Significant linkage and non-linkage of type 1 von Willebrand disease to the von Willebrand factor gene

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Summary

Significant linkage of types 2A and 2B von Willebrand disease (VWD) to the von Willebrand factor (VWF) gene have been reported, as well as mutations in the VWF gene. However, data for the partial quantitative variant are less consistent. An inconsistency of association between the type 1 VWD phenotype and genotype has been reported recently. We undertook linkage analysis of 12 families with definite or possible type 1 VWD patients. One family with classic type 1 VWD had a high lod score ($Z = 5.28$, $\theta = 0.00$). A total lod score of 10.68 was obtained for the four families with fully penetrant disease. In two families linkage was rejected, while three families did not show conclusive evidence of linkage. This study corroborates ABO blood group influence, especially in patients with mild deficiencies and/or incomplete penetrance. Indirect genetic analysis may be an option for diagnosing asymptomatic or presymptomatic type 1 VWD carriers, particularly in families showing higher penetrance. The study indicates defects of the VWF locus are to be expected in more than half of the families studied. However, as defects at different loci may be the cause of this phenotype, the results of the segregation analyses should be interpreted with caution, especially in studies involving small families, or mild expressions of the disorder or incomplete penetrance

Keywords: genetic linkage, von Willebrand disease, type 1 VWD, microsatellites, VWF gene.

INTRODUCTION

Von Willebrand disease (VWD) is currently one of the most frequent hereditary bleeding disorders diagnosed in adults. It is caused by quantitative or qualitative deficiencies of von Willebrand factor (VWF), or both. The real frequency of this disorder is difficult to determine because the most common variants generally show mild symptoms, and so are not detected unless the patient suffers trauma, undergoes surgery or experiences any situation that represents a challenge to his or her haemostatic system. Moreover, estimates of the prevalence of VWD are obscured by factors such as incomplete penetrance, variability of VWF levels in the general population, and the unreliability of some of the assays used in the diagnosis of this condition. The number of patients estimate with VWD has been estimated to be up to 1% of the general population (Rodeghiero et al, 1987; Werner et al, 1993). Current estimates, which take into account all the variants of the disorder, now calculate that at least 100 per million show symptoms of VWD (Sadler et al, 2000).

Under the revised classification (Sadler, 1994), which considers that VWD is caused by mutations at the VWF locus, more than the original 20 variants reported (Ruggeri, 1987) have been reduced to six. Type 1 VWD, the most common variant (60±80% of all cases), comprises partial quantitative deficiencies of VWF and is often difficult to diagnose. VWF circulates in plasma as a multimeric glycoprotein, is synthesized in the endothelial cells and megakaryocytes from a mRNA of 8¥8 kb, mediates the adhesion of platelets to the exposed subendothelium, and carries and stabilizes factor VIII. The VWF gene, located on chromosome 12, spans 178 kb and contains 52 exons (Mancuso et al, 1989), making it one of the most complex genes that encodes haemostatic proteins. Abundant detailed information on VWF and the molecular deficiencies that cause this disorder is available, fundamentally in its functional variants. However,

diagnosis of the disease still poses practical problems for clinicians, especially in type 1 VWD.

Significant linkage had been reported for subtype 2A and 2B VWD (Verweij et al, 1988; Iannuzzi et al, 1991; Murray et al, 1991). A large number of different mutations causing this and other qualitative variants, as well type 3 VWD, have established the involvement of the VWF gene in this disorder. However, no significant linkage has been reported for type 1 VWD, although in small families this phenotype had been observed to co-segregate with the VWF locus (Cumming et al, 1992; Inbal et al, 1992). In contrast, lack of association between the type 1 VWD phenotype and intragenic markers of the VWF gene has been reported in families from a population study (Castaman et al, 1999).

In our study, several indirect genetic analyses with intragenic markers of the VWF gene were performed on families with definite or possible type 1 VWD patients. The aim of these analyses was to ascertain the implication of the VWF locus in this variant. The subjects showed varying degrees of severity in both their bleeding symptoms and levels of VWF. ABO blood group (BG) influence on this variant was also studied, as well as the presence of haplotypes with a possible common origin.

PATIENTS AND METHODS

Patients

An indirect genetic analysis was performed on 10 families with dominant inheritance, and two with possible recessive transmission, one of them consanguineous. Four

families with only one member presenting analytical data and phenotype compatible with type 1 VWD were also included in the study of ABO BG presence. The patients were diagnosed as type 1 or possible type 1, in accordance with the Consensus Criteria for the Diagnosis of Type 1 VWD recommended by the International Society on Thrombosis and Haemostasis (unpublished observations). Basically, the laboratory tests were compatible with VWD type 1 if the levels of both VWF:RCo (ristocetin cofactor) and VWF:Ag (antigen) were < 2SD below the population mean and ABO blood type adjusted mean on >/= 2 determinations. The plasma VWF multimer distribution was normal, and the Agglutination Platelet Induced by Ristocetin (RIPA) did not indicate abnormal sensitivity to low concentrations of ristocetin. A significant mucocutaneous bleeding history requires at least two symptoms in the absence of a blood transfusion history, or one symptom requiring treatment with blood transfusion or one symptom recurring on at least three distinct occasions. The diagnosis was based upon criteria for VWF deficiency, symptoms and inheritance, all of which must be satisfied.

Coagulation studies

The functional, antigenic and multimeric assays were carried out as reported previously (Casaña et al, 1998). Briefly, ristocetin cofactor activity was assayed by adapting the old agglutination method with stabilized platelets to the Behring Coagulometer Timer. The protein was quantified using an enzyme-linked immunosorbent assay (ELISA), factor VIII activity was measured using the one-stage clotting assay, and the multimeric pattern was detected by luminography in 1¥2% and 2¥2% agarose gels (detection limit <0.1%). Because the normal ranges calculated in our laboratory were not BG and age adjusted, we assumed the lower level limits of VWF (36 and 48 for the VWF:Ag, and 50 and 75 for the VWF:RCo) reported for O and non-O BG subjects respectively (Gill

et al, 1987; Rodeghiero et al, 1987). The analytical data on the patients with phenotype compatible with type 1 VWD are summarized in Table I.

Genetic analyses

DNA was extracted from blood collected in EDTA using a standard method. For segregation analysis, the following microsatellites were commonly analysed: VNTR3, VNTR1 and VNTR2, named on the basis of their first description. These microsatellites are located on intron 40 of the VWF gene, and correspond to nucleotides 31/1640-1793, 31/1890-1991 and 31/2215-2380 respectively (Casaña et al, 1995). The other microsatellite analysed, VWP, is in the promoter region, and corresponds to nucleotides 1/1490-1665. The eight alleles detected in the marker VNTR3 ranged from 138 to 166 bp and were numbered in decreasing order, the eight alleles found in the first description. These microsatellites are located on intron 40 of the VWF gene, and correspond to nucleotides 31/1640-1793, 31/1890-1991 and 31/2215-2380 respectively (Casaña et al, 1995). The other microsatellite analysed, VWP, is in the promoter region, and corresponds to nucleotides 1/1490-1665. The eight alleles detected in the marker VNTR3 ranged from 138 to 166 bp and were numbered in decreasing order, the eight alleles found in the microsatellite VNTR1 ranged from 98 to 126 bp and were named according to repeats ATCT, the nine alleles observed in the polymorphism VNTR2 ranged from 150 to 182 bp and were designated in decreasing order (1b, 1a, 1….7). The seven alleles identified in the microsatellite VWP ranged from 178 to 188 bp and were also numbered in decreasing order. The Rsal and Hphl polymorphisms located, respectively, on exon 18 (15/292 A/G, T/A789) and exon 28 (24/672G/A, A/T1381) were routinely studied. The screenings for the mutations C1149R (3445T→C) and

C1130F (3389T→G) were performed by restriction analysis using AluI and ItaI respectively (Eikenboom et al, 1996). The mRNA was obtained as previously reported (Casaña et al, 2000).

Linkage analysis

As all the markers analysed were situated at the same locus, the different haplotypes were recorded as single alleles. The pairwise linkage analysis of the disease phenotype versus the VWF haplotype was performed following a mlink routine of version 5.1 of the Linkage Program (Lathrop et al, 1984).

RESULTS

Coagulation studies and VWF gene polymorphism segregation analyses were performed on 109 individuals from 12 unrelated families. Fifty-three patients showed laboratory data compatible with type 1 VWD, of which 42 could be considered as definite type 1 VWD and 11 as possible type 1 VWD. In all the patients the plasma multimeric structure showed the presence of all multimers, similar to those of normal plasma, in low resolution gels. No anomalies in the triplet structure were observed in high resolution gels.

A lod score of $Z_{max} = 5.28$ for a recombination frequency (θ) of 0.00 was obtained in family F1 (Fig 1). In F2 a lod score very near to the threshold of three, which is considered significant, was recorded. The lod scores obtained in the linkage analyses are summarized in Table II. A total Z_{max} score of 11.87 was obtained in the five families

that showed a positive lod score of over 1 (Table II). Assuming dominant inheritance, F6 did not show linkage with the VWF gene. No linkage was obtained in the consanguineous F7 either, assuming recessive or dominant inheritance (Table II, Fig. 2). In the other families with lod scores of under 1, it was not possible to establish linkage with the VWF gene, fundamentally on account of the small number of individuals studied or, in some patients, the mild expression of the phenotype, which hinders association with a haplotype.

An exhaustive analysis of the polymorphic alleles associated with the type 1 VWD phenotype showed that the patients from F8 and F9, the latter with probable recessive inheritance, shared the haplotype [6-(+)-1-5-6-6], corresponding to the markers VWP, *Rsa*I, *Hph*I, VNTR3, VNTR1 and VNTR2 respectively. In F5 a similar haplotype, varying only in the VWP microsatellite, was also associated with the phenotype. In the study of 190 non-related chromosomes, the haplotype, including alleles 5 or 6 of the VWP marker and the nucleus [5-5-6] of the microsatellites of intron 40, was found once (haplotype 6-1-5-6-6). That is, we observed an excess of haplotype 5/6-5-5-6 among the chromosomes linked to type 1 VWD (3 out of 10) versus the control group (1 out of 190). The Fisher test ($P > 0.0005$) was significant, which suggests that this haplotype may carry a common deficiency associated with mild type 1 VWD.

We have also observed that a large number of patients belong to the O BG. Of the 57 individuals with VWF levels compatible with type 1 VWD, the BG of 44 was known, 24 (55%) of them belonging to the O BG. According to figures released by the Blood Bank of the Community of Valencia, 44% of the donors belong to the O BG. The results of the comparison of the cases observed in the patients with the expected cases in the local population were not significant ($\chi^2 = 1.40$; $P = 0.24$). Although the frequency of the O BG in families with a dominant inheritance pattern and complete penetrance

was similar to that of the rest of the population (8/23; $\chi^2 = 0.54$; $P = 0.46$), those patients with mild VWF deficiency and/or incomplete penetrance mostly belonged to the O BG (15/21; $\chi^2 = 6.14$; $P = 0.01$). The comparison of the latter group versus the former was also significant ($P = 0.016$ for Fisher test). That is, blood group appears to be irrelevant in families with complete penetrance, but in families with a mild phenotype and incomplete penetrance it is a factor to be taken into account.

DISCUSSION

According to the data of segregation and linkage analyses, four of the families studied (F1, F2, F3 and F4) may be regarded as classic dominant type 1 VWD with high penetrance (Fig 1). The bleeding symptoms presented by the patients varied from mild to moderate, with the possible exception of some patients from F1 and F4, who occasionally experienced moderate to severe haemorrhage. The response to the vassopresin analogue (DDAVP) was satisfactory for each of the patients tested, and non-discrepant functional and antigenic levels of VWF over the normal values were obtained in each case. In these patients, therefore, a search for mutations in the VWF gene or promoter region may contribute to the identification of the molecular deficiency causing the disorder. In the seven patients from F2, a candidate mutation which segregates with the phenotype (T1156M) had already been detected in exon 26 of the VWF gene (Casaña et al, 2001a). Mutations C1149R and C1130F, which have a negative dominant effect on the VWF (Eikenboom et al, 1996; Castaman et al, 2000), were ruled out for the families included in the study (data not shown). In F9, the mutation C1227R in exon 28 of the VWF gene associated with the maternal allele was

identified (Casaña et al, 2001b). In F5, the results of the phenotype and segregation analyses (Fig 3) were compatible

with a transmission associated with the VWF gene with incomplete penetrance, as proposita III:2 showed normal VWF levels and had no bleeding history, even though she was a carrier of a risk haplotype. There is currently no known explanation for this particular case, although sometimes the presence of silent alleles may account for the variable expression and the analytical data observed in some families (Peerlinck et al, 1992). The analysis of exonic polymorphisms in cDNA obtained from platelet mRNA showed that proposita III:1 expressed the two alleles (data not shown), although this analysis could not be performed on the other affected members of the family. Other genetic changes found in the VWF gene or in other loci may be implicated in this variability. Several cases of compound heterozygosity have already been reported in type 1 VWD, as in the case of a carrier of a cytosine deletion in exon 18 of one of the alleles, and an amino acid change affecting the other allele (2680delC/P1266L) (Zhang et al, 1993). Another case concerns the detection of two mutations in exons 5 and 18 in different alleles (L150Q/R728Q) (Zhang et al, 1995). Recessive inheritance in type 1 VWD has also been reported in several families (Eikenboom et al, 1993, 1998).

Individuals II:2, III:1 and III:3 from F6 showed lower than normal ABO-BG-adjusted VWF levels on more than two occasions. They all presented mild bleeding symptoms, except individual III:3, who had no bleeding history. Assuming dominant inheritance, linkage of the phenotype to the VWF gene was rejected. The affected members of F7 presented very low levels of VWF and mild bleeding symptoms. Negative lod scores (±) were also obtained in this consanguineous family, which would appear to rule out recessive inheritance associated with the VWF gene. On the other hand, although dominant inheritance with incomplete penetrance cannot be dismissed, this possibility is

highly improbable as the disorder is expressed in one sibship descended from consanguineous parents (I:2 and III:1 would have to be asymptomatic carriers). Another possibility, although a highly improbable one, is that the father (I:1) may be compound heterozygous for mutations at the VWF gene, which have no phenotype expression unless associated with another mutation from the maternal allele. In view of this, the phenotypes presented by these two families are presumably occasioned by deficiencies in other loci.

In the other families, segregation analysis failed to provide conclusive evidence for the possible implication of the VWF gene because of their small size, non-participation of some family members in the study, and the presence of mild phenotypes (Fig 4). However, the fact that a similar haplotype was found in three families (F5, F8 and F9; Fig 3) would suggest that it might be a carrier of a common deficiency associated with type 1 VWD, and this possible ancestral relation might reinforce VWF gene implication in the bleeding diathesis presented by these patients.

As previously mentioned, the variability observed may be traceable in part to recessive inheritance or mutations affecting the expression of some of the alleles. It is also known that plasma VWF levels may be modified by genetic and environmental factors such as thyroid hormones, oestrogens or stress. The best-characterized genetic modifier is the ABO blood group, which accounts for approximately 30% of genetic effect (Nichols & Ginsburg, 1997). In our study the number of patients with O BG was regarded as significant in the milder deficiency group with variable expression of the gene, i.e. blood group may be irrelevant in families with complete penetrance, but might be a factor to take into account in cases with mild phenotypes and incomplete penetrance. Mutations affecting genetic modifiers might also cause type 1 VWD, even in the absence of a mutation at the VWF gene, which might be the case for the two families in

which no linkage was obtained (F6 and F7). Indeed, an ectopic expression in the vascular endothelial cells of the Galgt 2 gene (N-actylgalactosaminyltransferase) has recently been identified as the primary modifier of plasma VWF levels in a mouse strain (Mohlke et al, 1999), in which it was known that type 1 VWD did not segregate with the VWF gene locus. It is highly unlikely that the same alteration will appear in humans (Ginsburg, 1999). Similarly, any of the multiple enzymes involved in glycosylation or other stages of VWF biosynthesis may be implicated. Future studies of a large number of families that do not show linkage between VWD and the VWF gene may shed light on which these putative loci are, although such a study would constitute an arduous task on account of the large number of genes that might be involved. Nevertheless, the findings raise the question of naming the phenotypes which, while presenting similarities to type 1 VWD, do not show linkage. A simple solution to this question might be to retain the existing nomenclature and admit locus heterogeneity for this type.

The diagnosis of type 1 VWD is generally limited to phenotype analysis. Some findings suggest that recourse to adjusted scales of VWF levels may not be essential for diagnosis as bleeding symptoms depend on the VWF levels father than the ABO type (NituWhalley et al, 2000). Given the present difficulties involved in diagnosis, the characterization of the genetic molecular bases may be of great help. However, direct genetic analysis for type 1 VWD is not a practical option yet because screening the VWF gene and promoter region is both costly and time-consuming. Indirect genetic analysis, therefore, may be an alternative way of diagnosing asymptomatic and presymptomatic carriers, particularly in cases of high penetrance. However, caution needs to be exercised when interpreting the data of segregation analysis in view of genetic heterogeneity, variable expression of the gene and varying inheritance patterns.

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