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SHORT COMMUNICATION

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A novel *VWF* variant associated with type 2 von Willebrand disease in German Wirehaired Pointers and German Shorthaired Pointers

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Summary

Von Willebrand disease (VWD), caused by deficiency of the von Willebrand factor (VWF), is the most common bleeding disorder in humans and dogs. The complete cDNA encoding VWF of a German Wirehaired Pointer with type 2 VWD was sequenced, and we found four variants that alter the amino acid sequence. These variants were: c.1657T>G corresponding to p.Trp553Gly; c.1777G>A (p.Glu593Lys); c.4937A>G (p.Asn1646Ser) and c.5544G>A (p.Met1848Ile). A haplotype of the c.1657G, c.1777A and c.4937G alleles co-segregated with the VWF antigen level in a four-generation pedigree with the disease. Healthy dogs of the breed were found that were homozygous for the c.1777A or the c.5544A allele, indicating that these variants do not cause VWD. Dogs that were homozygous for the c.4937G allele and had no signs of a bleeding disorder were observed in the Chinese Crested dog breed. Thus, only the c.1657G variant was found in the homozygous state exclusively in VWD affecteds, and this variant is the strongest candidate to be the cause of VWD type 2 in the German Wirehaired Pointer breed. A screen of German Shorthaired Pointers indicated that the variant also segregates with VWD in this breed.

Keywords bleeding disorder, canine, dog, gene, mutation, single base extension

Von Willebrand factor (VWF) is a carrier for clotting factor VIII and a mediator of the initial adhesion of platelets after vessel injury (Johnson *et al.* 1988; Sadler *et al.* 1995). Von Willebrand disease (VWD) classification is based on VWF concentrations in plasma and on the presence of an abnormal spectrum of VWF multimers. Type 1, the most common type, is associated with a mild or moderate bleeding tendency caused by a low concentration of the VWF protein. Type 3, the most severe type, is characterized by the complete absence of VWF protein in the plasma. Type 2 also has severe clinical signs and is characterized by all variants in which the biochemical structure of the VWF protein is abnormal. Multimers of the protein normally present in plasma are absent in type 2 VWD (Sadler *et al.* 1995). Earlier, DNA sequence analysis showed that the type 2 disease in German Shorthaired Pointer dogs (GSP) was associated with homozygosity of the *VWF* variant c.4937A>G, corresponding to the amino acid variant p.Asn1646Ser of the encoded protein (Kramer *et al.* 2004). However, the complete coding sequence of the gene was not screened in that study.

We set out to analyze the complete coding sequence of the *VWF* gene of an affected German Wirehaired Pointer (GWP), a breed closely related to GSP. A skin biopsy was taken from the proband (Fig. 1), a privately owned dog presented to the University Clinic for Companion Animals with bleeding disorder (Van Dongen *et al.* 2001). RNA was isolated with a total RNA separation kit (Qiagen). Complementary DNA was synthesized with reverse transcriptase (Promega). Overlapping fragments of the complete coding region of the *VWF* cDNA were selected from the reference sequence NM_001002932.1 and amplified with the primers listed in Table S1. The PCR was performed under standard conditions with Platinum Taq Polymerase (Invitrogen). The DNA fragments were purified after agarose gel electrophoresis using QIAquick® gel extraction kit (Qiagen). The DNA sequence was analyzed with Big Dye Terminator v.3.0 chemistry and a Genetic Analyzer 3100 (Applied Biosystems). The comparison of this DNA sequence to the reference sequence of the canine *VWF* cDNA revealed five DNA sequence variations. The T- or C-residue at position 156 of the open reading frame of the cDNA (c.156C>T) did

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not translate into an amino acid change. The other four, namely c.1657T>G, c.1777G>A, c.4937A>G and c.5544G>A, resulted in the exchange of tryptophan for glycine at position 553 of the amino acid sequence (NP_001002932.1:p.Trp553Gly), of glutamic acid for lysine at position 593 (p.Glu593Lys), of asparagine for serine at position 1646 (p.Asn1646Ser) and of methionine for isoleucine at position 1848 (p.Met1848Ile) respectively. The residues correspond to exons 14, 15, 28 and 32 respectively of the *VWF* gene. The GenBank accession number of the *VWF* cDNA sequence of the affected GWP is KX661325.

A family of GWP dogs that segregated type 2 VWD with recessive inheritance has been described before (Van Dongen *et al.* 2001). The inheritance of the biochemical phenotype of the VWF antigen concentration is semi-dominant in GWP and GSP, and heterozygotes can be distinguished by ELISA (enzyme-linked immunosorbent assay) (Van Dongen *et al.* 2001; Kramer *et al.* 2004). With VWF in general, the range of antigen concentrations measured in heterozygous dogs sometimes overlaps with the range found in homozygous normal or affected dogs, and therefore we chose non-consecutive classes to type the dogs. Dogs with VWF antigen concentrations of 0–10%, 25–75% and 90% and above were considered to be homozygous affecteds, heterozygous carriers and homozygous normal dogs respectively. All dogs that were phenotyped by their VWF antigen level by ELISA could be classified. SNaPshot® reactions were developed for the observed missense *VWF* variations (Applied Biosystems). The primer sets for the PCRs and single base extension reaction for the SNaPshot® assay are listed in Table S2. The missense variations were analyzed in dogs of the VWD family, and haplotypes were deduced (Fig. 1). The VWF antigen levels co-segregated with the c.[1657G; 1777A; 4937G; 5544A] haplotype. The two-point LOD (logarithm of the odds) score for linkage of VWF levels with the polymorphism at position 4937, which was typed in all dogs of the pedigree, was 6.0 at recombination fraction 0, as calculated with MLINK (Lathrop *et al.* 1984). This high LOD score indicates that the variation of the VWF level in the GWP pedigree results from a variation in the *VWF* gene region.

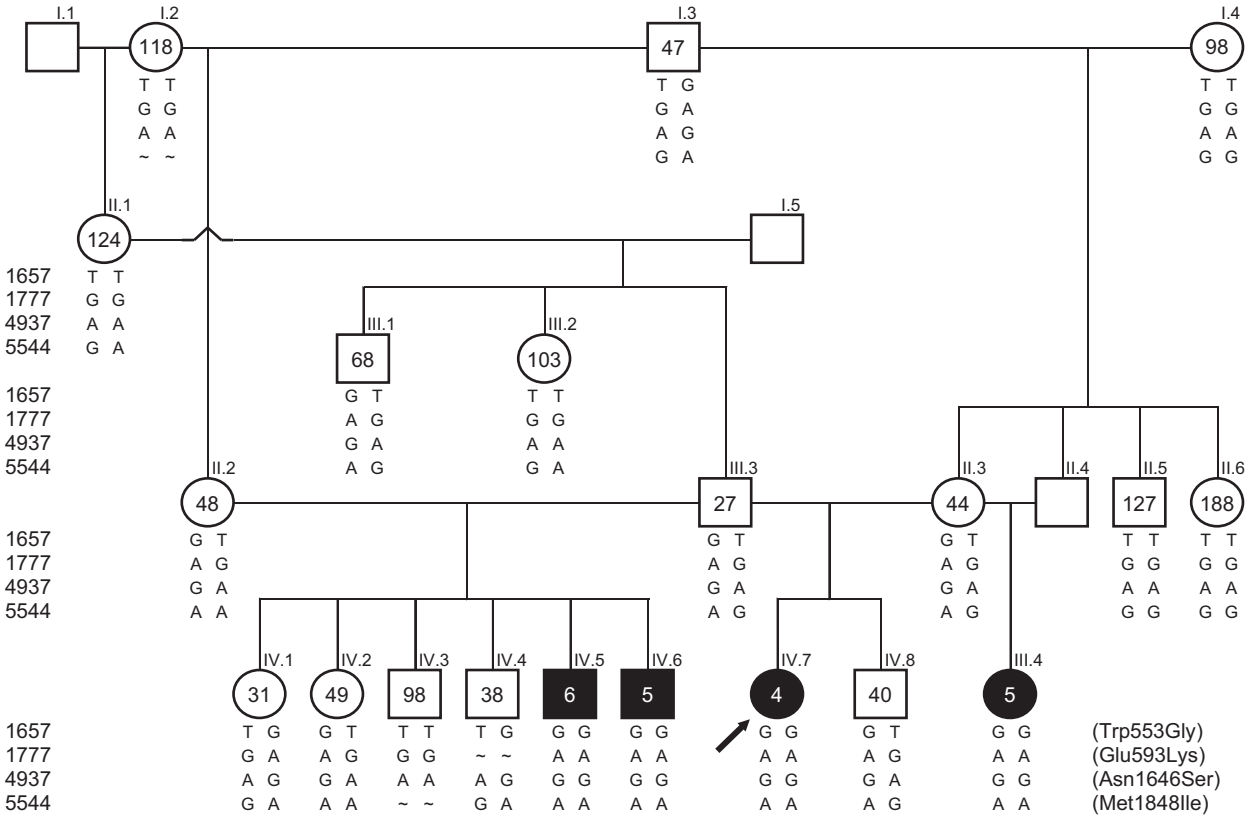

Figure 1 Co-segregation of the von Willebrand factor (VWF) level with a haplotype of the encoding gene in a family of German Wirehaired Pointers. The VWF levels were measured by ELISA and depicted as relative percentages within the symbols of the investigated dogs. The VWF levels of dogs I.1, I.5 and II.4 were not determined. The haplotypes of the polymorphisms c.1657T>G, c.1777G>A, c.4937A>G and c.5544G>A are shown below the symbols. The numbers in this notation refer to the nucleotide positions in the open reading frame of the cDNA. The corresponding amino acid variations are indicated between brackets. The semi-dominant phenotype based on VWF antigen concentration segregates with the GAGA haplotype. The proband with von Willebrand disease is indicated by an arrow. Dogs III.4, IV.5 and IV.6 were also affected. ~, not determined.

From the family data we concluded that the c.5544A variant could not cause VWD, because dogs II.2 and IV.2 were homozygous for this variant whereas these dogs had intermediate levels of VWF antigen (Fig. 1). Dogs II.1 and III.2 were heterozygous for the same variant, whereas their VWF antigen level was normal.

We typed 20 GWP dogs unrelated to the VWD family for the three remaining variations that were possibly directly involved in VWD. These dogs had no history of VWD, and their VWF antigen level was not measured. Two of the dogs were homozygous for the c.1777A variant (Table S3). So, two of the four amino acid variants found—the p.553Gly variant and the p.1646Ser variant—remained candidates for the cause of von Willebrand disease in the GWP breed. The POLYPHEN-2 tool predicted that the p.Trp553Gly mutation is probably damaging for VWF function with a score of 0.987 on a scale of 0–1 (Adzhubei *et al.* 2010). The p.Asn1646Ser mutation was predicted to be benign with a score of 0.001. In line with the POLYPHEN-2 outcome, the p.Trp553 residue was conserved in VWF of all vertebrates analyzed, whereas the p.Asn1646 was less well conserved (Fig. 2).

Both remaining candidate variations were included in a custom-designed assay dedicated to detection of known pathogenic variants in dogs. Application of the assay in routine diagnostic tests showed that the two variations were in complete linkage disequilibrium in the GWP and GSP breeds. Of the 194 GWP dogs tested, 20 were carriers of the c.1657G and c.4937G alleles. GWP dogs homozygous for these alleles were not observed. Of the 97 GSP tested, 26 dogs were carrier and two dogs were homozygous for both these alleles. Contact with the owner of one of the homozygous dogs confirmed that it had a history of severe bleeding incidents and died of internal bleeding, consistent with clinical signs of VWD type 2. The owner of the other homozygous GSP reported prolonged bleeding after injury and spontaneous nose bleeds, but the phenotype seemed less severe than that of the first dog. The screen for both variations confirms for the first time that the c.1657G allele fully segregates with the c.4937G allele and VWD in the GSP breed as it does in the GWP breed.

Genotyping of dogs from a wide variety of breeds showed that the c.4937G variant but not the c.1657G allele is present in the Chinese Crested dog breed. Of the 41 tested dogs of this breed, 14 were carriers and three were homozygous for the c.4937G allele. Owners of the Chinese Crested dogs that were homozygous for this variant were contacted, and none of the dogs had signs of a bleeding disorder. Remarkably, some commercial laboratories offer the VWD type 2 DNA test for the Chinese Crested dog breed. Our data indicate that, if this test is based solely on the earlier report of Kramer *et al.* (2004) that c.4937G is associated with VWD in GSP, it is likely to result in false positives.

Based on the POLYPHEN-2 predictions and the observation that only the c.1657G (p.553Gly) variant is exclusively found in the homozygous state in GWP and GSP dogs that are affected by VWD, we suggest that this mutation is a primary candidate causal mutation for the disease in the breeds. The position of the mutation corresponds to CFA27: g.38887211 of the reference genome CanFam 3.1. It is possible that the variant p.553Gly is not deleterious to the function of the protein on its own but acts in concert with the p.1646Ser variant to cause VWD in GWP and GSP dogs. Functional studies of *in vitro* synthesized gene products should be performed to establish the effect of each of the mutations separately on VWF activity.

This study underlines the importance of analyzing candidate disease genes comprehensively, especially in purebred animal populations. Observed nonsynonymous variants may well be in full linkage disequilibrium with other, possibly causal, gene variants in particular breeds. Therefore, it is important to categorize all coding variants in a disease gene and to explore the distribution of these variants across breeds, as has been done in this study.

Because VWF antigen assays cannot always distinguish heterozygous carriers from normal dogs, there is a need for a reliable DNA test. A DNA test has been used before to

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Dog       541 LAEPLVEDFGNAWKLLGACENLQKQHRDPC 1631 GPHANVQELEKIGWPNAPILIHDFEMLP
Man       541 ....R.........H.D.QD.....S... 1631 ..N.......R.........Q...T..
Cattle    539 .V.....H...S...RAD..D..E.PS... 1629 ..RVD.....RVS..QT..F.Q...R..
Pig       540 .V.....H.......H.D..D.R..PT... 1625 ..DVDM....RLS......F.Q...T..
Rat       541 .V....V........Q.D.PD.HR..S... 1631 .SR..L....R.SR.I...F.Q...T..
Chicken   538 .V.V.L.....S...NAD.QD.L..DN... 1618 T.R......RM.SQ..R..ILQSYST.I
Zebrafish 534 .V.TR..A...S..MNAD.DDV.M.NS...         no similarity detected
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Figure 2 Conservation of Von Willebrand factor (VWF) sequences that contain candidate causal mutations in German Wirehaired Pointers (GWP) with Von Willebrand disease (VWD). The canine protein sequences surrounding the variable residues were compared with VWF of a selection of vertebrates using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acids that are identical to those in dog are indicated by a dot. The residues p.Trp553 (W) and p.Asn1646 (N) are underlined and these are Gly (G) and Ser (S) respectively in GWP dogs with VWD. No similarity can be detected between the canine amino acid sequence surrounding p.Asn1646 and Zebrafish VWF. The accession numbers of the proteins used are NP_001002932.1 (dog), NP_000543.2 (man), NP_001192237.1 (cattle), NP_001233150.1 (pig), NP_446341.1 (rat), XP_004938134.2 (chicken) and NP_001268918.1 (zebrafish).

minimize the incidence of VWD type 3 in Dutch Kooiker dogs in a short period of time (Slappendel *et al.* 1998; Van Oost *et al.* 2004). From the numbers of carriers observed in the GWP and GSP breeds, we conclude that the use of the test for selection of breeding pairs is warranted.

Conflict of interests

CD and IL-D are employed by Laboklin Labor für Klinische Diagnostik GmbH & Co. KG that performs diagnostic DNA tests on a commercial basis.

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Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

Table S1 Oligonucleotide sequences for PCR amplification of canine VWF cDNA.

Table S2 Oligonucleotide sets for SNAPSHOT assay.

Table S3 Genotype frequencies of VWF variants in 20 unaffected GWP dogs.