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Nonsense mediated decay of VWF mRNA subsequent to c.7674-7675insC mutation in type3 VWD patients

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ABSTRACT

Von Willebrand disease (VWD), the most common genetic bleeding disorder, is caused by defects in Von Willebrand factor (VWF). Quantitative deficiencies of the protein lead to either VWD type3, the severe form of the disease or VWD type1 with milder clinical manifestation. Null alleles are the most common mutations in *VWF* gene causing type3. However, some of these mutations are not translated into the protein and are selectively degraded at mRNA level by nonsense-mediated decay (NMD) pathway. Here, we have studied a large VWD type3 pedigree with a premature termination codon (PTC) causing insertion mutation (c.7674-7675insC) in *VWF* exon 45. We further investigated the impact of the mutation on the VWF mRNA expression using a quantitative Real-time PCR assay and cDNA sequencing. The relative expression of the gene was significantly decreased in the patients' platelets (*Mean ratio* = 0.03 (0.01–0.05), $p = 0.001$) compared to their normal relatives. The heterozygote carriers of the mutation had lower than normal VWF mRNA levels (*Mean ratio* = 0.62 (0.29–0.91), $p = 0.006$). Direct sequencing of exon 45 on the platelet-derived cDNA in the carriers revealed only the wild-type allele confirming the decay of the mutation carrying allele. In conclusion, quantitative analysis of *VWF* gene expression showed that c.7674-7675insC mutation in *VWF* gene resulted in degradation of VWF mRNA via NMD. This pathway might play an important role in the pathogenesis of VWD characterized by quantitative deficiency of VWF due to reduced mRNA levels.

Introduction

Von Willebrand disease (VWD), the most common genetic bleeding disorder, is caused by defects in adhesive plasma glycoprotein, Von Willebrand factor (VWF). This factor is synthesized only in endothelial cells and megakaryocytes, although a cryptic transcript of the *VWF* gene is detectable in leucocytes [1,2]. Quantitative deficiencies of the protein lead to either VWD type3, the severe form of the disease or VWD type1 with milder clinical manifestation. VWD type2 is a result of qualitative defects of VWF [3].

Null alleles are the most common mutations in *VWF* gene causing type3. These mutations commonly cause lack of protein secretion by interrupting the reading frame and creating a premature termination codon (PTC) [4]. This can happen in all 52 exons of the gene and therefore, a wide variety of altered or truncated proteins might be generated. But, some of these mutations are not translated into the protein and are selectively degraded at mRNA level by nonsense-mediated decay (NMD) pathway [5]. NMD is a transcriptional regulatory mechanism which efficiently degrades mRNA with PTC to diminish the production of potentially deleterious truncated proteins. The role of this pathway in the processing and stability of the VWF mRNA following various mutations has been studied. However, the reported inconsistent data indicate that the pathway activation is dependent on the nature and the position of the mutation [2,6–8].

Here, we have studied a large type3 consanguine pedigree (Fig. 1). We found a PTC causing insertion mutation (c.7674-7675insC) in *VWF* exon 45 as the genetic defect. We further investigated the impact of the mutation on the mRNA expression and stability using a quantitative Real-time PCR assay and cDNA sequencing.

Material and methods

Subjects and sample collection

A type3 VWD consanguine family including three patients participated in this study (Fig. 1). The proband (III-1) was initially referred to the Genetic Clinic at Pasteur Institute of Iran. After obtaining an informed written consent, 13.5 ml of peripheral blood was collected from the subjects in tubes containing 1.5 ml of sodium citrate

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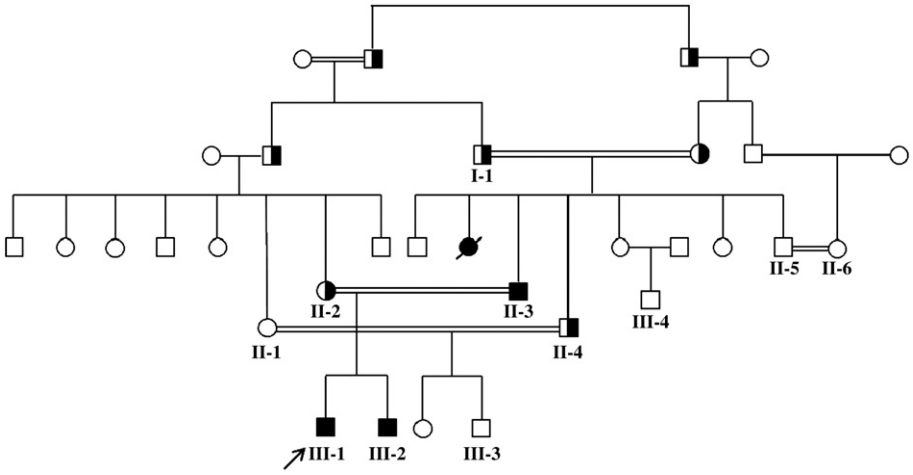

Fig. 1. The pedigree of the family affected by type3 VWD. Eleven members of a three-generation type3 VWD family were investigated. The mutation (c.7674-7675insC) was first detected by the sequencing of the whole *VWF* gene in the proband (III-1). The subjects including 3 patients, 3 carriers and 5 normal individuals were analyzed for the mutation in *VWF* gene as well as VWF mRNA expression level.

(0.109 M). Blood samples of unrelated normal individuals were also included in the study as calibrator controls.

The plasma VWF antigen (VWF:Ag) was measured using an Enzyme-linked immunosorbent assay (ELISA) [9]. The VWF activity (VWF:RCo) and coagulation FVIII activity (FVIII:C) were determined as described previously [10,11].

DNA extraction and mutation detection

Genomic DNA was extracted from 200 µl of peripheral blood using QIAamp mini kit (Qiagen). The concentration and purity of the purified DNA were determined by spectrophotometry (Nanophotometer™, Implen, Germany). High quality DNAs (A260/280 ≥ 1.8) were selected and kept at −20 °C until used for further analysis. The specification of the primers used for the amplification of mutation-containing fragment has been summarized in Table 1. The amplified fragments were sequenced on ABI 3130 Genetic Analyzer instrument. The sequencing data were verified using ChromasPro software and aligned on the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequence changes were confirmed on both strands.

RNA extraction and RT-PCR

Platelets and peripheral blood mononuclear cells (PBMCs) were isolated according to standard methods with minor modification as described previously [2,7]. Briefly, the blood samples were centrifuged at 150 × *g* for 20 min. The Platelet Rich Plasma (PRP) was removed from the upper phase and centrifuged at 1000 × *g* for 15 min to obtain platelets. To isolate PBMCs, the remaining blood samples were diluted (1:2) with PBS, overlaid on Ficoll-Plaque™ and centrifuged at room temperature at 400 × *g* for 30 min.

Platelet and PBMC pellets were treated with TRI Reagent® (SIGMA-Aldrich) as recommended by the manufacturer. The extracted RNA was further purified using RNeasy mini kit (Qiagen). The concentration and purity of the purified RNA were determined by spectrophotometry. High quality RNAs (A260/280 ≥ 1.8) were selected and kept at −80 °C until used for cDNA synthesis. Up to 1 µg RNA was converted to cDNA using Quantitect® reverse transcription kit (Qiagen) according to the manufacturer's instruction. To verify the integrity of the cDNA, a RT-PCR experiment was performed using *GAPDH* specific primers. The primers and TaqMan probes for RT-PCR and quantitation of *VWF* and *GAPDH* genes expression were designed by the Primer Express v.3.0 software (Applied Biosystems, Foster City, USA). *VWF* exon 45 on the cDNA was also amplified and undergone sequencing to determine the proportion of mutation-containing transcript in the subjects of the family. Relative allele-specific mRNA quantitation by cDNA sequencing was performed as described previously [7].

Quantitative TaqMan probe Real-time PCR assay

To improve the accuracy of the quantitation of VWF mRNA expression, we chose two target fragments spanning distinct exon–exon boundaries of the transcript as described before [12,13]. The Real-time PCR assay was performed on ABI 7300 system using SDS v.1.0.1 software. Each reaction mixture contained 5 µl cDNA, 5 pmol of each primer, 10 pmol of the specific probe and 12.5 µl of

Table 1
Characteristics of the primers and probes used for the quantitative Real-time PCR and RT-PCR assays.

Oligo name	Sequence (5′ > 3′)	Accession	Amplicon (bp)
VWF1-QRT-F	TCTGTGGATTCAGTGGATGCA	NM_000552	85
VWF1-QRT-R	CGTAGCGATCTCCAATTCCAA		
VWF1-QRT-P	FAM-CGCCAGGTCCAACAGAGTGACAGTGT-TAMRA		
VWF2-QRT-F	AGAAACGCTCCTTCTCGATTATTG	NM_000552	84
VWF2-QRT-R	TGTCAAAAAATTCCCCAAGATACAC		
VWF2-QRT-P	FAM-AGAGGCTCACTCTCTTGCCATTCTGGAAGT-TAMRA		
GAPDH-QRT-F	ACACCCACTCCTCCACCTTTG	NM_002046	112
GAPDH-QRT-R	TCCACCACCCTGTTGCTGTAG		
GAPDH-QRT-P	FAM-TGGCATTGCCCTCAACGACCACTT-TAMRA		
VWF-RT-EX45-F	AAGAGTGTCGGCTCCCAGTG	NM_000552	399
VWF-RT-EX45-R	CGTAGGCAAACATCTCCCACAAC		
VWF-PCR-EX45-F	TCCTGAGAGAAGAGCACATTCCC	NG_009072	386
VWF-PCR-EX45-R	AGATTTCGGTCCTATCCATTTCCC		