FRAMEWORK-DEPENDENT *IN VIVO* EXPRESSION OF TWO MISSENSE MUTATIONS (PRO^{134} →THR AND ALA^{244}→VAL) IN THE COAGULATION FACTOR VII GENE FROM A MALTESE KINDRED.

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INTRODUCTION

Coagulation factor VII (CFVII) is a trace vitamin K-dependent plasma glycoprotein (1) which, subsequent to activation, plays a pivotal role in the initiation of blood coagulation.

Hereditary CFVII deficiency (2) is a rare coagulopathy, inherited in an autosomal recessive manner with high penetrance and variable expression (3). On the other hand, increased CFVII coagulant activity has been identified as a risk factor for ischaemic heart disease (4).

Traditionally, CFVII functional variants have been described on the basis of diverse activation patterns of the extrinsic pathway when thromboplastins from brains of different mammalian species are employed in the clotting assay (5-7). In the last few years, 24 gene mutations have been reported.

In this report, we describe two additional molecular variants which were identified in a Maltese kindred with hereditary CFVII deficiency, and, established partial haplotypes which could account for the diversity in levels of activity both among wild-type and mutant CFVII proteins.

PATIENTS

The proband was a four year old girl without any history of bleeding episodes. CFVII deficiency was diagnosed during pre-operative coagulation studies prior to tonsillectomy. Additional coagulation and molecular studies were performed in cases I-1 to I-4, II-1, II-2, and III-1 to III-3 (refer to pedigree in Fig 1) after informed consent was obtained.

METHODS

CFVII coagulant activity (CFVII:C) was measured by the one-stage

clotting assay utilizing a panel of thromboplastins prepared by acetone drying of porcine, bovine, human and rabbit brain tissue. CFVII antigen levels (CFVII:Ag) were measured by an enzyme immunoassay kit.

Genomic DNA from members of the kindred was analysed for the presence of gross deletions, insertions or re-arrangements by Southern analysis.

Exons 2 to 8 of the parental CFVII alleles and their respective intron-exon boundaries were amplified as seven fragments by PCR and sequenced. Sequencing of fragments in which the sequence obtained differed from the normal CFVII sequence was repeated from a new, separate PCR.

Confirmation of mutations identified by sequencing and genotype analysis of other family members was performed by restriction endonuclease digestion or by allele-specific oligonucleotide hybridization analysis.

RESULTS

CFVII activity and antigen levels. The levels of CFVII activity using rabbit brain thromboplastin and the levels of CFVII antigen are given in the pedigree of Fig 1. Activity and antigen levels obtained for cases II-1 (mother), II-2 (father), III-1(proband's sister), III-2 (proband) and III-3 (proband's brother) suggested that the parents and the sister could be heterozygotes whereas the proband and her brother were homozygotes or double heterozygotes. CFVII coagulant activity levels obtained for cases I-1 (maternal grandmother), I-3 and I-4 (paternal grandparents) were in the range of normal, while that for case I-2 (maternal grandfather) was suggestive of heterozygosity.

One-stage CFVII assays were also performed with a panel of thromboplastins from four species on the presumed heterozygotes, II-1, II-2 and III-1 and the presumed homozygotes / double heterozygotes I II-2 and III-3. The results are given in Fig 2. It can be seen that the profiles of CFVII activity of cases II-1, III-1 and II-2 differed from each other. A striking feature is that the profile of the proband (III-2) was very similar to that of her brother (III-3). The existance of two distinct patterns for the parents (compare II-1to II-2) argues in favor of double heterozygosity (rather than homozygosity) for individuals III-2 and III-3.

Southern analysis. A gross CFVII gene re-arrangement was excluded since the sizes and intensity of the genomic restriction fragments appeared identical in control and patient DNA.

Identification of mutations by sequence analysis. Case II-2 was shown to be heterozygous for a missense mutation at nucleotide 8,906 (numbering as in O'Hara *et. al.*; 1987). This point mutation, designated CFVII Malta I, is a C $\rightarrow$ A transversion. Case II-1 was shown to be heterozygous for a missense mutation

at position 10,648, resulting in a C → T transition. This mutation has been named CFVII Malta II. Both index cases, that is II-1 and II-2 were also shown to be heterozygous for the previously described G→A transition at position 10,976 and C→T transition at position 7,880.

Confirmation of mutations and genotype analysis in family members. Restriction analysis and ASOH analysis confirmed the base pair substitutions identified by sequencing in the index cases and identified the genotype of other members of the kindred.

Segregation analysis of the four base pair substitutions gave linkage phases consistent with the four alleles of Table 1 and the genotypes of Fig 1.

DISCUSSION

Molecular analysis of CFVII sequences from members of a Maltese kindred with CFVII deficiency revealed the presence of two wild-type gene frameworks (wild-type framework 1 and wild-type framework 2), and two new mutations (CFVII Malta I; 8,906 C→A, and CFVII Malta II; 10,648 C→T). Interactions between the four different alleles which are characterized by the sequences shown in Table 1, could account for the phenotypic heterogeneity in coagulant activity and antigen levels among family members (Fig 1).

Frameworks constitute fixed sequence variations that form the backgrounds upon which mutations leading to specific phenotypes occur (8). Rather than displaying scattered sequence differences between individuals, the gene exhibits polymorphism limited to common sequence types. Some such frameworks such as those occurring in the β-globin gene (8) and perhaps also CFTR (9) predate racial divergence and are considered ancient, while the mutations are a more recent event.

The CFVII wild-type framework 2 allele is associated with Gln instead of Arg at amino acid residue 353 of the CFVII protein and results in a moderately decreased CFVII antigen and coagulant activity (10,11). Compared with wild-type framework 1 homozygotes, wild-type framework 2 heterozygotes have about 75% activity while wild-type framework 2 homozygotes have about 50% activity, as observed in cases I-1, and I-4 (Fig 1). Consequently, it can be anticipated that the functional effects of mutations in CFVII alleles could depend on whether the mutation occurred on either CFVII framework 1 or framework 2 alleles. It appears from the limited studies reported that the major effect of the framework 2 allele is to decrease the proportion of the circulating zymogen that is activated (11). It has a heterozygous incidence of 10% in the UK and the USA (10) and is clearly detectable also in Mediterranean populations (this study and ref. 12).

The CFVII Malta I mutation occurred on a framework 1 gene. It is a C→A transversion at nucleotide 8,906 in exon 6 (numbering as in O'Hara *et. al.*; 1987) and predicted a proline to

threonine replacement at amino acid 134. Proline^{134} is located just after the second potential growth factor domain and immediately prior to the single interchain disulfide bond.

The CFVII Malta II mutation occurred on a CFVII framework 2 gene. It is a C $\rightarrow$T transition in a CpG dinucleotide occurring at position 10,648 in exon 8. CpG dinucleotides are known to be frequent sites of mutation. This base substitution predicted the replacement of alanine at position 244 by valine. Alanine^{244} is the second residue downstream of the aspartic acid which forms part of the catalytic triad His-193, Asp-242, Ser-344. A mutation analogous to CFVII Malta II has been detected in CFIX (13) in which In Ala^{271} $\rightarrow$Val is associated with severe deficiency of CFIX coagulant activity (CFIX:C $\cong$ 1%).

The two mutations occurred in four different genotypes among five family members and meaningful comparisons with respect to genotype-phenotype relationships are consequently limited. A useful comparison can however be made between cases II-2 and III-1 (refer to Fig 2). Both are CFVII Malta I - FW1 heterozygotes, but, while case II -2 has wild-type CFVII framework 2 on the allele *in trans* with CFVII:C of 27% (rabbit thromboplastin) and CFVII:Ag of 41%, case III-1 has wild-type CFVII framework 1 *in trans* with higher levels of CFVII:C (46%; rabbit thromboplastin) and CFVII:Ag (73%). These data concur with previous reports (10,11) that framework 2 is associated with a decrease in both coagulant activity as well as in antigen level.

Another comparison can be made between cases II-1 and III-1. Both are heterozygotes for the wild-type CFVII framework 1 allele, but case II-1 has CFVII Malta II - FW2 *in trans* whereas case III-1 has the CFVII Malta I - FW1 allele *in trans* together with slightly lower CFVII:C (except with rabbit thromboplastin) and higher CFVII:Ag levels. This implies that the CFVII Malta II mutation is less severe than the CFVII Malta I, or that the wild-type CFVII framework 1 allele has a dominant position on CFVII expression as shown above, or both.

The CFVII deficiencies documented in this study are the only cases that have been observed thus far in the population of Malta (365,000). This incidence is well within the reported worldwide incidence of 1 in 500,000 (18), even though the actual worldwide as well as local frequency may actually be somewhat higher since those with a mild and at times even with a substantial deficiency could go undetected.

The occurrence of alternate gene frameworks in the context of which specific mutations occurred may be related to the phenotypic variability of coagulation disorders and perhaps also the *in vivo* expression of other genes. It can be seen that interpretation of genotype-phenotype relationships may be complicated by the differential effects of sequence variations beyond the immediate site of a mutation.

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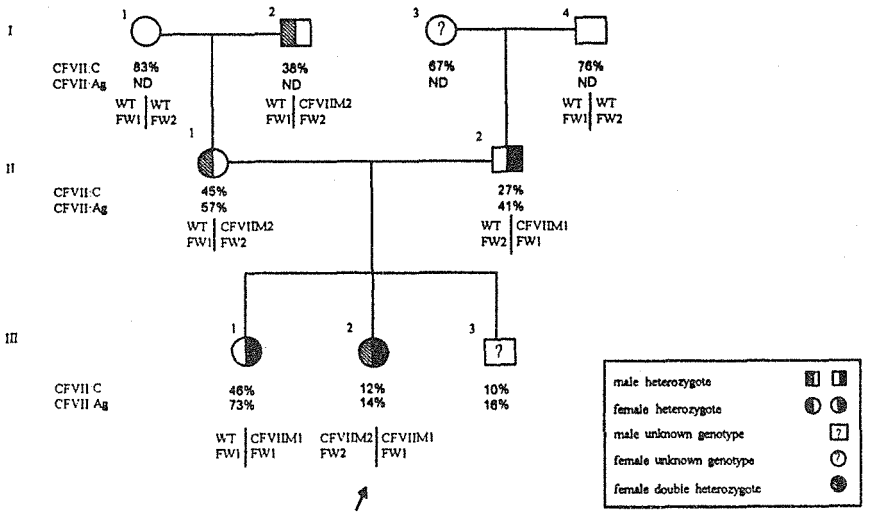

Fig 1. Pedigree of kindred including percentage CFVII coagulant activity values utilizing rabbit brain tissue thromboplastin (normal range 60-150%), CFVII antigen levels (normal range 70-130%) and genotype analysis results. CFVIIM1 = CFVII Malta I; CFVIIM2 = CFVII Malta II; WT = wild-type; FW1 = framework 1; FW2 = framework 2; ND = not done.

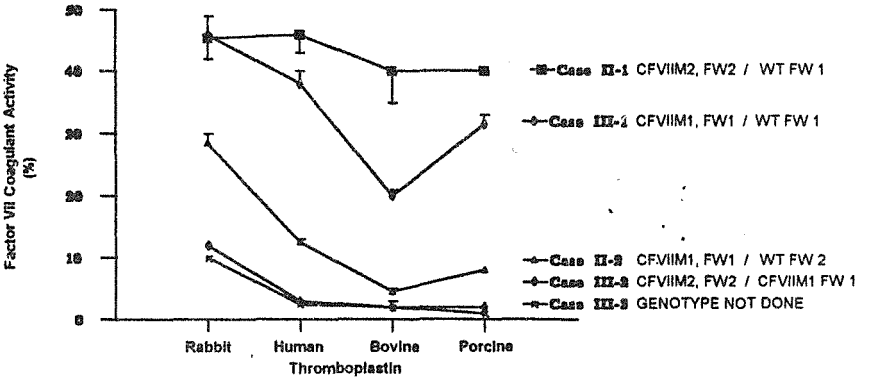

Fig 2. Profiles of CFVII coagulant activity obtained with thromboplastins from rabbit, human, bovine, and porcine brain tissue.

Nucleotide position	7,880	8,906	10,648	10,976
Wild-type framework 1	C	C	C	G
Wild-type framework 2	T	C	C	A
FW1; CFVII Malta I	C	A	C	G
FW2; CFVII Malta II	T	C	T	A

Table 1. The four different alleles that segregated in the kindred.