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Identification and characterisation of mutations associated with von Willebrand disease in a Turkish patient cohort

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Summary

Several cohort studies have investigated the molecular basis of von Willebrand disease (VWD); however these have mostly focused on European and North American populations. This study aimed to investigate mutation spectrum in 26 index cases (IC) from Turkey diagnosed with all three VWD types, the majority (73%) with parents who were knowingly related. IC were screened for mutations using multiplex ligation-dependent probe amplification and analysis of all von Willebrand factor gene (*VWF*) exons and exon/intron boundaries. Selected missense mutations were expressed *in vitro*. Candidate *VWF* mutations were identified in 25 of 26 IC and included propeptide missense mutations in four IC (two resulting in type 1 and two in recessive 2A), all influencing VWF expression *in vitro*. Four missense mutations, a nonsense mutation and a small in-frame insertion resulting in type 2A were also identified. Of 15 type 3 VWD IC, 13 were homozygous and two compound heterozygous for 14 candidate mutations predicted to result in lack of expression and two propeptide missense changes. Identification of intronic breakpoints of an exon 17–18 deletion suggested that the mutation resulted from non-homologous end joining. This study provides further insight into the pathogenesis of VWD in a population with a high degree of consanguineous partnerships.

Keywords

large-scale deletion; multiplex ligation-dependent probe amplification; mutation analysis; recessive 2A; von Willebrand disease

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Conflicts of interest
None declared.

Introduction

von Willebrand disease (VWD) is a common autosomally inherited bleeding disorder resulting from mutations in the von Willebrand factor (*VWF*) gene. VWD is divided into three distinct types; types 1 and 3 result from mild-moderate and severe reductions in circulating plasma VWF respectively while type 2 results from specific functional defects influencing VWF (1). Type 2 VWD is further subdivided into types 2A, 2B, 2M and 2N based on the nature of the particular functional defect (1). Population studies have estimated the prevalence of VWD to be between 1% and 1 in 10,000 (2, 3), with the majority of reported cases being diagnosed with type 1 (4). Studies performed during the past two decades have investigated the molecular basis of VWD in several populations to ascertain the mechanisms involved in the pathogenesis of this disorder and to understand how these mechanisms differ between VWD types. The majority of these studies have focused on European and North American populations where type 1 VWD cases predominate in the clinical population. However, studies of ethnic populations with a greater degree of consanguineous marriage suggest that recessively inherited forms of VWD are more prevalent (5, 6).

The aim of this study was to investigate and characterise VWD pathogenesis in a cohort of patients from Turkey. Given the high levels of consanguineous marriage reported (7, 8), it was hypothesised that recessively inherited forms of VWD would be more prevalent in this cohort and that their investigation would therefore provide additional insight into the mechanisms associated with these forms of VWD.

Materials and methods

Study population and phenotypic analysis

Following informed consent, peripheral venous blood samples and plasma were obtained in Turkey from 26 families diagnosed with VWD. The majority of index cases (IC; 73%) had parents who were either 1st cousins (n=14), 1st cousins once removed (n=1) or 2nd cousins (n=4). Only 7 IC had parents who were not knowingly related (#5, 11, 12, 17, 21, 25 and 26). Skin bleeding times (BT) were determined using a Simplate-II device (General Diagnostics, Morris Plains, NJ, USA). Blood samples and plasma were sent to Aarhus, Denmark for basic phenotypic and multimer analysis and to Sheffield, UK for genotyping and mutation detection. VWF plasma levels (VWF:Ag), ristocetin cofactor activity (VWF:RCo) and factor VIII coagulant activity (FVIII:C) were measured as previously described (9). Ristocetin induced platelet aggregation (RIPA) was performed on platelet-rich plasma using standard methods with a final ristocetin concentration of 1.2 mg/mL (10). Plasma multimers were analysed via electrophoresis on 2% or 3% (w/v) sodium dodecyl sulphate (SDS)-agarose gels and colorimetric staining (11). Four additional families from the cohort were omitted from the study due either to lack of DNA (n=2) or to insufficient phenotypic information (n=2).

Genotyping and mutation analysis

DNA was extracted from citrated blood using standard methods for all 26 IC, available family members (n=102) and 100 Caucasian healthy control individuals (HC). Genotypes were determined for ABO blood group and two $(ATCT)_n$ short tandem repeats (STR) in intron 40 (12, 13) in all 26 families as previously described (14). In addition, single nucleotide polymorphism (SNP) genotypes were determined via direct DNA sequence analysis where relevant. Initially, mutation analysis of the *VWF* promoter and all 52 exons/flanking intronic sequence was performed in IC using chemical cleavage mismatch analysis (CCMA) or conformation-sensitive gel electrophoresis (CSGE) (9). Standard PCR amplification using redesigned primers (sequences and reaction conditions available on request) followed by direct DNA sequence analysis of each amplicon was subsequently performed to confirm mutations identified and to identify additional mutations not detected using CCMA or CSGE.

Multiplex ligation-dependent probe amplification (MLPA)

MLPA, a technique based on sequence-specific probe hybridisation to genomic DNA (15), was used to screen IC and 5 HC for copy number variation (CNV) in *VWF*, utilising VWF-specific kits (P011-A1/P012-A1 and P011-B1/P012-B1; MRC-Holland b.v., Amsterdam, The Netherlands). Fragment size analysis was performed using an ABI 3730 DNA sequencer and Peak Scanner™ software v1.0 (Applied Biosystems Europe BV, Warrington, UK). Calculation of copy number ratios for each exon-specific amplicon was performed in comparison with HC using the gene dosage analysis method developed by the National Genetics Reference Laboratory (Manchester, UK; http://www.ngrl.org.uk/Manchester/publications/MLPA%20Spreadsheets).

Characterisation of the exon 17–18 deletion

The size of the exon 17–18 deletion was estimated using Southern blot analysis utilising standard procedures. Briefly, 10 μg DNA digested overnight with *Eco*RI was electrophoresed on a 0.8% (w/v) agarose gel for 18 h, blotted on Hybond-N nylon membrane (Amersham Pharmacia Biotech UK Ltd., Bucks) and VWF specific bands detected using a full length VWF cDNA probe labelled with ^{32}P α-dCTP (Amersham). Long-range PCR (primer sequences and reaction conditions available on request) utilising the Expand Long Template PCR System (Roche Diagnostics Ltd., Burgess Hill, UK) followed by direct DNA sequence analysis was used to determine breakpoints. Multiplex PCR was designed to detect the exon 17–18 deletion utilising standard *Taq* polymerase (deletion-specific forward primer: 5′-GGAAGTTGGGATGAGGTTCA-3′; wild-type forward primer: 5′-GTATCTGCTCCTGCCATCGT-3′; reverse primer: 5′-ATTCCCTGCAGCATCTCTGT-3′).

Expression of recombinant VWF containing p.S49R or p.R324Q

The expression vector containing wild-type full length VWF cDNA (pSVH vWF1; WT) was obtained as described (9). The expression vector containing c.147C>A (pVWF-S49R) was constructed via mutagenesis of shuttle vector pXbaI, which contained ~450 bp of WT (encompassing exons 2–4) obtained by *Xba*I digestion cloned into pGEM-3Z (Promega UK

Ltd., Southampton, UK). The expression vector containing c.971G>A (pVWF-R324Q) was constructed via mutagenesis of shuttle vector pKpnI, which contained ~4.5 kb of WT (encompassing exons 4–28) obtained by *Kpn*I digestion cloned into pGEM-3Z. Site-directed mutagenesis of pXbaI and pKpnI was performed using the GeneEditor™ system (Promega) and mutagenic oligonucleotides 5′-TTGATGGGAGAATGTACAGCT-3′ (c.137_157 of *VWF*; pXbaI) and 5′-GTCAGGAGCAATGCGTGGATG-3′ (c.962_982 of *VWF*; pKpnI). Mutant fragments were sub-cloned back into WT VWF cDNA and sequence analysis confirmed the presence of each mutation in the p.VWF-S49R and p.VWF-R324Q vectors.

Expression of WT, p.VWF-S49R and p.VWF-R324Q in COS-7 cells, steady-state analysis of VWF secretion and multimer analysis of secreted VWF concentrated from COS-7 growth medium following transfection with WT or p.VWF-S49R was performed as described (9). Multimer analysis of secreted VWF concentrated from COS-7 growth medium following transfection with WT and/or pVWF-R324Q was performed in Hamburg, Germany via electrophoresis on medium resolution (1.5% w/v) SDS-agarose (16).

In silico analysis

The effect of candidate missense mutations on VWF was predicted using three online protein prediction tools: Align-Grantham Variation Grantham Deviation (A-GVGD) (17), PolyPhen-2 (18) and Sorting Intolerant From Tolerant (SIFT) (19). The effect of candidate splice mutations was predicted using six online prediction tools (20). Multi-species protein alignment was performed using ClustalW2 (21). All *in silico* analysis was performed in June 2011. Sequence numbering followed Human Genome Variation Society nomenclature with ‘ A’ of the first methionine termed nucleotide 1 (nucleotide RefSeq NM_000552.3; amino acid RefSeq NP_000543.2).

Results

Families from all regions of Turkey were referred to the Pediatric Hematology Unit, Ankara for diagnosis. Bleeding history in the IC, family history of bleeding, bleeding time (BT), VWF:Ag, VWF:RCo and FVIII:C levels were taken into account when diagnosing VWD. BT comprised part of the initial diagnosis of patients with IC considered to have VWD if BT was ≥6 min. IC were initially diagnosed with type 1 VWD (n=5), type 2 VWD (n=6) and type 3 VWD (n=15), but final diagnosis was altered in five IC following detailed phenotypic and mutation analysis (Table 1). Mutation analysis identified 24 different mutations in 25/26 (96%) of IC, each of which segregated with disease phenotype (Table 1).

Type 1 VWD

Mutations were identified in all five IC initially diagnosed with type 1 VWD. Four of the mutations resulted in missense alterations within the VWF propeptide; p.S49R, p.R273W and p.R324Q. The p.R273W mutation was recessively inherited in two IC, both of whom had a severe type 1 phenotype (VWF:Ag 6 and 9 IU/dL, VWF:RCo 6 and 4 IU/dL respectively). The other two mutations were dominantly inherited and associated with a mild type 1 phenotype. The remaining IC was heterozygous for a p.Y1456* nonsense mutation.

Previous functional analyses investigating the influence of the p.R273W mutation on VWF storage and secretion have confirmed that it causes severe intracellular retention of VWF *in vitro* (14, 22, 23), however the other two propeptide mutations had not been previously characterised. Both mutations were shown to affect poorly conserved VWF residues (Fig. S1) and *in silico* analysis predicted that both mutations would be benign. *In vitro* characterisation of recombinant VWF containing each mutation highlighted that both mutations had a significant influence on VWF expression (Fig. 1). Expression of p.S49R resulted in a significant increase in the amount of VWF secreted from the cell compared to WT (100%) both when expressed in homozygous form (139%) and heterozygous form (109%; Fig. 1). Conversely, expression of p.R324Q caused a significant retention of VWF within the cell compared to WT (100%) when expressed in homozygous (113%) and heterozygous (106%) forms (Fig. 1). Neither IC had abnormal multimers (Table 1; Fig. 1); however when p.S49R was expressed alone *in vitro*, a reduction in high molecular weight multimers was observed. Multimer profiles following *in vitro* expression of p.R324Q were normal (Fig. 1).

Type 2 VWD

IC diagnosed with type 2 VWD were initially classified as having either 2A or 2N VWD. The two IC with type 2N were homozygous for missense mutations located in the D′ (p.C788R) and D3 (p.C1225G) domains respectively. When compared against normal plasma (100%), patient plasma had reduced FVIII binding capacity (VWF:FVIIIB) similar to that observed in plasma from a known type 2N patient homozygous for p.T791M (p.C788R homozygote 0% (heterozygote 49%) vs. 1.3%; p.C1225G homozygote 7.1% vs. 7.1%) (9, 24). Previous investigations have also shown that these mutations impair VWF secretion and FVIII binding *in vitro*, confirming that both account for the observed disease phenotype (9, 23). Mutations were identified in three of the four IC with type 2A VWD. Each had a classic dominantly inherited 2A phenotype. One IC had the common p.R1597W missense mutation located in the VWF A2 domain (25). The other two IC had novel mutations; a p.L1657H missense mutation in the A2 domain and a small 9 bp duplication resulting in a three amino acid insertion in the A1 domain (p.Y1271_C1272insYFY) respectively. A p.L1657I mutation has been previously described in type 2A VWD (26) and substitution of p.C1272 has been reported several times in type 2A (25).

Despite performing MLPA and sequence analysis of all 52 exons and closely flanking intronic sequence, no mutation was identified in the remaining IC diagnosed with type 2A VWD. Notably, STR/SNP genotyping of the IC and their parents was consistent with an autosomal recessive inheritance pattern (data not shown) and the multimer profile observed in the IC correlated well with multimer patterns reported in type 2A(IIC) patients (Fig. 2) (27).

Type 3 VWD

Mutations were identified on both alleles in all IC diagnosed with type 3 VWD, the majority of whom were homozygous for a single point mutation (13/15; 87%). Most candidate mutations (n=16) were predicted to result in null alleles and included nonsense (n=6), frameshift (n=4) and splice mutations (n=3) plus a deletion of exons 17–18, whilst two were

missense mutations. Only four of these mutations had been reported previously (Table 1) (25, 28–30).

In silico analysis of the three candidate splice mutations predicted that they would result in exon skipping (n=2) or intron retention (n=1) resulting in a frameshift (Table 1). A homozygous deletion of exons 17–18 was identified during PCR analysis of VWF in IC #3 through lack of amplification of these exons. Southern blot analysis of genomic DNA highlighted a novel ~8 kb band (Fig. 3A) indicating that the deletion encompassed the *Eco*RI restriction enzyme site within intron 18 and at least part of intron 16. MLPA analysis confirmed the deletion was recessively inherited (Fig. 3B) and long-range PCR followed by direct DNA sequencing mapped the breakpoints (c.2186+2253_2442+3683del, predicted to result in p.C729Wfs*29) and indicated that the deletion was 9683 bp in size (Fig. 3C).

Two type 3 VWD IC were homozygous for missense mutations in the VWF propeptide. One IC had the p.R273W mutation which has been characterised and shown to result in intracellular retention (14, 22, 23). The remaining IC had a novel p.R34G mutation. p.R34 is completely conserved across species (Fig. S1) and *in silico* evolutionary conservation analysis predicted that the mutation would have a detrimental effect on the VWF protein.

Phenotype of heterozygous type 3 VWD parents

Both parents were available for all fifteen type 3 VWD IC. Only three individuals (10%) had increased bleeding times (6, 7 and 13 min), but VWF:RCo and VWF:Ag were reduced below the normal range in 15 (52%) and 13 (43%) of individuals analysed respectively (Table 2). Mean VWF:RCo/VWF:Ag ratio was 1.0 for all individuals analysed. None of these 30 heterozygous carriers of 16 different mutations had abnormal multimer profiles (Table 2). Fourteen individuals reported one or more bleeding symptoms (47%), but their VWF levels were not significantly different to those of 16 individuals who did not report symptoms (Table 2). It has previously been observed that heterozygous carriers of type 3 VWD mutations have significantly raised ratios of FVIII:C to VWF:Ag (31, 32). In this cohort, FVIII:C/VWF:Ag ratio (mean 1.4) was increased (1.5–2.3) in 43% of type 3 VWD carriers. Notably an increased ratio was not observed in splice mutation carriers (mean FVIII:C/VWF:Ag ratio 0.9) with four of four carriers having normal ratios, whereas in nonsense/frameshift mutation carriers 11 of 21 (52%) had raised ratios (mean FVIII:C/VWF:Ag ratio 1.5; Table 2).

Discussion

To date there have been few reports concerning VWD in populations with high rates of consanguinity. In this study, a cohort of 26 Turkish IC diagnosed with VWD types 1–3 and their family members had phenotypic and genotypic investigation of their VWD to understand the mutations responsible and the consequences of those mutations. Prolonged BT in all IC may have enhanced the selection of cases with significant bleeding, leading to the high detection rate for mutations in this cohort. At the time of recruitment VWF collagen binding (VWF:CB) assays were not routinely performed, so unfortunately VWF:CB data (which might have allowed additional characterisation of VWD phenotype) was unavailable for this cohort.

Four of the five type 1 VWD mutations were missense and all occurred in the VWF propeptide. p.R273W has previously been shown to result in intracellular retention (14, 22, 23), and *in vitro* characterisation carried out in this study suggests that both the p.S49R and p.R324Q mutations also affect VWF expression. In addition to the three missense mutations reported here, 36 missense mutations have been reported within the VWF propeptide to date (25, 33). Six of these (p.D141Y, p.G160W, p.N166I, p.C275R/S and p.R760C) have also been shown to affect VWF expression *in vitro* when expressed in the heterozygous form (25, 33). In comparison to these mutations and p.R273W, the effect of p.S49R and p.R324Q on VWF expression is not as pronounced. However, all these previously reported mutations affect VWF residues completely conserved across species, unlike p.S49R and p.R324Q, suggesting that disruption of p.S49 and p.R324 residues is likely to be less detrimental.

In vitro expression of recombinant p.S49R showed a significant increase in VWF secreted from the cell compared with WT both when expressed in the homozygous form or when co-expressed in the heterozygous form with WT, although in both cases there was no significant difference in VWF retained within the cell (Fig. 1). The IC had a normal multimer profile, however homozygous expression of recombinant p.S49R appeared to indicate a loss of high molecular weight multimers (multimer analysis of recombinant p.S49R/WT was not performed). p.S49R may therefore interfere with the VWF multimerisation process within the cell resulting in only low molecular weight forms of VWF being secreted. Increased secretion of VWF has not been previously reported, however mutations affecting amino acid substitutions in proximity to p.S49 have not been functionally characterised (25, 33). Although VWF:Ag level in the IC (58 IU/dL) was within the normal range observed in this study (46–146 IU/dL), the IC suffered from frequent epistaxis and prolonged bleeding from superficial wounds. The IC also had blood group O and combined with p.S49R this may be sufficient to explain the mild bleeding phenotype observed. In addition, p.S49R was not present when screened in 35 HC and 35 unrelated Turkish family members, and has not been reported by HapMap (34), the 1000 Genomes Project (35) or in African-American HC (36), which suggests that this variant is unlikely to be a polymorphism.

Expression of recombinant p.R324Q resulted in a significantly reduced VWF secretion and increased intracellular retention of VWF both when expressed in homozygous or heterozygous forms, although recombinant p.R324Q still formed normal multimers (Fig. 1). p.R324Q is therefore likely to result in type 1 VWD through impaired secretion of VWF, similar to several other type 1 VWD mutations (37).

Interestingly, in this cohort, p.R273W was initially associated with both recessive type 1 and type 3 VWD. Of note, a recessive type 2A VWD patient, compound heterozygous for missense mutations p.C275R and p.P1337L, has been described with a similar phenotype to both IC initially diagnosed as recessive type 1 (VWF:Ag 7 IU/dL, VWF:RCo 2 IU/dL, FVIII:C 32 IU/dL, BT 11 min, absent high molecular weight multimers); the p.C275R mutation resulting in intracellular retention of VWF and contributing to the abnormal multimer profile (38). These two IC may therefore be more accurately classified as having recessive 2A VWD (despite IC #2 having a VWF:RCo/VWF:Ag ratio of 1.0 as the ratio may be misleading when VWF:RCo is <10 IU/dL) and re-examination of the multimer

profile in one of these IC supported a reclassification. The difference in phenotype between the three p.R273W IC cannot be explained by ABO blood group, as both the type 3 and one of the type 2A IC had blood group A (14). Heterozygous carriers of this mutation did not report a bleeding phenotype (Table 2). It is possible that this phenotypic difference between IC is due to other genetic influences, for example polymorphic variation within the *VWF* locus or other genes (39, 40). However, DNA sequence analysis of *VWF* did not reveal differences between *VWF* haplotypes in the IC (14).

One IC (initially classified as type 1) was heterozygous for a p.Y1456* nonsense mutation and had a severe phenotype (VWF:Ag 15 IU/dL, VWF:RCo <3 IU/dL, BT >20 min, abnormal multimers). This nonsense mutation has previously been reported in a compound heterozygous type 3 VWD patient and the familial carrier of this mutation had normal VWF levels and no bleeding symptoms (41). A further unidentified mutation in addition to this nonsense mutation must occur in this IC to explain the severe phenotype observed and this IC could also be more accurately classified as having recessive 2A VWD. No other mutation was identified and no further DNA is available for this IC. Linkage analysis indicated that the IC inherited the mutation from their father, from whom no DNA or phenotypic data was collected.

Two IC were homozygous for novel missense mutations (p.C788R and p.C1225G respectively) and initially diagnosed with type 2N VWD based on impaired VWF:FVIIIB results (9), despite both IC having FVIII:C levels similar or higher than those recorded for VWF:Ag and VWF:RCo. Interestingly, one of these mutations (p.C1225G) has recently been identified in two German patients classified with type 2A VWD because the mutation had no detectable effect on VWF:FVIIIB (42). As mentioned by Yadegari and colleagues (42), it seems likely that p.C1225G may belong to a group of mutations that have pleiotropic effects on VWF function, and p.C788R may also fall into this category.

This study failed to identify a mutation in IC #4 who had an apparent recessive type 2A(IIC) VWD phenotype and in whom pedigree analysis indicated homozygous inheritance. Although rare, several 2A(IIC) mutations have been reported clustered within the D2 domain encoded by exons 11–17 (25, 43) and the phenotype in the IC resembles those previously reported. Despite analysis of DNA from the IC using both MLPA and direct DNA sequencing, no evidence of CNV or any mutation in exons 1–52 was identified. The possibility that the IC had a homozygous deep intronic mutation within this region affecting mRNA splicing cannot be dismissed (44). To result in a 2A(IIC) phenotype, a candidate mutation would have to result in the production of an in-frame altered VWF protein disrupting VWF multimerisation. An intronic mutation leading to creation of a cryptic splice site and introduction of an additional in-frame exon is a possible explanation, similar to recently reported *F8* intronic mutations causing mild haemophilia A (45). Unfortunately, mRNA from the IC was unavailable and further analysis would therefore need to focus on analysis of genomic DNA for variation in introns 10–17, which range in size from ~750 bp up to 6 kb. DNA sequence analysis of those introns ≤1.2 kb in size (i.e. introns 11, 12 and 14) revealed no candidate mutation in the IC.

The majority of IC had type 3 VWD. In total, 16 different mutations were found in this group, 87% predicted to result in a null allele (6 nonsense, 5 frameshift and 3 splice), comparable with other type 3 studies (43). A homozygous p.P2063S missense change was initially identified in IC #9. Although p.P2063S has a reported population frequency of 2.5% (46) and was previously considered to be a mutation (25), it does not influence VWF expression and is now considered to be a polymorphism (37). Recent DNA sequence analysis identified a homozygous p.Q1734* nonsense mutation in IC #9 (Table 1), providing further indication that p.P2063S is not responsible for the type 3 phenotype. No VWF inhibitor history was reported and no *de novo* mutations were identified in any of the type 3 VWD IC.

A previously unreported exon 17–18 deletion was mapped to breakpoints within intron 16 (c.2186+2253) and intron 18 (c.2442+3683) allowing design of a specific multiplex PCR to detect this deletion (Fig. 3D). Previously reported large deletions in type 3 VWD have been shown to occur via homologous recombination involving *Alu* repetitive elements (43), however breakpoints for this deletion occurred within a L2b long interspersed nuclear element (LINE) and within a MIRb short interspersed nuclear element (SINE) respectively. Notably, it has been shown that non-homologous recombination can occur between different repetitive elements via a mechanism of non-homologous end-joining due to microhomology around the breakpoints (47), most recently in CNV events observed in haemophilia A (48). The sequence at the exon 17–18 deletion breakpoints has CC dinucleotide microhomology and there are motifs associated with DNA recombination located within 75 bp of each breakpoint, i.e. deletion hotspot consensuses, DNA polymerase arrest sites and DNA polymerase a/b frameshift hotspots (Fig. 3C) (49, 50). The deletion is therefore proposed to have resulted from non-homologous end joining. It leads to introduction of a premature stop codon (p.C729Wfs*29) and is likely to result in nonsense mediated decay, and thus a null allele.

In conclusion, this study provides further insight into the pathogenesis of VWD. The observations of recessive type 2A VWD, including type 2A(IIC), and a high proportion of type 3 VWD cases in this study highlights the contribution of consanguinity to VWD and provides additional insight into the mechanisms associated with VWD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is known about this topic?

- von Willebrand disease (VWD) is a common autosomally inherited bleeding disorder with a population prevalence between 1% and 1 in 10,000.
- In Europe and North America, dominantly inherited forms of VWD, especially type 1 VWD, predominate in the clinical population.
- Studies of ethnic populations with a greater degree of consanguineous marriage suggest that recessively inherited forms of VWD are more prevalent.

What does this paper add?

- Investigation of a cohort of VWD families demonstrating a high proportion of consanguineous marriage identifies a significant proportion of rare VWD forms confirming the contribution of consanguinity to VWD severity.
- Eighteen novel VWD mutations were identified in this patient cohort including three missense mutations in the von Willebrand factor propeptide and a large deletion of exons 17–18.
- Investigation of these novel mutations and rarer forms of VWD provides further insight into the pathogenesis of VWD and the associated disease mechanisms.

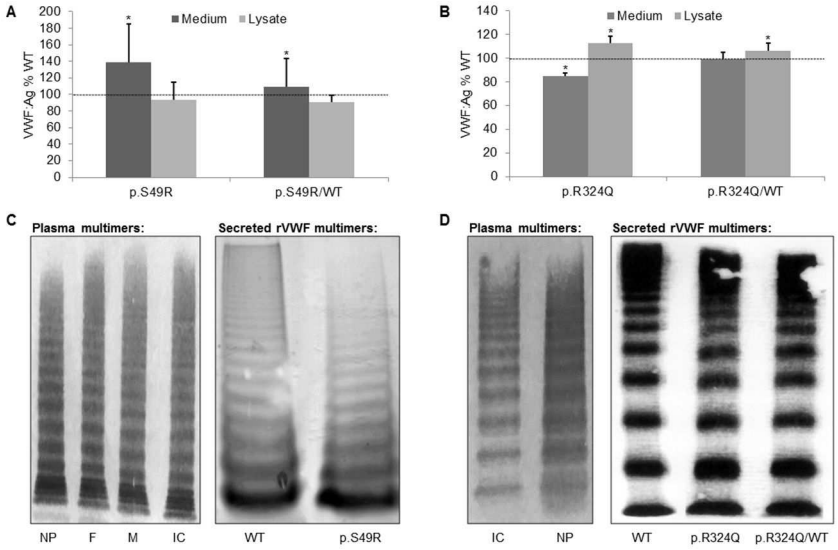


Figure 1.

Recombinant VWF (rVWF) expression in COS-7 cells (mean values from six independent experiments). A and B) Mutant rVWF (p.S49R and p.R324Q respectively) secreted into the culture medium and retained within the cell relative to WT rVWF only (100%). C and D) Comparison of patient multimer profile (electrophoresed on 2% SDS-agarose) and multimer profile of secreted rVWF (1.5% SDS-agarose) observed for p.S49R (rVWF in homozygous state only) and p.R324Q respectively (IC, index case; F, father; M, mother; NP, normal plasma; *$p<0.05$, two-tailed Student' s t-test).

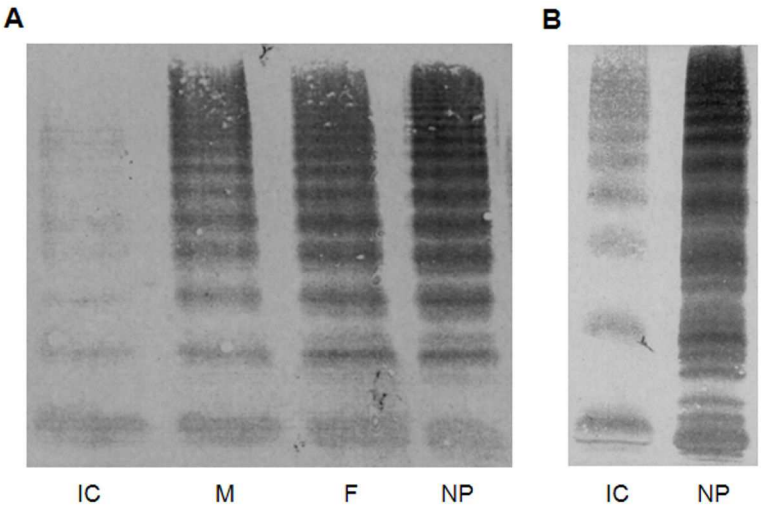
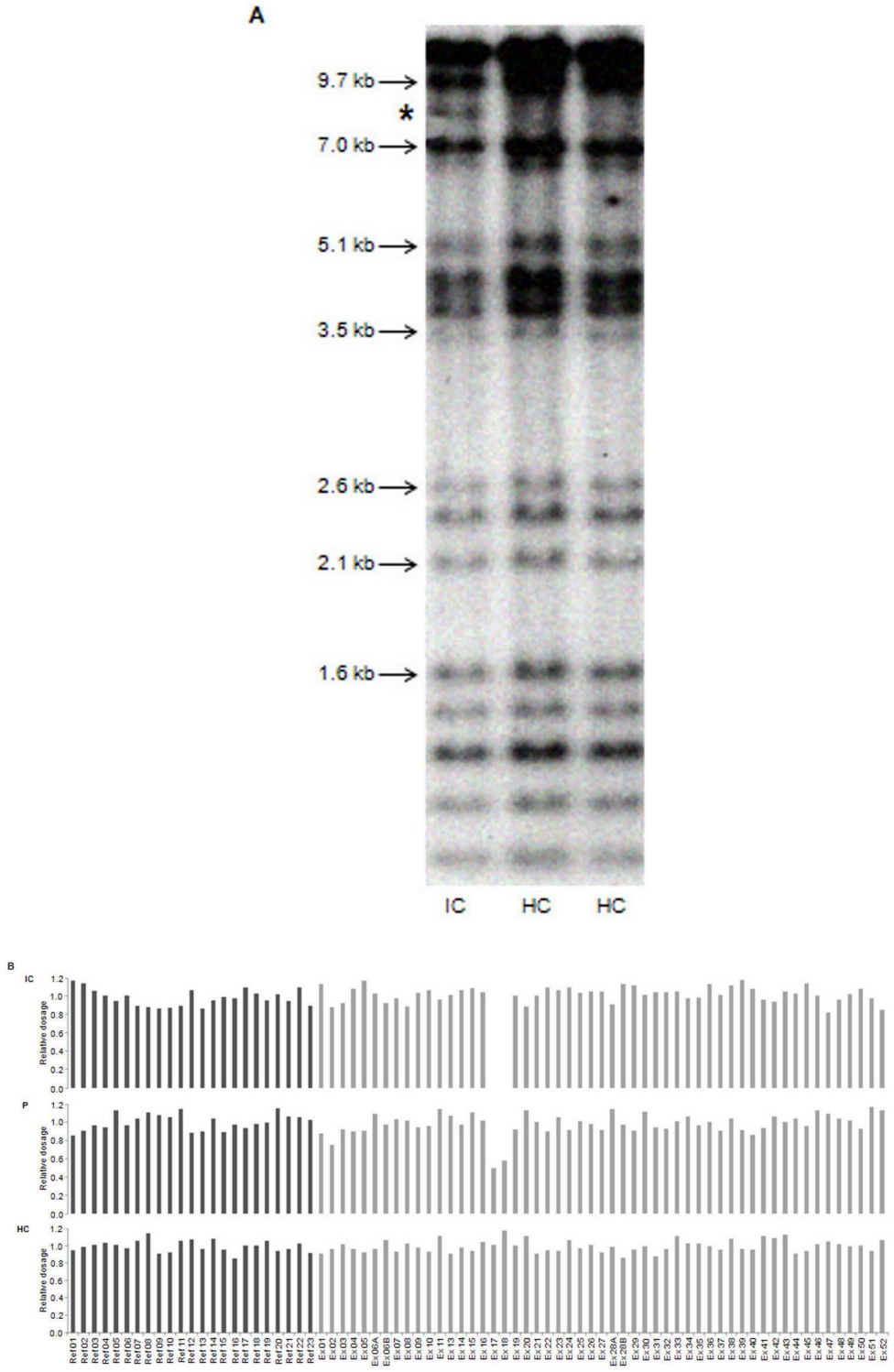


Figure 2.

Multimer profiles observed in IC #4 with 2A(IIC) VWD and their parents (M, mother: VWF:Ag 60 IU/dL, VWF:RCo 36 IU/dL, FVIII:C 98 IU/dL, no bleeding symptoms; F, father: VWF:Ag 26 IU/dL, VWF:RCo 30 IU/dL, FVIII:C 107 IU/dL, moderate bleeding symptoms) when electrophoresed on 2% (A) or 3% (B) SDS-agarose (NP, normal plasma).



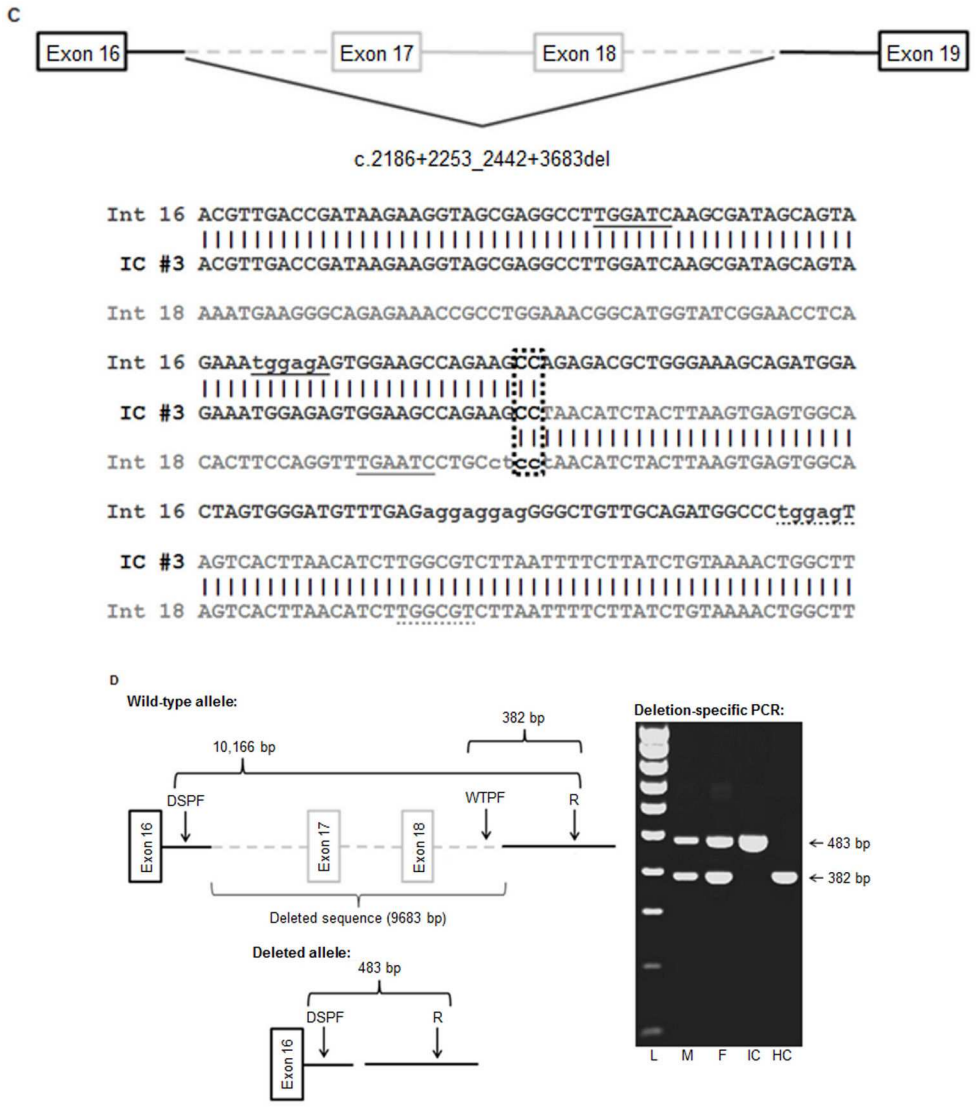


Figure 3.

Characterisation of the exon 17–18 deletion. A) Southern blot analysis of *Eco*RI digested DNA highlighting the novel ~8 kb fragment (*) observed in IC #3 but neither HC. B) Confirmation of the exon 17–18 deletion following dosage analysis by MLPA in the index case (IC), a heterozygous parent (P) and a healthy control (HC). C) Deletion breakpoints in intron 16 and intron 18 highlighting 2 bp microhomology (dotted border), deletion hotspot consensus sequences (solid underline), DNA polymerase arrest sites (lowercase) and DNA polymerase a/b frameshift hotspots (dotted underline) close to the breakpoint junctions. D) Multiplex PCR designed to detect the exon 17–18 deletion (DSPF, deletion-specific forward primer; WTPF, wild-type forward primer; R, reverse primer) further confirming the homozygous inheritance in the index case (IC) and heterozygous inheritance in both parents (F and M).

Table 1

Candidate mutations and phenotype in the 26 Turkish VWD index cases following all analyses

VWD type	IC[a]	Candidate mutation(s)[b]		VWF:RCo (IU/dL)	VWF:Ag (IU/dL)	VWF:RCo/ VWF:Ag	FVIII:C (IU/dL)	ABO genotype	Bleeding time (min)	RIPA (1.2 mg/mL)	Multimers[c]
		Allele 1	Allele 2								
1	26	c.147C>A (p.S49R)	-	ND	58	-	ND	O/O	8	R	NM
1	22	c.971G>A (p.R324Q)	-	56	48	1.2	70	O/O	10	R	NM
2A	4	-	-	<3	11	0.3	10	O/O	>20	ND	2A(IIC)[f]
2A[d]	2	c.817C>T (p.R273W)	c.817C>T (p.R273W)	6	6	1.0	20	O/O	15	No agg	ND
2A[d]	8	c.817C>T (p.R273W)	c.817C>T (p.R273W)	4	9	0.4	21	A/A	15	No agg	2A(unspecified)[f]
2A	17	c.3806_3814dup (p.Y1271_C1272insYFY)	-	12	35	0.3	20	ND	>20	R	2A(sm)[f]
2A[d]	12	c.4368C>G (p.Y1456*)	-	<3	15	0.2	18	O/O	>20	No agg	2A(unspecified)[f]
2A	25	c.4789C>T (p.R1597W)	-	17	35	0.5	40	A/O	15	R	2A(IIA)[f]
2A	21	c.4970T>A (p.L1657H)	-	7	15	0.5	10	A/O	>20	No agg	2A(IIA)[f]
2A[e]	18	c.2362T>C (p.C788R)	c.2362T>C (p.C788R)	10	18	0.6	15	O/O	10	R	Dimers[g]
2A[e]	24	c.3673T>G (p.C1225G)	c.3673T>G (p.C1225G)	<3	7	0.4	22	B/B	>20	R	Dimers[g]
3	15	c.100C>G (p.R34G)	c.100C>G (p.R34G)	<3	2	NA	1	B/O	>20	No agg	Ab
3	6	c.171C>A (p.C57*)	c.171C>A (p.C57*)	3	<1	NA	2	B/O	>20	No agg	ND
3	20	c.817C>T (p.R273W)	c.817C>T (p.R273W)	<3	<1	NA	9	A/A	>20	No agg	Ab
3	23	c.1730-1G>C (p.T577Sfs*10)	c.7448dupA (p.Y2483*)	<3	<1	NA	2	A/A	>20	No agg	ND
3	3	c.2186+2253_2442+3683del (p.C729Wfs*29)	c.2186+2253_2442+3683del (p.C729Wfs*29)	ND	<1	-	4	A/O	>20	No agg	Ab
3	1	c.2443-1G>C (p.V815Cfs*15)	c.2443-1G>C (p.V815Cfs*15)	<3	<1	NA	8	B/O	>20	No agg	ND
3	5	c.2641delC (p.L881Sfs*28)	c.7130dupC (p.H2378Afs*13)	3	<1	NA	4	O/O	>20	No agg	Ab
3	19	c.2733dupT (p.K912*)	c.2733dupT (p.K912*)	<3	3	NA	5	O/O	>20	No agg	Ab
3	11	c.3385_3386delAG (p.S1129Lfs*12)	c.3385_3386delAG (p.S1129Lfs*12)	<3	<1	NA	5	A/O	>20	No agg	Ab
3	13	c.4413dupC (p.D1472Rfs*40)	c.4413dupC (p.D1472Rfs*40)	<3	1	NA	9	A/O	>20	No agg	Ab
3	14	c.5053+1G>T (p.D1685Tfs*8)	c.5053+1G>T (p.D1685Tfs*8)	<3	2	NA	5	A/O	>20	No agg	Ab
3	9	c.5200C>T (p.Q1734*)	c.5200C>T (p.Q1734*)	ND	<1	-	2	O/O	>20	No agg	ND
3	7	c.7176T>G (p.Y2392*)	c.7176T>G (p.Y2392*)	ND	<1	-	4	O/O	>20	No agg	Ab

VWD type	IC[a]	Candidate mutation(s)[b]		VWF:RCo (IU/dL)	VWF:Ag (IU/dL)	VWF:RCo/ VWF:Ag	FVIII:C (IU/dL)	ABO genotype	Bleeding time (min)	RIPA (1.2 mg/mL)	Multimers[c]
		Allele 1	Allele 2								
3	10	c.7176T>G (p.Y2392*)	c.7176T>G (p.Y2392*)	ND	<1	-	4	A/O	>20	No agg	Ab
3	16	c.7603C>T (p.R2535*)	c.7603C>T (p.R2535*)	<3	<1	NA	<1	A/O	>20	No agg	Ab
			Normal range:	50–172	46–146	-	52–140	-	<6	-	-

Ab, absent; No agg, no aggregation at 1.2mg/mL ristocetin; NM, normal multimers; R, reduced; ND, not determined; NA, not appropriate to calculate.

[a] *In vitro* characterisation of IC 2, 8, 18, 20 and 24 has previously been reported (9, 14);

[b] Mutations c.2443-1G>C, p.Y1456*, p.D1472Rfs*40, p.R1597W, p.H2378Afs*13 and p.R2535* previously reported (25, 28–30, 41);

[c] Multimers electrophoresed on 2% SDS-agarose;

[d] Previously classified as type 1 VWD;

[e] Previously classified as type 2N VWD

[f] Based on multimer phenotypes described by Budde *et al.* (16);

[g] Only dimers visible.

Table 2

Phenotype in type 3 VWD mutation carriers

Mutation type	Mutation	VWF:RCo (IU/dL)	VWF:Ag (IU/dL)	VWF:RCo/ VWF:Ag	FVIII:C (IU/dL)	FVIII:C/ VWF:Ag	ABO genotype	Bleeding time (min)	RIPA (1.2 mg/mL)	Multimers[a]	Bleeding symptoms[b]
Missense	p.R34G	60	72	0.8	90	1.3	B/O	3	R	ND	E
		65	86	0.8	90	1.0	O/O	7	R	ND	B, E, M, P
	p.R273W	60	47	1.3	70	1.5	A/A	3	ND	NM	None
		ND	79	ND	ND	ND	A/A	ND	ND	NM	None
Nonsense	p.C57*	50	33	1.5	47	1.4	B/O	2	R	NM	None
		36	42	0.9	98	2.3	A/O	4	R	NM	None
	p.K912*	24	36	0.7	60	1.7	O/O	5	N	ND	None
		26	44	0.6	50	1.1	O/O	6	N	ND	None
	p.Q1734*	30	39	0.8	60	1.5	O/O	3	R	NM	None
		52	47	1.1	110	2.3	O/O	4	R	NM	None
	p.Y2392*	20	30	0.7	30	1.0	O/O	4	ND	NM	Mild
		30	30	1.0	60	2.0	O/O	5	ND	NM	Mild
		82	47	1.7	100	2.1	O/O	5	R	NM	Mild
		59	57	1.0	59	1.0	A/O	13	R	NM	Mild
	p.Y2483*	46	60	0.8	94	1.7	A/O	1	R	NM	S
	p.R2535*	75	118	0.6	100	0.8	A/O	4	R	NM	None
		35	134	0.3	105	0.8	A/O	5	R	NM	None
Frameshift	p.C729Wfs*29	43	45	1.0	90	2.0	A/O	2	R	NM	E
		48	74	0.6	100	1.4	A/B	3	R	NM	None
	p.L881Sfs*28	38	41	0.9	25	0.6	O/O	5	R	NM	Mild
	p.S1129Lfs*12	21	36	0.6	60	1.7	O/O	4	R	NM	None
		84	64	1.3	65	1.0	A/O	5	R	NM	None

Mutation type	Mutation	VWF:RCo (IU/dL)	VWF:Ag (IU/dL)	VWF:RCo/ VWF:Ag	FVIII:C (IU/dL)	FVIII:C/ VWF:Ag	ABO genotype	Bleeding time (min)	RIPA (1.2 mg/mL)	Multimers[a]	Bleeding symptoms[b]
Splice	p.D1472Rfs*40	72	66	1.1	100	1.5	A/A	2	N	NM	None
		70	64	1.1	102	1.6	O/O	4	ND	ND	D, M
	p.H2378Afs*13	76	54	1.4	55	1.0	A/O	3	N	NM	None
	c.1730-1G>C	100	62	1.6	ND	ND	A/O	3	N	NM	None
	c.2441-1G>C	104	51	2.0	43	0.8	A/O	2	N	NM	Mod
		35	43	0.8	38	0.9	B/O	5	R	NM	Mod
	c.5053+1G>T	32	40	0.8	37	0.9	A/A	3	R	NM	Mod
		24	41	0.6	40	1.0	A/O	4	No agg	NM	Mod
	Normal range:	50–172	46–146	ND	52–140	ND	-	<6	-	-	-

No agg, no aggregation at 1.2mg/mL ristocetin; N, normal; NM, normal multimers; R, reduced; ND, not determined.

[a]Multimers run on 2% SDS-agarose.

[b]Bleeding symptoms reported by referring clinician: B, bruising; D, dental bleeding; E, epistaxis; M, menorrhagia; P, post-partum bleeding; S, surgical bleed; Mild; mild bleeding; Mod, moderate bleeding.