotypes among the subjects. However, it should be mentioned that the genetic linkage of the two polymorphisms, make them equally effective in the VWF biology.

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