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Genetics

Mutations of von Willebrand factor gene in families with von Willebrand disease in the Åland Islands

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ABSTRACT **Patients with von Willebrand disease in four families in the Åland Islands, including the original family that was described in 1926 by the Finnish physician von Willebrand, were screened for mutations in the Swedish "hot-spot" regions (exons 18, 28, 32, 43, and 45) of the von Willebrand factor gene. One cytosine deletion in exon 18 was detected in each of these families. Linkage analysis and genealogical studies suggest that the deletion present in these four families probably has an origin in common with the mutations in the Swedish patients. Apart from the deletion in exon 18, two close transitions (G → A at S1263 and C → T at P1266) in exon 28 on the same chromosome were identified in one individual who married into the original family and in his two children. The transitions could be due to a recombination between the von Willebrand factor gene and its pseudogene.**

In 1926, Erik von Willebrand from Helsinki described a severe bleeding disorder in a family (family S) living in Föglö, an island in the Åland Archipelago, situated between Finland and Sweden, the shortest distance between Åland and Sweden being 20 km (1). The propositus was a 5-year-old girl, who later bled to death during her fourth menstrual period. She had a normal coagulation time, but the bleeding time was prolonged, despite a normal platelet count. All but one of her 11 siblings had bleeding symptoms, as did both of her parents, who were third-degree cousins, and many members of both families. Four of the proband's sisters had died of uncontrollable bleedings in early childhood; three died from gastrointestinal bleeding and one from bleeding after she bit her tongue in a fall. The dominating symptoms were bleedings from the mucous membranes, such as from the nose, the gingivae after tooth extractions, the uterus, and the gastrointestinal tract. In contrast to the findings in hemophilia, hemarthroses seemed to be rare.

von Willebrand extensively reviewed the literature on similar patients and, after further investigations of the symptoms and laboratory findings in this family, he suggested that the heredity was autosomal dominant (1, 2). During 1930s to 1950s von Willebrand and other investigators published reports on the findings in this and other bleeder families living on the Åland Islands (3, 4).

In the beginning of the 1950s it was shown that the levels of factor VIII (FVIII, then mainly referred to as antihemophilic globulin or antihemophilic factor) were low (less than 0.10 unit/ml) in males and females suffering from a severe inherited bleeding disorder with symptoms similar to those described by von Willebrand (for reviews see refs. 5–7). In 1957 Nilsson *et al.* (7) described six Swedish families having an autosomal dominant hemorrhagic diathesis with similar features; the FVIII level and the prolonged bleeding time were restored to normal by infusion of the plasma factor concentrate fraction I-0 (8), which stopped the hemorrhages (6, 7, 9). The bleeding-time-correcting activity was not identical with FVIII or purified fibrinogen (6, 7, 9), suggesting that the prolonged bleeding time was due to the lack of an unknown plasma factor (later shown to be the von Willebrand factor; vWF) and not to a defective capillary wall or to a primary defect in platelet function (refs. 6, 7, and 9; see also refs. 10 and 11). The patients were subsequently shown to suffer from von Willebrand disease (vWD) type III. The Swedish team also showed that the surviving siblings of the original Åland family with bleeding symptoms had the same laboratory manifestations as the parents and siblings of the Swedish probands who had bleeding symptoms (12). At the same time Jürgens *et al.* (13) investigated the same patients and also demonstrated a decreased FVIII activity. Thus the disease originally described by von Willebrand was considered to be identical with that found in the Swedish patients.

In 1979–1981, Nyman *et al.* (14–16) showed that the bleeder families in the Åland Islands could be divided into several categories. The survivors in family S had the characteristics of vWD type I—i.e., decreased levels of vWF and ristocetin cofactor activity in addition to normal or decreased levels of FVIII; the platelet aggregation was normal (14).

The genomic structure of the vWF gene was published in 1989 by Mancuso *et al.* (17). It spans 178 kb and encodes a precursor protein of 2813 amino acids. The coding sequence is an 8.7-kb mRNA containing 52 exons. Point mutations in the vWF gene of patients with vWD can be screened for by using the polymerase chain reaction (PCR), followed by direct sequencing.

In the present study, four families with vWD, including some living members of the original family (family S) described by von Willebrand in the Åland Islands, were investigated for the mutations which were recently demonstrated in the families with vWD in Sweden (18, 19). One cytosine deletion in exon 18 was detected in the families. The only living patient with vWD type III in the Åland Islands was found to be homozygous for the deletion. Furthermore, two close transitions in exon 28, located on the same chromosome, were detected in one member of family S, who married into the family, and in his two children.

MATERIALS AND METHODS

Patient Materials. The study was approved by the Ethics Committee of Karolinska Hospital. During March and April 1992, blood samples for DNA analyses were drawn from individuals in four families.

Family S (the original family) from Föglö. Individuals II:2, II:3, II:4, II:5, II:6, III:1, III:2, III:3, and III:4 were investigated (Table 1; see Fig. 1 for pedigree).

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Abbreviations: FVIII, factor VIII; vWF, von Willebrand factor; vWFAg, vWF antigen; vWD, von Willebrand disease.

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Table 1. Results of coagulation investigations in family S of Föglö in 1957, 1976/1977, and 1992

Family member	Sex	Born	Relative amount of coagulation factor*									Blood group†
			1957	1976/1977				1992				
			FVIII	FVIII	vWFAg	FVIII/ vWFAg	Ristocetin cofactor	FVIII	vWFAg	FVIII/ vWFAg	Ristocetin cofactor	
I:1	M	1876	0.93	—	—	—	—	—	—	—	—	
I:2	F	1882	0.51	—	—	—	—	—	—	—	—	
II:1	M	1912	—	0.81	0.80	1.01	0.73	—	—	—	—	
II:3	F	1915	0.53	0.87	0.56	1.55	0.46	—	—	—	—	A+
II:4	M	1916	0.35	0.65	0.35	1.86	0.32	0.72	0.41	1.76	0.54	O+
II:5	F	1922	—	—	—	—	—	0.96	0.86	1.12	0.65	
II:6	M	1927	—	—	—	—	—	1.10	0.67	1.64	0.81	A+
III:3	M	1943	—	0.39	0.34	1.15	0.42	0.58	0.54	1.07	0.67	O+
III:4‡	F	1946	—	0.69	0.20	3.45	0.30	—	—	—	—	A+

*In 1957 and 1976/1977 FVIII, vWF antigen (vWFAg), and ristocetin cofactor activity were measured in units/ml against a pooled reference plasma from healthy donors; in 1992 they were determined in international units/ml against the World Health Organization standard.
†II:2 and III:1 (Fig. 1) have blood group O.
‡Only III:4 has a prolonged bleeding time.

Table 2. Coagulation analyses in the subjects of the other Åland families, S-B, E, and I, which have a common ancestor in 1650

Family	Member	Sex	Born	Relative amount of coagulation factor								Bleeding time*	vWD type
				1976/1977				1992					
				FVIII	vWFAg	FVIII/ vWFAg	Ristocetin cofactor	FVIII	vWFAg	FVIII/ vWFAg	Ristocetin cofactor		
S-B	HS	M	1928	0.45	0.54	0.83	0.24	1.09	0.63	1.73	0.59	P	I†
E	BoE	M	1939	0.67	0.39	1.71	—	—	—	—	—	N	I
I	II:1‡	M	1990	—	—	—	—	0.03	<0.06	—	<0.05	P	III
	I:1	F	1964	—	—	—	—	0.85	0.57	1.49	0.50	P§	I
	I:2	F	1961	—	—	—	—	1.17	0.70	1.67	0.59	N	I(?)¶
	I:3	M	1958	—	—	—	—	0.92	0.45	2.04	0.52	N	I

*P, prolonged; N, normal.
†This patient also has a cyclooxygenase defect.
‡Coagulation factors were analyzed at the Finnish Red Cross Transfusion Service, Helsinki.
§13 min (normal < 7 min).
¶The ratio FVIII/vWF > 1.6 indicates vWD type I (24).

Family E from Vårdö. The subject, BoE, has clinically fairly severe vWD type I (Table 2 and refs. 4, 12, and 13).

Family S-B from Lumparland. The subject, HS, has vWD type I and a cyclooxygenase defect (see Table 2 and refs. 12, 13, and 16).

Family I from Lemland. Four members of this newly detected family were investigated (see Table 2 and Fig. 2). The 2-year-old boy (II:1), the only patient with severe vWD type III at present residing on the Åland Islands, had recently been treated with blood transfusions after gastrointestinal bleeding. The diagnosis was therefore made on DNA extracted from his hair follicles.

Blood Coagulation Analyses. Various Ivy methods have been used for a number of years for measuring the bleeding time. Thus, for the sake of simplicity, the bleeding time is reported to be normal or prolonged. FVIII activity was analyzed by almost identical methods during all these years, using hemophilia A plasma as a substrate in a recalcification assay. Over the years, the vWFAg has been measured by different methods, such as Laurell electroimmunoassay, us-

Table 3. Oligonucleotides for PCR and sequencing

Exon	Number*	Location†	Primer sequence (5′→3′)	Exon size, bp	Annealing temp., °C
18	Vi17-u	15/148	TGTGGAAGGTAGGTCCATTA	268	55
	Vi18-d	15/416	ACAAGAAAACTGAAGGGCAG		
28	Vi27-u	7523	TGTGGGAATATGGAAGTCATTG	544	60
	Ve28-1d	8067	CAGGGCGGTCGATCTTGCTGAA		
	Ve28-2d	8462	GTCCGATCCTTCCAGGACGAAC	939	60
	Ve28-1u	8424	ATGGTTCTGGATGTGGCGTTC		
	Vi28-d	9076	GTATCTTGGCAGATGCATGTAGC	653	60
32	Vi31-u	13457	TGAACATCTTCCTCATAGGGCTGA	473	55
	Vi32-d	13928	CCATGAACAGAAACTTAAAG		
43	Vi42-u	32/24	CTTCTGTGTAGTAGGTGCTAA	269	55
	Vi43-d	32/293	CTCTGATAGCTGCAGGCATG		
45	Vi44-u	34/118	CCTGTGGTGGGACTTACATGTTA	388	60
	Vi45-d	34/506	TCAGGAGCCAAAAGTGGAAAGAG		

*V, vWF gene; i, intron; e, exon; u, upstream; d, downstream.
†Location of the 5′ nucleotide refers to genomic sequencing data of the vWF gene (fragment/sequence number) (17). Underlined sequence refers to the vWF pseudogene sequence (21).

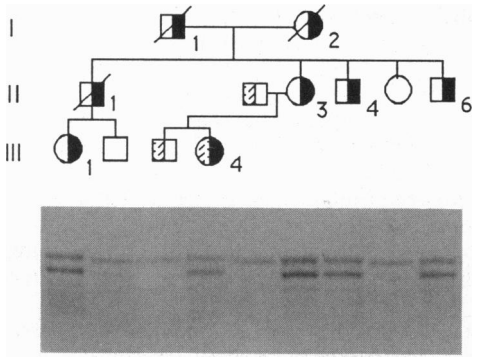

FIG. 1. Deletion pattern analysis in family S. (*Upper*) Pedigree. Individuals represented by half-solid symbols are heterozygous for the deletion in exon 18. Half-shaded symbols represent individuals heterozygous for the missense mutation in exon 28. Patient III:4 has both the deletion and the missense mutation. Individual II:2 comes from the same island as family S, though from another village. (*Lower*) Electrophoresis in denatured 6% polyacrylamide gel.

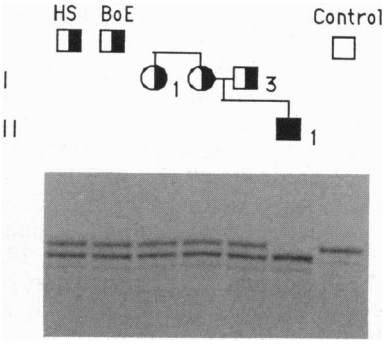

FIG. 2. Deletion pattern in family I and in the two members investigated of families E and S-B. Individuals represented by half-solid symbols are heterozygous for the deletion in exon 18. The individual represented by the solid symbol (II:1) is homozygous for the deletion in exon 18; his DNA was extracted from hair follicles.

ing rabbit antibodies, and more recently by ELISA, using goat polyclonal antibodies. The ristocetin cofactor activity was determined by employing formaldehyde-fixed platelets.

PCR, Asymmetric PCR, and Sequencing. Total genomic DNA was isolated from lymphocytes. PCR was performed under standard conditions (19); there were 35 cycles at 94°C for 1 min, at 55–60°C for 1 min, and at 72°C for 1 min with a final extension for 7 min at 72°C (Table 3). Single-stranded DNA was synthesized by asymmetric PCR (19) and was sequenced with Sequenase T7 DNA polymerase.

To investigate the $(GT)_n$ polymorphism and to screen for the one cytosine deletion in exon 18, a size-detecting method similar to that for $(CA)_n$ was used (22).

Reagents. *Taq* DNA polymerase was purchased from Perkin–Elmer/Cetus; oligonucleotide primers were from Symbicom (Umeå, Sweden); and Sequenase T7 DNA polymerase was from United States Biochemical.

RESULTS AND DISCUSSION

Samples from the four families were screened for mutations with PCR, followed by direct sequencing of the "hot-spot" region in exons 18, 28, 32, 43, and 45 (refs. 18 and 19; unpublished data; Table 3). No mutations were detected in exons 32, 43, and 45.

In the original family S, one cytosine deletion in exon 18 was identified. Sequencing of this fragment showed that the deletion occurred in a stretch of six cytosines, which has been found to be a hot spot in the majority of the vWD type III patients in Sweden (19). The mutation interrupts the reading frame and results in an early translational stop. Five subjects (II:3, II:4, II:6, III:1, and III:4; Fig. 1) in this family, who had vWD type I (Table 1), were found to be heterozygous for the deletion. The deceased subject II:1 must also have carried the same deletion, because his daughter (III:1) has it. In the second generation, at least four subjects (II:1, II:3, II:4, and II:6) carry the deletion. These results indicate that the deletion originates from the parents (I:1 and I:2), who are supposed to be heterozygous. All five of the girls who died

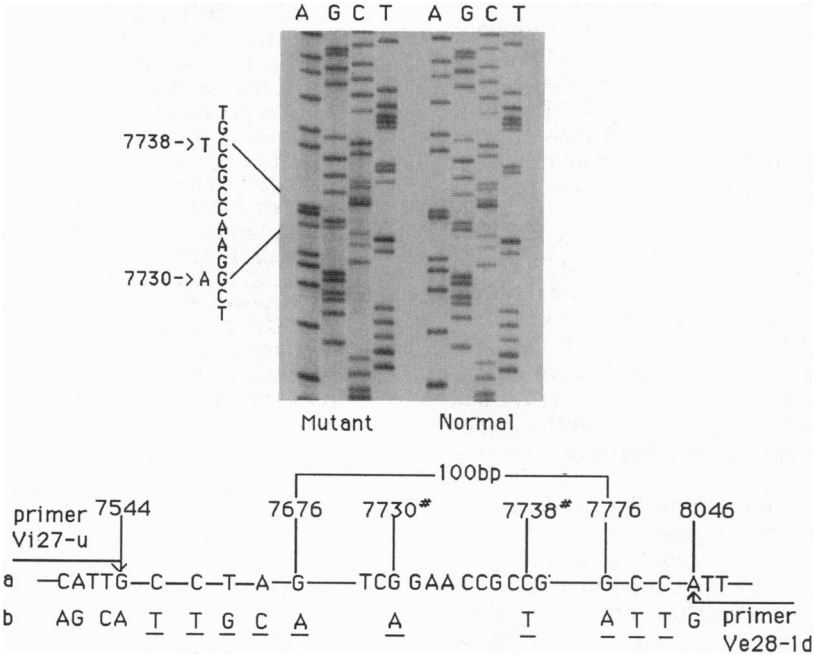

FIG. 3. (*Upper*) Sequencing with the primer Ve28-1d. (*Lower*) Difference between the vWF gene and its pseudogene in the PCR fragment (Vi27-u to Ve28-1d). Line a, the vWF gene sequence. Line b, the pseudogene sequence. Only differences are shown. Number in the sequence refers to the vWF pseudogene sequence (21); #, location of the transitions, S1263 (7730) and P1266 (7738).

from uncontrolled bleeding were probably homozygous for the deletion.

In family I, the proband, II:1, with vWD type III, is homozygous for the deletion in exon 18, while his parents (I:2 and I:3) and his maternal aunt (I:1) are heterozygous (Fig. 2). His father and his maternal aunt have vWD type I, but we did not detect a decreased level of the vWF in his mother (I:2). The two type I subjects investigated in families E and S-B were found to be heterozygous for the deletion (Fig. 2).

In family S, besides the deletion in exon 18, two transitions, one at S1263 and one at P1266 in exon 28 on another chromosome (Fig. 3), were identified in two siblings (III:3 and III:4). The transition G → A at S1263 is a neutral (TCG → TCA) mutation and the other, C → T at P1266, results in an amino acid change of proline to leucine (CCG → CTG). The transitions should be located on the same chromosome, since they were also detected in the father of III:3 and III:4, who married into the family. It is interesting that these transitions are also present in the vWF pseudogene sequence (17, 21). The DNA sequence, 55 bp upstream of S1263 and 39 bp downstream of P1266 in the fragment (Fig. 3), was found to be identical with the vWF gene sequence. The transitions were not found in 45 unrelated individuals (90 chromosomes). Since the vWF gene and its pseudogene are 97% identical, the mutant fragment probably originates from a recombination (21). If that is true, the fragment involved in the DNA recombination could be less than 100 nucleotides long. Another possible explanation for these transitions might be that the region which is involved is highly methylated, as these two transitions can be explained by an mC → T mutation at the CG dinucleotide.

Individual III:3, who has only the transitions, has lower levels of the plasma vWF and was earlier diagnosed as having vWD type I. Subject III:4 (Fig. 1), with the deletion in exon 18 and the transitions, has a prolonged bleeding time, a high ratio (3.45; normal range 0.6–1.6) of FVIII/vWF (Table 1), and lower plasma vWF (0.20 international unit/ml) compared with the other family members (II:3, II:4, II:6, and III:1), who have only the deletion in exon 18. In spite of the fact that she has blood group A [which is known to give higher vWF levels (20)], she is more affected than the other Swedish type I individuals, who were found to be heterozygous for the exon 18 cytosine deletion (unpublished data). The substitution is close to another substitution which we found in one Swedish vWD type III patient (unpublished data). The variation in phenotype may reflect the difference in the genotype. However, as the vWF is an extremely large protein and the levels of plasma vWFAg can vary under different conditions in the same individual (23), it is difficult to determine the effects of the substitution.

Since the deletion in exon 18 occurs in a stretch of six cytosines, the primer with the base mismatch at the 3′ end can easily slip, and a one-base loop is formed in the middle of the primer when it is annealed with the template in the PCR. For this reason a size-detecting method based on PCR was used to screen for the deletion in exon 18. Compared with oligonucleotide hybridization, this method is safe, easy, and quick. Screening for the deletion in members of vWD families and in patients suspected of having vWD can be of diagnostic value.

Linkage analyses of a highly informative $(GT)_n$ repeat marker in the vWF gene promoter region were performed. Eight different alleles (A1 to A8 according to the repeat numbers) were detected in the Swedish population (22). The results of the present study suggest that the mutant allele (with the deletion in exon 18) in the S and I families is linked to A6 $[(GT)_{21}]$ and that the normal allele is linked to A4 $[(GT)_{23}]$. Similar results were obtained in most of the Swedish vWD type III patients (unpublished data), which is not surprising since the Åland Islands are situated very near Sweden. The E, S-B, and I families have been shown to be related as far back as the 1650s. With regard to the relation between the S family and the others, only anecdotal evidence has hitherto been found. It should be possible to find if there is a relation between the Åland families and the Swedish families because in the Scandinavian countries all inhabitants are registered in the parish where they were born or died.

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