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Genotype/Phenotype Correlations for Coagulation Factor XIII: Specific Normal Polymorphisms Are Associated With High or Low Factor XIII Specific Activity

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Factor XIII is a transglutaminase essential for normal hemostasis. We have studied the plasma FXIII levels and FXIII activity in 71 individuals and found these to be normally distributed. FXIII specific activity is also normally distributed. However, we show that FXIII activity is not directly dependent on FXIII levels, and individuals with low FXIII levels may have high FXIII activity and vice versa. We have determined the FXIIIA genotype in these individuals to assess whether the variation observed in FXIII specific activity is dependent on specific polymorphisms in the FXIIIA gene. Our data show that the Leu34 and Leu564 variants give rise to increased FXIII specific activity, while the Phe204 variant results in lower FXIII specific activity. We also report preliminary evidence that the Phe204 polymorphism may be associated with recurrent miscarriage. Overall, we have identified 23 unique FXIIIA genotypes. Certain specific FXIIIA genotypes consistently give rise to high, low, or median FXIII specific activity levels, while others appear to have little or no consistent influence on the FXIII phenotype. These genotype to phenotype relationships are discussed in light of the growing interest in the role of FXIII in clinical problems involving an increased thrombotic tendency.

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FXIII IS A TRANSGLUTAMINASE, which plays an essential role cross-linking fibrin in the final stages of the blood coagulation pathway. Low levels of FXIII have been reported in a number of clinical conditions, including inflammatory bowel disease (both Crohn's disease and ulcerative colitis), as well as several types of malignancy.[1-3] Increased transglutaminase activity is found in the Alzheimer's disease brain.[4] In addition, tissue transglutaminase is now known to be the autoantigen of celiac disease.[5] Several studies have also indicated that FXIII is important in stimulation of connective tissue cells and the processes of inflammation and wound healing.[6-9] Inherited homozygous FXIII deficiency results in serious bleeding complications, inefficient wound healing, and a high risk of miscarriage in deficient females.[10,11]

The distribution of FXIII is now known to be almost ubiquitous.[12] Intracellular FXIII exists as a homodimer of two A subunits, while circulating FXIII is an A_2B_2 tetramer.[13] The A subunit constitutes the catalytic moiety and the B subunit is thought to play a role in stabilization of the A subunit. On activation by thrombin and Ca^{2+}, the A and B subunits dissociate. The A subunit is then cleaved to produce the catalytically active form of the protein, FXIIIA*.[14] FXIIIA* catalyzes the Ca^{2+}-dependent formation of intermolecular ϵ-(γ-glutamyl) lysine bonds between fibrin molecules leading to stabilization of the clot structure.[15] FXIIIA* can also cross-link fibronectin, vitronectin, collagen, and lipoprotein(a) in the extracellular matrix.

Deficiency of both the FXIII A and B subunits has been described. However, in the majority of cases, inherited autosomal recessive FXIII deficiency is due to defects in the gene for the FXIIIA subunit.[16-18] Hence, the FXIIIA subunit has been studied much more extensively than the FXIIIB subunit. The FXIIIA gene is now known to be highly polymorphic.[16] The known, normal FXIIIA gene polymorphisms are presented in Table 1.

A number of methods have previously been used to determine the activity of FXIII in plasma. These are based on measurements of clot stability,[19] ammonia production,[20] and incorporation of labelled amines into either polymers of casein[21,22] or fibrin.[23] We describe a modification of the activity assay of Song et al,[23] based on measuring incorporation of biotinylcadaverine into fibrinogen to determine the plasma FXIII activity. We also present an enzyme-linked immunosorbent assay (ELISA) for determination of FXIII levels. Both assays are performed in 96-well microtiter plates for speed and for ease of handling of large sample numbers.

In this report, we show that FXIII activity is not directly dependent on FXIII levels and discuss the variation we have observed in FXIII specific activity in 71 unrelated healthy individuals. We also present the FXIIIA gene sequence variations we have found in 113 individuals (comprising 36 normal men, 42 normal women, and 35 women who have suffered three or more recurrent miscarriages), who are all normal with respect to FXIII, from the United Kingdom. This variation in genotype is then compared with the variation in FXIII specific activity, in each individual in each group, to assess the influence of specific amino acid changes (inferred from the genotype data) and the combination of FXIIIA polymorphisms on specific activity. We describe the first examples of associations between normal FXIIIA variants and high or low FXIII specific activity. We therefore provide the first evidence of a genetic basis for the wide variation seen in normal plasma FXIII levels and activities. Women who are homozygously deficient in FXIII are known to suffer spontaneous abortion.[11] We now present data, which suggests an association between one specific FXIIIA normal genetic variant and risk of recurrent miscarriage in women who are otherwise normal with respect to plasma FXIII.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. Sheep anti-human FXIII was obtained from The Binding Site (Birmingham, UK) and rabbit anti-human FXIII was

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purchased from Calbiochem (La Jolla, CA). Biotinylcadaverine was obtained from Pierce & Warriner (Chester, UK). Standard normal plasma (Behring Standard Human Plasma) was obtained from Behring Diagnostics (Milton Keynes, UK).

Subjects. Blood samples were analyzed from 113 consenting, healthy, unrelated individuals who do not suffer from FXIII deficiency. The 113 individuals are subdivided as follows: 36 normal (N) men, 42 N women, and 35 women who have suffered three or more recurrent miscarriages. The mean age of these individuals was 30.3 years ± 5.5 years (standard deviation), age range, 18 to 49 years. Ethical approval from The Leeds (East) Medical Ethics Committee was obtained before initiating this study.

Sample processing. Blood was collected in citrated Monovette tubes (Sarstedt, UK). The plasma was separated by centrifugation, aliquoted, and flash frozen in liquid nitrogen for storage at −70°C. The buffy coat, containing the peripheral blood mononuclear cells (PBMCs), was also collected and stored at −70°C for isolation of genomic DNA.

FXIII ELISA. FXIII levels were determined using an ELISA. Ninety-six–well microtiter plates (Corning, High Wycombe, UK) were coated with 100 µL/well of sheep anti-human FXIII antisera (1:1,000 dilution of 10 mg/mL antisera, in 200 mmol/L citrate/phosphate buffer, pH 3.0) for 1 hour at 37°C. The plates were blocked for 1 hour at 37°C using 200 µL/well of blocking buffer (1% bovine serum albumin in 0.5 mol/L NaCl, 20 mmol/L Tris.HCl, pH 7.5, containing 0.01% Tween-20 and 0.02% azide). The plate was then washed twice with blocking buffer. For each test plasma sample and the normal plasma standard, a range of dilutions was performed in blocking buffer ensuring that the dilution range covered both the maximum and the minimum possible response. A total of 100 µL of diluted plasma samples was then loaded into each well, in triplicate, and the plate incubated for 2 hours at 37°C. The plate was washed twice with blocking buffer, then twice with washing buffer (0.5 mol/L NaCl, 20 mmol/L Tris.HCl, pH 7.5, containing 0.01% Tween-20 and 0.02% azide) before applying 100 µL of rabbit anti-human FXIII antisera (1:1,000 dilution in blocking buffer) to each well. After a further incubation for 1 hour at 37°C, the plate was rinsed once with blocking buffer followed by two rinses with washing buffer. A total of 100 µL of anti-rabbit IgG conjugated to alkaline phosphatase (1:20,000 dilution in blocking buffer) was added to each well and the plate incubated for 1 hour at 37°C. The plate was then washed twice with washing buffer.

To each well, 100 µL of alkaline phosphatase substrate, p-nitrophenol phosphate (pNPP; 1 mg/mL in 1 mol/L diethanolamine, pH 9.8, containing 0.5 mmol/L $MgCl_2$), was added and the color development performed at 37°C. The reaction was stopped by the addition of 100 µL of 4 mol/L NaOH. The absorbance was then read at 405 nm.

Calculation of FXIII levels. The relative amount of FXIII in each plasma sample was determined using a relative quantitation method comparing 50% maximum binding (IC_{50}) using dilutional analysis for each plasma sample. In this method, it is important that the maximum and minimum responses are achieved. The absorbances at which these two responses occur are then designated 100% and 0% response, respectively, and the absorbances observed at all other dilutions of plasma are calculated as a percentage of the maximum response. Figure 1A shows a graph of percentage response versus dilution of plasma. A sigmoidal fit is applied and the dilution at which the IC_{50} is achieved is then calculated. The relative FXIII level in each test plasma sample is determined by the ratio of the IC_{50} of the test plasma to the IC_{50} of standard normal plasma.

FXIII activity assay. FXIII activity was determined using a modification of the fibrinogen and biotinylcadaverine assay described previously.[23] Ninety-six–well microtiter plates were coated with fibrinogen (100 µL/well of a 40-mg/mL solution in TBS; 40 mmol/L Tris.HCl, pH 8.3, 140 mmol/L NaCl, 0.02% azide) for 40 minutes at 25°C. The plate was then blocked with 0.5% nonfat dried milk in TBS (NFDM). After 20 minutes at 37°C, the plate was washed with TBS and the following reaction components were added; 50 µL of TBS, 10 µL of 0.5 mmol/L dithiothreitol (DTT), 10 µL of 40 mmol/L biotinylcadaverine, 10 µL of a 1:10 dilution of test plasma, 10 µL of 1 mol/L $CaCl_2$, and finally 10 µL of thrombin solution (200 U/mL in TBS). The reaction was performed at room temperature and stopped at various time points by the addition of 200 µL of 200 mmol/L EDTA. For t = 0, EDTA was added to the wells before the addition of the reagents. The plate was then rinsed with TBS and 100 µL of streptavidin-alkaline phosphatase (SAAP) conjugate (1:100 dilution of a 1 mg/mL solution in NFDM) was added to each well. After incubation for 1 hour at 37°C, the plate was washed twice with TBS containing 0.01% Triton X100, followed by two rinses with TBS. The color development step was performed as described for the FXIII ELISA above.

For each plasma sample, the initial rate of generation of product is determined (Fig 1B), and the activity is calculated as a percentage of the rate observed with standard normal plasma.

Statistical analysis. This was performed using SPSS (SPSS UK, United Kingdom) and Clump statistics software (Dave Curtis; www.hgmp.mrc.ac.uk). The analysis performed on the distribution of FXIII levels, activity, and specific activity was done using the Kolmogorov-Smirnov "Goodness of Fit" Test which compares a given distribution with a normal distribution.

Genotype analysis. Genomic DNA was isolated from PBMCs using standard procedures. Exons 2, 5, 12, and 14 of the FXIIIA gene, carrying the known normal polymorphisms, were amplified by polymerase chain reaction (PCR) as described previously.[16] PCR products from exons 2, 5, and 14 were denatured and then subjected to single-strand conformational polymorphism (SSCP) analysis in GeneGel Excel polyacrylamide gels (122 × 110 × 0.5 mm; Pharmacia Biotech, St Albans, UK) cooled continuously at 15°C, using the Pharmacia Biotech PhorGene electrophoresis unit. After electrophoresis at 600 V for 90 minutes, the gels were silver-stained using the Pharmacia Biotech silver staining kit. PCR products showing mobility shifts in SSCP analysis were sequenced as described previously.[16] The sequence variation at codon 564 (leucine or proline) was determined by amplification refractory mutation system-PCR (ARMS-PCR)[17] using the forward primer (dTTGCCTGTCATTATCTCTGG) with both a leucine specific reverse primer (dCTTCTTGAAYTCTGCCTTGA), and proline specific reverse primer (dCTTCTTGAAYTCTGCCTTGG) in separate PCRs. The sequence variation at codon 567 was determined by EcoRI restriction analysis of the exon 12 PCR product.

RESULTS

Factor XIII levels. An ELISA assay has been developed to determine the levels of FXIII in plasma. Figure 1A shows the dilution profiles for plasma samples from three different individuals and the method for determining the relative levels in each. It is clearly possible to distinguish plasma samples containing different concentrations of FXIII. The plasma FXIII levels were determined in 73 unrelated individuals. Levels were found to vary between 47.9% and 243.9% of that of the standard normal plasma with a mean at 105% ± 37.64% standard deviations (Fig 2A). This variation was analyzed and found to be consistent with a normal distribution (Fig 2A). The range of FXIII levels was compared between the three groups studied and no differences were found. The range of FXIII levels found is comparable to results we obtained previously from 34 normal, unrelated individuals using a rocket immunoelectrophoresis assay (unpublished results, 1983). Our results also compare favorably with other, smaller studies of Murdock[24] (mean, 95; range, 60 to 130, n = 24) and Shainoff[25] (mean, 112; range, 50 to 200, n = 12).

FXIII activity. The FXIII activity in plasma was determined by monitoring the rate of incorporation of biotinylated cadaver-

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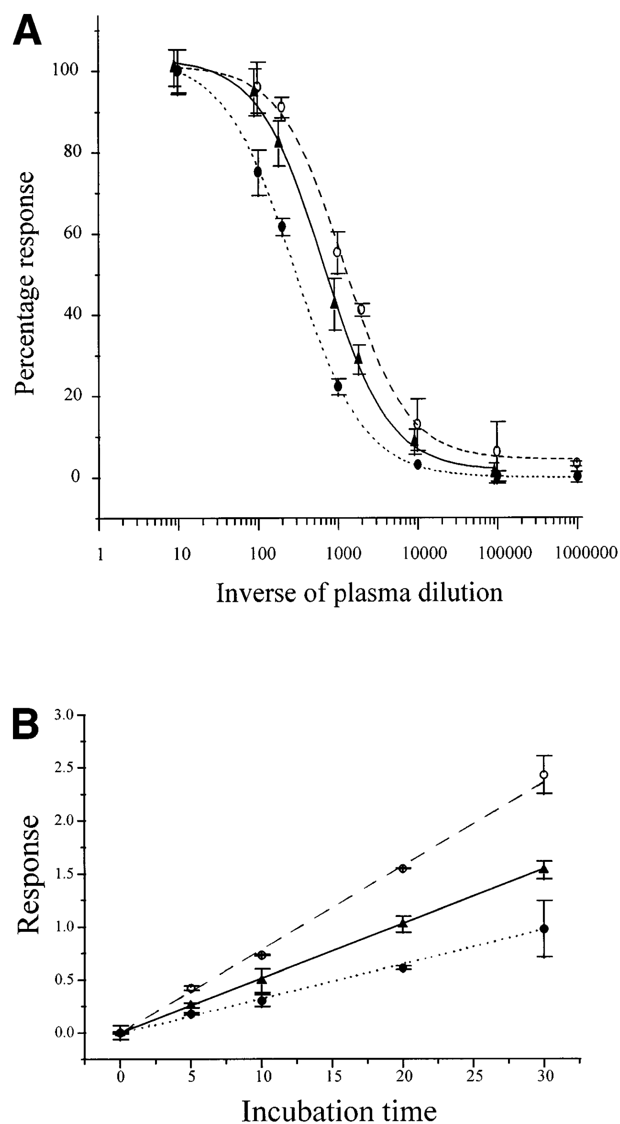

Fig 1. (A) The percentage response at different dilutions, assayed in triplicate, of three separate plasma samples. The FXIII level in each of the plasma samples is determined by extrapolating the inverse of the dilution at the IC_{50} and calculating the FXIII level as a percentage of the IC_{50} observed with standard normal plasma. (B) Initial rates of incorporation of biotinylated cadaverine into fibrin in three separate plasma samples showing the different rates detected for each of these samples. Each sample was assayed in triplicate for every time point.

ine into fibrin. Figure 1B shows that a difference in the rates of incorporation can be detected in different plasma samples. When the plasma FXIII activity was investigated in a population of 72 unrelated individuals, it was found to range between 53.2% and 221.3% of the standard normal plasma value (Fig 2B). The mean was calculated to be 105% ± 28.56% standard deviations. This variation was also found to indicate a normal distribution (Fig 2B). Again, no difference was found in the range of FXIII activities between the three groups studied. The range that we have found for FXIII activity is similar to the 47% to 250% range we had previously determined when measuring the rate of incorporation of dansylcadaverine into casein and comparing it with normal pooled plasma (data not presented). Our data are also comparable to the results of Wagner et al,[26] who found the FXIII activity range to be between 0.51 to 1.52 of the pooled plasma value (in U/mL for n = 64).

FXIII specific activity. The FXIII specific activity was calculated for all the individuals studied. Figure 2C shows the distribution of specific activity in 71 unrelated individuals and this is compared with a normal distribution. There was no significant difference in the range of specific activities found in the three groups studied. The overall FXIII specific activity range was found to be between 0.50 and 2.13, with the value for standard normal plasma being set at 1.0. The mean was 1.08 ± 0.40 standard deviations. Both the Kolmogorov-Smirnov Test (Fig 2A through C) and the normal probability plot (Fig 2D) suggested that the FXIII specific activities we have found in our study fit a normal distribution.

FXIIIA genotype. Analysis of the FXIIIA gene exons 2, 5, 12, and 14 was performed in 113 individuals. The polymorphisms Arg77Gly, Arg78Lys, and Phe88Leu in exon 3 were not examined because these have been identified only at the amino acid or cDNA level and have never been reported at the genomic DNA sequence level (Table 1). We have sequenced exon 3 of the FXIIIA gene in 25 individuals and have not found any to carry the codon 77, 78, or 88 variations.

The frequency of each of the normal sequence variations is presented in relation to the number of chromosomes studied (Tables 2 and 3) and to numbers of homozygotes and heterozygotes for each amino acid change (Table 4). Although the frequency of Leu at codon 34 is about 20% (Table 2), only one individual from 113 (0.88%) was found to be homozygous for Leu34 (Table 4). Also, there were no homozygotes found for Phe at codon 204 or Ile at codon 650 (Table 4). The GAG sequence at codon 567 was only observed in Pro564 homozygotes and Pro564/Leu564 heterozygotes, but not in Leu564 homozygotes. Nine different haplotypes were identified from 88 individuals, representing 176 chromosomes, who were either homozygous for each polymorphism or heterozygous at only one locus (Table 3). The Val34-Tyr204-Pro564-Val650-Glu651 (11111) haplotype appears to be the most common. Twenty-three unique genotypes were identified in the population studied, suggesting a high degree of genetic variability (Table 5). Again the genotype Val34/Val34-Tyr204/Tyr204-Pro564/Pro564-Val650/Val650-Glu651/Glu651 (11111) is the most common followed closely by Val34/Leu34-Tyr204/Tyr204-Pro564/Pro564-Val650/Val650-Glu651/Glu651 (31111) and Val34/Val34-Tyr204/Tyr204-Pro564/Leu564-Val650/Val650-Glu651/Glu651 (11311).

The genotype frequencies for each of the polymorphisms were then compared between the different groups studied (Tables 2 and 4). The Phe204 polymorphism appears to be more common in the recurrent miscarriage (RM) group (Tables 2 and 4). A significant difference for Phe204 frequency was discovered when the RM data were compared with all normals (78 N men and women; $P = .017$), as well as when the RM data were compared with the N women group ($P = .053$). There were no significant differences found for codons 34, 564, 650, and 651 between N men versus N women, N men versus all women (N

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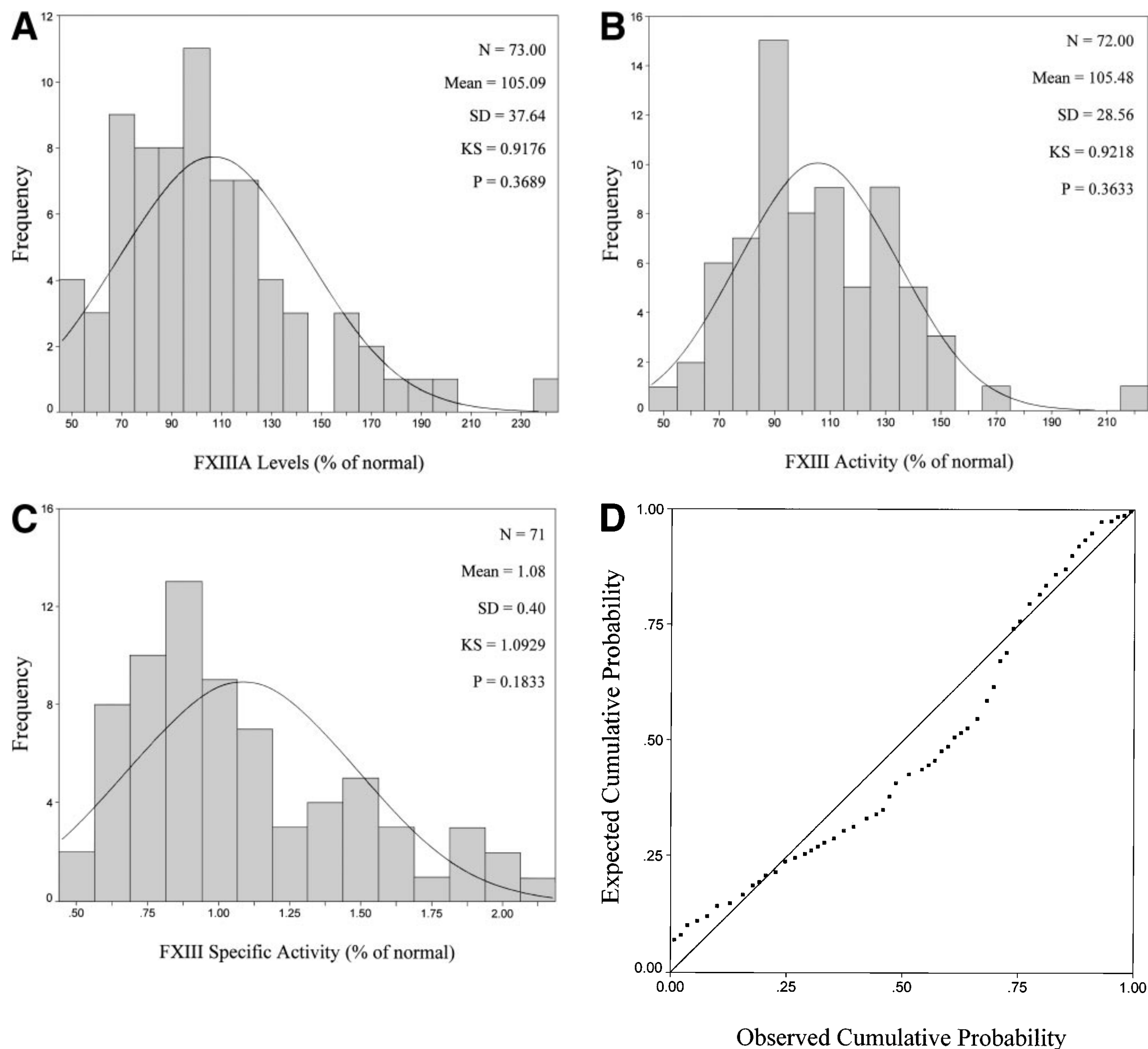

Fig 2. FXIII activity, levels, and specific activity (SA) within the normal study population. The Kolmogorov-Smirnov Goodness of Fit Test was used to test the hypothesis that the values obtained for FXIII levels, activity, and SA match a normal distribution. The value of the Kolmogorov-Smirnov test is based on the largest absolute difference between the observed and the theoretical cumulative distributions. The two-tailed probability value was used to decide whether the null hypothesis should be rejected. This is the probability of obtaining results as extreme as the ones observed, in either direction, when the null hypothesis is true. Because the two-tailed *P* values obtained are much higher than .05, the null hypothesis is not rejected, and hence the variation observed in FXIII levels, activity, and SA fits a normal distribution. For (A) to (C) the normal distribution curve is also plotted for comparison. (A) This histogram illustrates the variation seen in plasma FXIII levels in 73 unrelated individuals comprising 22 normal men, 37 normal women, and 14 RM women. (B) The distribution of plasma FXIII activity in 72 unrelated individuals comprising 23 normal men, 35 normal women, and 14 from RM group. (C) FXIII SA was defined as FXIII activity per unit FXIII level, this being 1.0 (ie, 100% activity/100% level) for the standard normal plasma. SA of plasma FXIII in 71 unrelated individuals (comprising 22 normal men, 35 normal women, and 14 RM women) mean age, 30.2 years, ± 5.5 standard deviation; age range, 18 to 48. (D) A normal probability plot was used to determine whether FXIII SA is normally distributed. The cumulative proportion for a single numeric variable is plotted against the cumulative proportion expected if the sample were from a normal distribution. Because the points all cluster around a straight line, this suggests that FXIII SA has a normal distribution within the population studied. SD, standard deviation; N, numbers studied; KS, Kolmogorov-Smirnov Test; *P*, two-tailed probability value.

women + RM women), N women versus the RM group, and the RM group versus all normals (N men + N women).

FXIIIA genotype/phenotype correlation. This analysis also includes some individuals from families we have studied previously,[16] known to be heterozygous for FXIIIA deficiency alleles and these were therefore not presented as part of the normal population study (Fig 2). The influence of each specific FXIIIA polymorphism on FXIII specific activity was assessed (Table 4). Alleles coding for Leu34, Tyr204, or Leu564 are associated with high FXIII specific activity. The effects of the

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Table 1. Known Normal Polymorphisms in the FXIIIA Gene Coding Region

Exon	No. of Amino Acid Residues	Codon	Amino Acid
2	34	GTG	Val
		TTG	Leu
3	77	Not known	Arg
			Gly
3	78	Not known	Arg
			Lys
3	88	CTC	Leu
		TTC	Phe
5	204	TAT	Tyr
		TTT	Phe
12	564	CCG	Pro
		CTG	Leu
12	567	GAA	Glu
		GAG	Glu
14	650	GTT	Val
		ATT	Ile
14	651	GAG	Glu
		CAG	Gln

The nucleotide polymorphism at codon 88 has only been reported at the cDNA level.

polymorphisms at codons 650 and 651 appear to be almost minimal to normal FXIII specific activity. The FXIII specific activity found for each of the different genotypes (representing codons 34, 204, 564, 650, and 651) is presented in Table 5 and Fig 3. It is clear that specific genotypes can be associated with a tendency to give rise to FXIIIA molecules with high, median, or low specific activities (Fig 3). Some FXIIIA genotypes appear to have no influence on the resultant FXIII specific activity, the same genotype resulting in variable FXIII specific activity, indicating there may be other factors additional to the genotype, which also affect this (Fig 3). The influence of Leu564 and Pro564 on FXIII specific activity is also clearly visible in Fig 3. Leu564 is associated with a higher FXIII specific activity compared with Pro564.

DISCUSSION

We have developed reliable assays for the determination of both the activity and the levels of FXIII in plasma. These have enabled us to study the variation in FXIII specific activity in a population of 71 unrelated individuals. There are no previous reports in the literature on determination of the specific activity of FXIII in different individuals. We now show that the specific activity in different individuals does vary considerably, while displaying a normal distribution for the population. Our data show that FXIII levels are not directly indicative of FXIII activity. Hence, individuals may have very high levels of the FXIII protein, but very low levels of FXIII activity, and vice versa.

Our genotyping data confirm the high genetic variability of the FXIIIA gene. The frequency of each of the amino acid polymorphisms at residues 34, 204, 564, 650, and 651 we have found in the normal UK population (normal men and women) correlates with those found for other smaller white populations studied (Finnish, n = 26; Russian, n = 21; and German, n = 14).[27] Genotype frequency analysis between the three groups studied shows that the Phe204 residue is significantly more common in the RM group compared with normal men and women. This is also true when our RM data for Phe204 are compared with the study of Suzuki et al,[27] who report only one Phe204 allele in 128 normal alleles from the white population, giving $P = .013$.

Table 3. Haplotypes Identified From 88 Individuals Who Were Either Homozygous for All of the FXIIIA Normal Polymorphisms or Were Heterozygous for Only One Polymorphism

	Sequence Haplotype	Frequency (176)	Frequency %
1	11111	88	50.00
2	11211	35	19.90
3	11112	21	11.93
4	21111	17	9.66
5	11212	5	2.84
6	12111	3	1.70
7	21112	3	1.70
8	21212	2	1.14
9	21211	2	1.14

The haplotype codes are explained in Table 2 and are given 5′ to 3′ for codons 34-204-564-650-651. For example, haplotype 21212 = Leu34-Tyr204-Leu564-Val650-Gln651, and haplotype 21112 = Leu34-Tyr204-Pro564-Val650-Gln651. An individual with these two alleles would be heterozygous at codon 564 only.

Table 2. Frequency of the Normal FXIIIA Polymorphisms Found at Codons 34, 204, 564, 567, 650, and 651 in 113 Individuals, Representing 226 Chromosomes

Amino Acid Residue	Genotype	N Men (72)	N Women (84)	RM Women (70)	Frequency (226)	Numerical Code
34 (Exon 2)	GTG	60	71	55	186	1
	TTG	12	13	15	40	2
204 (Exon 5)	TAT	71	83	65	209	1
	TTT	1	1	5	7	2
564 (Exon 12)	CCG	49	61	54	164	1
	CTG	23	23	16	62	2
567 (Exon 12)	GAA	66	74	61	201	—
	GAG	6	10	9	25	—
650 (Exon 14)	GTT	69	83	70	222	1
	ATT	3	1	0	4	2
651 (Exon 14)	GAG	58	69	51	178	1
	CAG	14	15	19	48	2

The frequency is given for number of alleles.
Abbreviations: N, normal; RM, recurrent miscarriage.

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Table 4. The Frequency of Homozygous and Heterozygous Individuals for Each of the FXIIIA Normal Polymorphisms

Exon	Amino Acid Residue	Genotype	Amino Acid	N Men (36)	N Women (42)	RM Women (35)	Total Frequency (113)	FXIII Specific Activity Mean ± SD	Numerical Code
2	34	GTG/GTG	Val/Val	25	29	20	74	1.098 ± 0.423 (53)	1
		TTG/TTG	Leu/Leu	1	0	0	1	1.73 (1)	2
		GTG/TTG	Val/Leu	10	13	15	38	1.285 ± 0.476 (22)	3
5	204	TAT/TAT	Tyr/Tyr	35	41	30	106	1.173 ± 0.454 (71)	1
		TTT/TTT	Phe/Phe	0	0	0	0	—	2
		TAT/TTT	Tyr/Phe	1	1	5	7	0.990 ± 0.307 (5)	3
12	564	CCG/CCG	Pro/Pro	19	23	21	63	1.079 ± 0.440 (37)	1
		CTG/CTG	Leu/Leu	6	4	2	12	1.341 ± 0.485 (9)	2
		CCG/CTG	Pro/Leu	11	15	12	38	1.207 ± 0.435 (30)	3
14	650	GTT/GTT	Val/Val	33	41	35	109	1.163 ± 0.452 (72)	1
		ATT/ATT	Ile/Ile	0	0	0	0	—	2
		GTT/ATT	Val/Ile	3	1	0	4	1.200 ± 0.417 (4)	3
14	651	GAG/GAG	Glu/Glu	25	33	21	79	1.157 ± 0.467 (52)	1
		CAG/CAG	Gln/Gln	3	6	5	14	1.120 ± 0.392 (9)	2
		GAG/CAG	Glu/Gln	8	3	9	20	1.220 ± 0.375 (15)	3

Correlation of each polymorphism with FXIII specific activity (SA) found for that polymorphism. The numbers in parentheses in the FXIII SA column refer to numbers of individuals with that FXIII SA.

Abbreviations: N, normal; RM, recurrent miscarriage.

It has been known for many years now that autosomal recessive FXIII deficiency in women leads to spontaneous miscarriage unless the patients receive plasma FXIII supplementation through FXIII concentrate injections.[10,11] It is also well established that only 0.5% to 2% of normal FXIII levels are sufficient to achieve normal hemostasis.[11] It is therefore very interesting to find the Phe204 polymorphism to be associated with RM, particularly when this variant gives rise to almost normal FXIII specific activity when assaying for fibrin cross-linking. It is possible that FXIII may cross-link substrates other than fibrin during its role in the maintenance of pregnancy, and the Phe204 association with recurrent miscarriage may correlate with FXIII structure and may indeed be independent of its level and activity. In fact, three-dimensional analysis of the FXIIIA protein molecule[28] in this region using the *RASMOL* software (Roger Sayle; www.umass.edu/microbio/rasmol/) shows that the polar side chain of Tyr at position 204 is able to form a hydrogen bond with Arg333, while Phe, with a nonpolar side chain, at position 204 is unable to form this interaction. This has implications for the final FXIIIA structure achieved by the two variants. In addition, from the structure, it appears that the hydrogen bond between Tyr204 and Arg333 has to be broken before the molecule can be activated. This may be the basis of the difference observed in FXIII specific activity between alleles with Phe204 and Tyr204.

Table 5. The Frequency of the FXIIIA Genotypes Identified in 113 Individuals Analyzed and Correlation of Each Genotype With FXIII SA Found for That Genotype

	FXIIIA Genotype	Frequency (113)	FXIII Specific Activity Mean ± SD
1	11111	24	0.94 ± 0.32 (16)
2	11112	5	0.82 ± 0.17 (3)
3	11113	4	1.11 (1)
4	11133	1	0.67 (1)
5	11211	8	1.34 ± 0.54 (6)
6	11213	1	0.84 (1)
7	11311	16	1.36 ± 0.51 (13)
8	11312	4	1.02 ± 0.32 (3)
9	11313	6	1.09 ± 0.32 (4)
10	13111	3	1.16 ± 0.49 (2)
11	13311	2	0.80 ± 0.07 (2)
12	13313	1	1.02 (1)
13	21212	1	1.73 (1)
14	31111	17	1.13 ± 0.64 (7)
15	31112	3	1.64 (1)
16	31113	3	1.59 ± 0.46 (3)
17	31133	2	1.52 ± 0.16 (2)
18	31211	2	1.44 (1)
19	31311	6	1.18 ± 0.58 (4)
20	31312	1	1.2 (1)
21	31313	1	1.41 (1)
22	31333	1	1.09 (1)
23	33111	1	ND

The numbers in parentheses in the FXIII SA column refer to numbers of individuals with that FXIII SA. The genotype codes are 5′ to 3′ for codons 34-204-564-650-651 and are explained in Table 4. For example, genotype 31312 = Val34/Leu34-Tyr204/Tyr204-Pro564/Leu564-Val650/Val650-Gln651/Gln651.

Abbreviation: ND, not done.

The comparative frequency of each polymorphism analyzed in the UK population of normal men and women is Leu564 > Gln651 > Leu34 > Ile650 > Phe204. The presence of five amino acid polymorphisms in apparently normal FXIIIA genes translates into 32 possible haplotypes. Although we could identify nine unique FXIIIA haplotypes in the UK population, we were unable to determine complete haplotypes for all of the people studied because 25 individuals proved to be heterozygous at more than one locus.

The genotype to phenotype correlations clearly indicate that Leu34, Leu564, and Tyr204 are associated with high FXIII specific activity. Activated FXIII (FXIII*) stabilizes the fibrin gel through two sets of ε (γ-glutamyl) lysine covalent bonds: two in the γ-chains and four in the α-chains, giving rise to γ-chain dimers and high molecular weight α-chain polymers. The structure of the α-polymer is further complicated by cross-linking to it of other plasma proteins including α2-plasmin inhibitor and fibronectin. In vitro studies have shown

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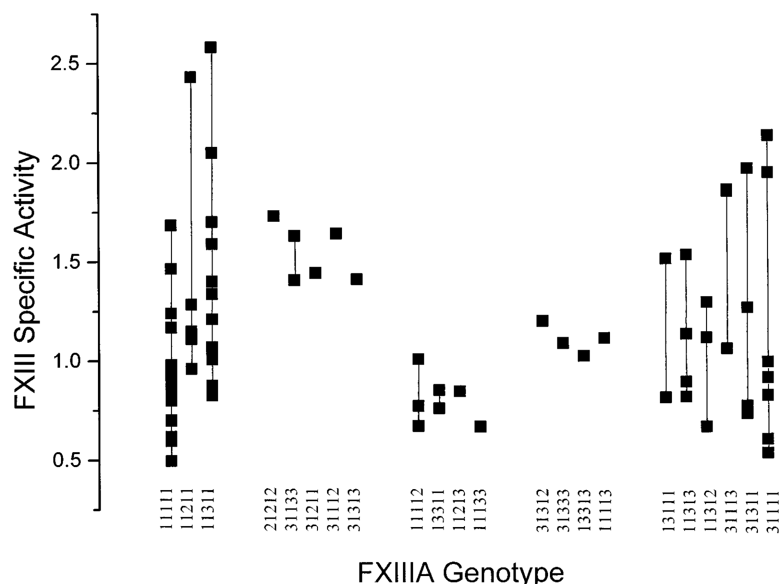

Fig 3. The range of FXIII specific activity (SA) observed for the different FXIIIA genotypes presented in Table 5. The genotype codes, explained in Table 4 and in the legend to Table 5, are 5′ to 3′ for codons 34-204-564-650-651. Individuals with FXIII SA of 2.43 and 2.58 (genotypes 11211 and 11311) are known to be heterozygous for FXIIIA deficiency and were not included in the normal population presented in Fig 2. Therefore, only one allele of genotype 11211 in both of these cases is probably responsible for the FXIII SA observed.

that the rate and extent of α-polymer formation are critically dependent on and significantly influenced by FXIII concentrations above those found in normal plasma.[29] This may be clinically significant in the context of thrombus removal. Also, the local FXIII concentration is increased at sites of injury due to platelet aggregation and degranulation. In such regions in vivo, formation of large fibrin polymers may significantly influence pathological thrombotic and thromboembolic phenomena, particularly in individuals with high plasma FXIII concentrations. This would be of particular concern for individuals who carry the Leu34, Tyr204, Leu564 variants either alone or particularly in combination on both alleles.

A very recent report has suggested that the Val34 FXIIIA haplotype may be associated with myocardial infarction (MI).[30] However, no FXIIIA protein data were presented from the study groups. We actually find the Leu34 variant to result in higher FXIII specific activity and therefore would expect Leu34 to be associated with MI in individuals who have high levels of the protein. Without FXIIIA protein data from the study of Kohler et al,[30] it is difficult to understand the basis of the association they suggest. Clearly, further work is required to resolve this question.

Apart from the actual cross-linking reaction, factors that influence the activity of the FXIIIA enzyme in plasma include the efficiency of enzyme activation and the specificity and efficiency of substrate binding. The Leu34 polymorphism is three residues away from the activation peptide cleavage site and may contribute toward more efficient activation of FXIII. The Leu564 residue lies on the exposed surface of the barrel 1 domain.[28] This may play a role in substrate binding. It is possible that these single amino acid changes in the protein sequence lead to minor changes in protein structure, which account for the variation observed in FXIII specific activity. This may be especially true for FXIIIA molecules with Leu564 compared with Pro564. Proline is an amino acid with properties very different from leucine and can significantly affect the three-dimensional structure of the protein.

Interestingly, the variable levels of other components of the coagulation pathway have also been found to have a genetic basis. Examples include factor V and factor VII levels,[31,32] high levels of both plasminogen-activator inhibitor-1, and plasma fibrinogen, which are associated with an increased risk of MI,[33,34] and elevated plasma prothrombin levels, which are associated with an increase in venous thrombosis (VT).[35] It is probable that the risk of cardiovascular problems, due to increased thromboembolic activity, is much higher for individuals who carry specific polymorphisms, which give rise to high activity proteins for more than one component of the blood clotting pathway, the effects probably being additive. It is therefore important that when assessing genetic risk for thrombotic problems such as MI and VT, all of the variable coagulation components are considered, including FXIII.

Although there is a clear effect of the FXIIIA genotype on FXIII specific activity, there are probably other factors, such as the levels of FXIIIB subunit in plasma, which may also influence the final levels and activity of plasma FXIII. FXIII levels, activity, or specific activity were not found to be dependent on age. Furthermore, there were no differences observed in either plasma FXIII levels or its activity (or in FXIIIA genotypes) between men and women. Plasma FXIII levels are becoming accepted as an important marker of disease activity in various inflammatory conditions including Crohn's disease, ulcerative colitis, and bacterial infection, as well as in patients with benign and malignant gynecological tumors.[1-3] Also, transglutaminase activity is increased in the Alzheimer's disease brain, and tissue transglutaminase has been identified as the autoantigen of celiac disease.[4,5] As yet, there is no evidence whether FXIIIA may also be involved in the covalent cross-linking of gliadin in celiac disease.

In conclusion, our findings strongly suggest that for accuracy of diagnosis, the determination of both FXIIIA activity and levels is important in clinical conditions for which FXIII may be used as a diagnostic marker of disease activity. The assays we have described provide a simple, but accurate and reproducible, means of determining the plasma FXIII activity, FXIII level, and therefore the FXIII specific activity, in large numbers of

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individuals. The studies we have presented provide the first evidence of a genetic basis for the massive variation that is observed in plasma FXIII levels and activity. They also define specific genotypes, which are associated with FXIII variability and these may serve as valuable tools for molecular diagnostics in the growing number of clinical situations where FXIII is recognized as an important marker. Our finding that the Phe204 FXIII variant may be associated with RM in women who otherwise have normal plasma FXIII highlights the importance of defining a structure/function relationship for FXIII in its additional biological roles.

NOTE ADDED IN PROOF

Since the submission of this article, Kangsadalampai and Board published a report on the factor XIIIA Val34Leu polymorphism.[36]

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