Simultaneous genotyping of coagulation factor XI type II and type III mutations by multiplex real-time polymerase chain reaction to determine their prevalence in healthy and factor XI-deficient Italians

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ABSTRACT

Background

Factor XI deficiency is a rare autosomal recessive coagulopathy, which is, however, common among Ashkenazi Jews, in whom the so-called type II (E117X) and type III (F283L) mutations account for 98% of alleles. In non-Jewish populations, a higher level of allelic heterogeneity has been reported. However, the type II mutation was found in individuals from England, Portugal, and Italy, and haplotype analysis confirmed its Jewish origin. The aims of this study were to develop a rapid and accurate assay for the simultaneous detection of type II/type III mutations and to determine the frequency of these mutations in a large Italian population of healthy individuals and in a cohort of factor XI-deficient Italian patients.

Design and Methods

Type II and III mutations were detected using a newly developed multiplex four-color real-time polymerase chain reaction assay. Haplotype analysis was performed by either DNA sequencing or fragment-length analysis.

Results

Both type II and type III mutations were found among 3879 healthy Italians with an allele frequency of 0.00064 and 0.00051, respectively. Among the 31 analyzed factor XI-deficient patients, the type II mutation was found in three individuals in the homozygous state and in eight individuals in the heterozygous state (one compound heterozygote type II/III). Haplotype analysis revealed the Jewish origin of both mutations.

Conclusions

The newly developed assay is highly specific and reliable (0.02% false positives); and offers a useful means for the molecular diagnosis of factor XI deficiency. Type II and III mutations are present in the Italian population and should be searched for first in factor XI-deficient patients.

Key words: factor XI deficiency, type II and type III mutations, multiplex real-time PCR, Jewish founder effect.

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Introduction

Coagulation factor XI (FXI) is the zymogen of a serine protease (activated FXI, FXIa) that contributes to hemostasis through activation of factor IX (FIX).[1] FXI is primarily produced by hepatocytes. It circulates in plasma as a disulfide-linked homodimer composed of two identical 80-kDa polypeptide chains,[2] and is complexed with high molecular weight kininogen.[3] The human FXI gene (*F11*) comprises 15 exons, and spans about 23 kb on the long arm of chromosome 4 (4q35.2).[4] Hereditary FXI deficiency (MIM+264900) is an injury-related bleeding disorder that was first described as a hemophilia-like syndrome by Rosenthal *et al*.[5] However, unlike in the hemophilias, spontaneous bleeding is rare in FXI deficiency, even in patients with severe deficiency;[6] bleeding usually occurs only after trauma or surgery, particularly at sites where there is local fibrinolysis.[7] Moreover, bleeding manifestations are not well correlated with the plasma levels of FXI activity and bleeding episodes can vary widely among patients with similar FXI levels.[8] Patients with very severe FXI deficiency can develop a FXI inhibitor, affecting FXI activation by thrombin or activated factor XII or inhibiting FIX activation by activated FXI.[9]

Hereditary FXI deficiency is generally transmitted as an autosomal recessive trait, and both sexes are affected; however, cases of dominant transmission have also been reported.[10,11] Although the disease is rare in most populations (prevalence 1 case in 10^6 individuals), it is frequent in Ashkenazi Jews, in whom a heterozygosity rate of 9% was discerned and severe deficiency (FXI activity <15U/dL) was estimated to occur in 1 in 450 individuals.[12] Two mutations, designated as type II (E117X) and type III (F283L), account for 98% of alleles in this population.[13] Haplotype analysis demonstrated that these two mutations are of ancient origin and arose from distinct founders: the type II mutation is common in Ashkenazi and Iraqi Jews (allele frequencies 0.0217 and 0.0167, respectively) and in Palestinian Arabs, and therefore probably originated in a Jewish founder approximately 2500 years ago, before the major divergence of these groups.[12] In contrast, the type III mutation (allele frequency 0.0254) is more recent, and is confined to Ashkenazi Jews.[14] In non-Jewish populations, a higher level of allelic heterogeneity has been reported, and 152 causative mutations have been described;[15] however, three of these mutations occur in specific population groups: Q88X is found in French families from Nantes,[16] C38R in French Basques,[17] and C128X in English families.[18] Haplotype analyses confirmed that the frequency of these mutations is due to founder effects. In particular, the C128X mutation is found in 2% of individuals screened for hemochromatosis in the UK,[18] and in 2% of normal individuals from the north-west of England, suggesting that severe FXI deficiency in the UK may be more common than previously thought.[19] The frequency of the C128X mutation is similar to that of the C38R mutation, which is present in 1% of French Basques.[17,20] The type II mutation has been reported in non-Jewish individuals from England,[21] Portugal,[22] and Italy;[23] in all cases haplotype analysis indicated the Jewish origin of the mutation.

In this study, we analyzed the frequency of type II and type III mutations in the Italian population, evaluated using a newly developed multiplex four-color real-time polymerase chain reaction (PCR) assay. We also investigated the frequency of these two mutations in 31 Italian patients with severe or mild FXI deficiency.

Design and Methods

Subjects

All individuals gave their informed consent before blood withdrawal, in accordance with the Ethics Committee of the University of Milan and with the principles of the Helsinki Declaration. The control population consisted of 3879 unrelated healthy Italian individuals (3444 men and 435 women) originating from different regions of the country; their mean age at the time of blood withdrawal was 48 years. None of the volunteers had a personal or familial history of FXI deficiency; all were referred for a routine complete blood count. Thirty-one unrelated Italian patients, referred between 2002 and 2006 to the Milan and Vicenza Centers because they were affected by mild/severe FXI deficiency were also analyzed; their main demographic and clinical characteristics are summarized in Table 1. DNA from three additional Israeli patients was used to set up the genotyping assay: one patient was homozygous for type II mutation, one was homozygous for type III mutation, and one was double heterozygous for type II/III mutations.

Measurement of FXI coagulant activity and antigen level

Platelet-poor plasma was obtained from FXI-deficient patients by centrifugating blood at 2000g for 10 min; plasma aliquots were stored at -80°C until use. FXI coagulant activity was measured by a one-stage method based on a modified partial thromboplastin time, with use of lyophilized immunodepleted human plasma lacking FXI (Hemoliance, Salt Lake City, UT, USA). FXI antigen was assayed using an in-house enzyme-linked immunosorbent assay (ELISA) with a goat anti-human FXI polyclonal antibody and a peroxidase-conjugated anti-human IgG (Affinity Biological Inc., Hamilton, Ontario, Canada). FXI levels were expressed in both tests as percentages of levels in pooled normal plasma from healthy individuals. Normal ranges for both FXI coagulant activity (FXI:C) and antigen (FXI:Ag) levels were 70 to 130%. The detection limits of the FXI functional and immunological assays were 1% and 0.1%, respectively.

DNA samples

Peripheral venous blood was collected in a 1:10 volume of 0.11 M trisodium citrate, pH 7.3. Genomic DNA was extracted from whole blood using a standard salting-out procedure. DNA was quantified spectrophotometrically. Quantitated samples were stan-

dardized for concentration (80 ng/μL for the source, 2.5 ng/ μL for the working dilution) and arrayed into 96-deep well plates (Eppendorf, Hamburg, Germany). In order to control for possible plate mix-ups or plate rotations, each 96-well plate included: (i) two water controls in unique and asymmetric positions; (ii) two to four known duplicate samples and (iii) type II and type III homozygous and heterozygous DNA samples, in duplicate. For real-time PCR we used 7.5 ng of DNA, and samples were transferred from deep-well to 96-well twin-tec semiskirted PCR plates (Eppendorf) using the automated pipetting system epMotion 5075LH (Eppendorf). DNA was dried overnight at 42°C, and plates were stored at room temperature until use.

Genotyping of type II and type III mutations by multiplex real-time PCR

Oligonucleotide primers (Table 2) were designed with Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA) and purchased from Sigma-Genosys (Haverhill, Suffolk, UK). Taqman probes were labeled with FAM/Black Hole Quencher 1 (*F11*-type-II-wt), HEX/Black Hole Quencher 1 (*F11*-type-II-mut), CY5/Black Hole Quencher 2 (*F11*–type-III-wt), and Texas Red/Black Hole Quencher 2 (*F11*-type-III-mut). Multiplex PCR experiments were run in a 15 μL final volume containing 7.5 μL 2x Premix Ex Taq (Takara Biomedicals, Shiga, Japan), 200 nM *F11*-type-II primers, 400 nM *F11*-type-III primers, 100 nM *F11*-type-II-wt probe, 400 nM *F11*-type-II-mut probe, 75 nM *F11*-type-III-wt probe, and 200 nM *F11*-type-III-mut probe.

Each PCR mix was prepared in a single tube and distributed into 96-well PCR plates, with the use of the epMotion 5075LH automated pipetting system. After DNA denaturation at 95°C for 30 sec, 45 cycles of a two-step PCR protocol were carried out in an iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) as follows: denaturation at 95°C for 5 sec and annealing/extension at 56.5°C for 45 sec. Fluorescence was measured during the PCR annealing/extension step. Allele discrimination was performed using the iQ5 Cycler software (Bio-Rad).

DNA sequencing

Sequencing reactions were performed directly on PCR products purified either by ammonium acetate precipitation, or by using Multi Screen PCR plates (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Sequence analysis was carried out on both strands by means of the BigDye Terminator Cycle Sequencing kit (version 3.1) (Applied Biosystems, Foster City, CA, USA) and samples were run on an automated ABI-3130xl DNA genetic analyzer (Applied Biosystems).

Haplotype analysis

Two single nucleotide polymorphisms [one in intron A (-231C>T) and one in intron E (*Hha*I polymorphism)] and two dinucleotide repeat polymorphisms [one in intron B $(CA)_{8-13}$ and one in intron M $(AT)]_{7-11}$ of *F11* were analyzed.[14,24,25] Polymorphisms in introns A, E,

Table 1. Characteristics of patients with FXI deficiency.

Patients	City of origin	Gender	Present age (years)	FXI: C (%)	FXI: Ag (%)	Severity of symptoms	Genotype for type II & type III mutations
1	Parma	M	13	20	46	mild	wt
2	Cesena	F	39	31	42	asympt.	type II het.
3	Cesena	F	41	33	44	asympt.	wt
4	Trento	M	69	1.2	5.5	asympt.	wt
5	Trento	F	63	<1	3	asympt.	wt
6	Bari	M	15	48	33	mild	type II het.
7	Vicenza	M	68	51	-	asympt.	wt
8	Parma	F	41	20	32	moderate	wt
9	Padova	M	40	57	-	asympt.	wt
10	Vicenza	F	40	38	57	asympt.	wt
11	Venice	F	47	28	68	asympt.	wt
12	Vicenza	F	64	26	39	moderate	wt
13	Vicenza	M	37	34	35	moderate	type II het.
14	Vicenza	F	26	44	15	mild	type II het.
15	Vicenza	F	36	54	64	mild	wt
16	Trento	M	24	3.5	3	very mild	wt
17	Cesena	M	16	37	-	asympt.	wt
18	Udine	F	34	26	-	mild	wt
19	Vicenza	M	35	34	-	asympt.	wt
20	Milan	M	39	<1	0.4	mild	wt
21	Milan	F	58	<1	0.4	mild	type II hom.
22	Reggio E.	M	64	<1	0.2	moderate	type II hom.
23	Reggio E.	M	35	<1	5	moderate	type II/other mutation compound het.
24	Milan	M	32	<1	0.5	asympt.	type II hom.
25	Milan	F	18	<1	0.3	mild	wt
26	Italy	M	32	<1	-	asympt.	wt
27	Italy	F	39	<1	-	asympt.	wt
28	Como	M	71	2	1.7	moderate	type II/type III compound het.
29	Varese	F	68	<1	<0.1	-	wt
30	Italy	M	-	<1	<0.1	asympt.	type II/other mutation compound het.
31	Bari	F	-	2	-	asympt.	type II/other mutation compound het.

Asympt. = asymptomatic; het. = heterozygous; hom. = homozygous. The dash means: no information available.

Table 2. Sequences and temperature of melting (Tm) of PCR primers and Taqman probes.

Oligo name	Sequence (5'→3')	Tm (°C)
F11 type II-F	TGTGGACCTAGACATGAAGG	53.2
F11 type II-R	GACGTCATCCGTGCATCT	53.6
F11 type III-F	AGGGAGGGTCTCACTCTG	53.1
F11 type III-R	GCAGCACAATATCCAGTTC	52.1
F11 type II wt-probe	6FAM-TTGGCATT**C**TTGAGCACTC-BQ1	52.5
F11 type II mut-probe	HEX-CTTGGCATT**A**TTGAGCACTC-BQ1	51.8
F11 type III wt-probe	CY5-CTCCCAAGA**A**ATCAGTGTCA-BQ2	51.0
F11 type III mut-probe	Texas Red-CTCCCAAGA**G**ATCAGTGTCA-BQ2	52.0

BQ1: black hole quencher 1; BQ2: black hole quencher 2; the polymorphic position is shown in bold.

and B were genotyped by sequencing the relevant PCR product. The microsatellite marker in intron M was genotyped as previously described.[23] In haplotype construction, seven additional single nucleotide polymorphisms, identified by a previous mutational screening in six FXI-deficient Italian patients, were included: -138A>C in intron A, 429T>C in exon 5, -361C>T in intron E, 801A>G in exon 8, 1191T>C in exon 11, 1812G>T and 1839G>A in exon 15. The primer sequences and PCR conditions used have been described elsewhere.[23]

Statistical analysis

Allele frequencies were calculated by the gene-counting method. χ^2 analysis with Yates' correction was used to test for departure of genotype frequencies from those expected according to the Hardy-Weinberg equilibrium.

Results

Multiplex four-color real-time PCR set-up

Type II and type III mutations were simultaneously detected by a newly developed four-color multiplex real-time PCR assay. To set up the method, DNA from three Israeli patients (one homozygous for the type II mutation, one homozygous for the type III mutation, and one double heterozygous for the two mutations) was used in duplicate. DNA from three healthy individuals (wild type for the whole *F11* coding sequence) was also used as negative control.

The assay sensitivity was determined for different amounts of starting DNA template. Multiplex PCR amplifications were optimized using 15, 10, 7.5, 5, and 2.5 ng of human genomic DNA. Reliable and reproducible results were obtained with all starting quantities of DNA.

Examples of the obtained amplification curves are shown in Figure 1. The allele discrimination software automatically assigns the genotypes using an algorithm that considers the signal (end-point relative fluorescent units, RFU) generated by replicates of homozygous mutant and double heterozygous mutant controls and plots the data as a diagram (Figure 2). Type II and type III alleles assigned by the software were double-checked in all cases by visual examination of the corresponding amplification curves.

Genotyping for type II and type III mutations in the Italian population

Genotyping of 3879 unselected Italian individuals showed the presence of both mutations. Five individuals were heterozygous for type II mutation (allelic frequency: 0.00064), and four individuals were heterozygous for type III mutation (allelic frequency: 0.00051). We did not find any homozygotes or double heterozygotes for the two mutations. Type II and type III genotype distributions are in accordance with a population in Hardy-Weinberg equilibrium (χ^2=0.002, p=0.99919

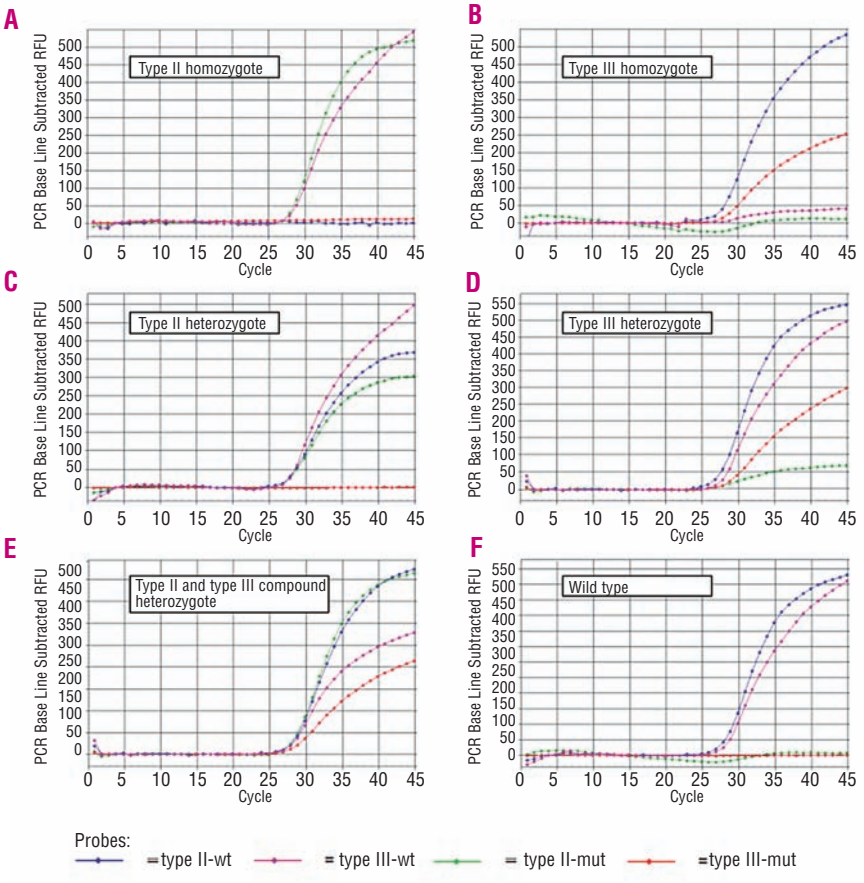

Figure 1. Results of the four-color multiplex real-time PCR. Six examples of amplification curves obtained with the 5' nuclease assay developed to screen for the type II and type III mutations are shown (A-F). The plots show that each wild-type probe does not generate any cross-hybridization with the corresponding mutant PCR product neither do the two mutant probes with the wild-type amplicons, demonstrating the specificity of the assay. (A) A type II homozygous individual showing a fluorescent signal for *F11*-type-II-mut probe (◆) and *F11*-type-III-wt probe (◆). (B) A type III homozygous individual showing a fluorescent signal for *F11*-type-III-mut probe (◆) and *F11*-type-II-wt probe (◆). (C) A type II heterozygous individual showing a fluorescent signal for *F11*-type-II-mut probe (◆), *F11*-type-II-wt probe (◆), and *F11*-type-III-wt probe (◆). (D) A type III heterozygous individual showing a fluorescent signal for *F11*-type-III-mut probe (◆), *F11*-type-III-wt probe (◆), and *F11*-type II-wt probe (◆). (E) A type II/type III double heterozygous individual showing a fluorescent signal for all four probes. (F) A wild-type individual showing a fluorescent signal only for the two wild-type probes.

and χ^2=0.001, p=0.9993, respectively). The frequency of type II and type III mutations observed is consistent with a FXI-deficiency prevalence of 1:10[6] in the Italian population.

Frequency of type II and type III mutations in FXI-deficient patients

Among the 31 patients with FXI deficiency (16 with mild and 15 with severe deficiency), three individuals were found to be homozygous for the type II mutation (patients #21, 22 and 24), four were found to be heterozygous for the type II mutation (patients #2, 6, 13 and 14), three were compound heterozygotes for the type II mutation and another mutation (patients #23, 30 and 31), and one patient was double heterozygous for type II/type III (patient #28). Overall, of a total number of 46 mutant alleles in this cohort of 31 patients, 14 were type II (30.4%) and one was type III (2.2%).

Assay performance

To evaluate the performance of the four-color multiplex assay, DNA from a total of 86 healthy controls and all 31 FXI-deficient patients was blindly tested by sequencing *F11* exons 5 and 9, where type II and type III mutations are located. The concordance between the results of the multiplex Taqman assay and DNA sequencing was 100%. Moreover, the type II and type III mutations identified in the Italian population were confirmed by direct sequencing, showing only one false positive (0.02%). These data demonstrate that the four-color multiplex Taqman assay is a specific and reproducible method for detecting type II and type III mutations in *F11*.

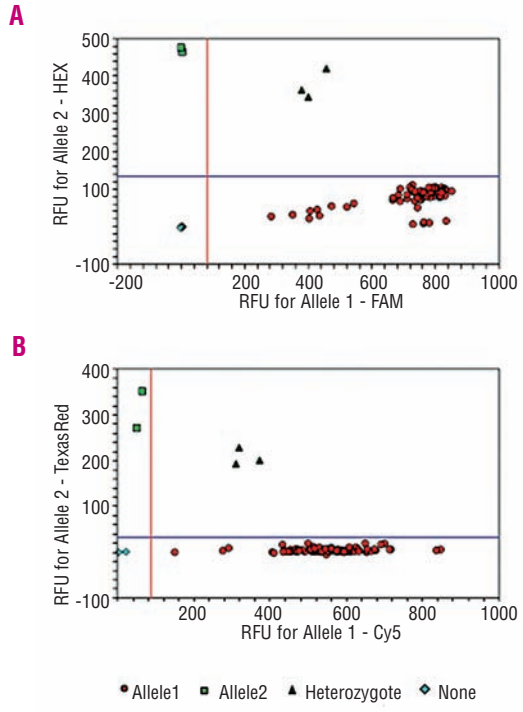

Figure 2. Allelic discrimination of type II and type III mutations. (A) Plot of fluorescence of FAM (*F11*-type-II-wt probe, allele 1) *vs.* HEX (*F11*-type-II-mut probe, allele 2). Four clusters are clearly distinguished: wild-type genotypes (●), heterozygous genotypes (▲), homozygous mutant genotypes (■), and no template controls (◆). (B) Plot of fluorescence of Cy5 (*F11*-type III-wt probe, allele 1) *vs.* Texas Red (*F11*-type-III-mut probe, allele 2). Four clusters are clearly distinguished, see above for description. In both panels, normalized fluorescence values (RFU) for the two alleles are plotted on the x and y axes.

Haplotype analysis

To determine whether the type II and type III mutations identified stemmed from a common founder, 11 genetic markers were analyzed. These included four intragenic polymorphic sites [-231C>T in intron A, $(CA)_{8-13}$ in intron B, *Hha*I polymorphism in intron E, and $(AT)_{7-11}$ in intron M], previously reported to define two different haplotypes shared by Jewish FXI-deficient patients carrying the type II mutation [-231C, $(CA)_{11}$, -431G (*Hha*I), $(AT)_9$] or the type III mutation [-231T, $(CA)_{10}$, -431G (*Hha*I), $(AT)_9$][14] (nomenclature according to Bolton-Maggs *et al*).[18] Genotyping was performed both by DNA sequencing and by restriction fragment-length polymorphism analysis. The type II mutation segregates with the [-231C, $(CA)_{11}$, -431G (*Hha*I), $(AT)_9$] haplotype, previously reported in Ashkenazi, Iraqi, Yemenite, Syrian, and Moroccan Jewish patients,[14] while the type III mutation segregates with the [-231T, $(CA)_{10}$, -431G (*Hha*I), $(AT)_9$] haplotype, evolved more recently and confined to Ashkenazi Jews[14] (Table 3). These findings suggest that both mutations are of Jewish origin.

Discussion

We report an analysis of the frequency of FXI type II and type III mutations in 3879 unrelated healthy Italian individuals, not selected for coagulation defects, and in 31 patients affected by severe (n=15) and mild (n=16) FXI deficiency. To genotype such a large number of individuals, a four-color multiplex real-time PCR assay based on 5' nuclease chemistry[26] was developed, allowing for the simultaneous detection of type II and type III mutations. This test offers numerous advantages over more traditional methods for mutation detection (allelic-specific PCR, PCR and restriction fragment-length polymorphisms analysis, PCR and sequencing, etc.), including speed and simplicity of the method, reduced labor, and reduced risk of cross-contamination. The multicolor multiplex assay is also amenable to automation and high-throughput processing, representing an attractive method for diagnostic laboratories processing a substantial number of specimens or for rapid screening for FXI deficiency prior to surgery, a condition that can cause excessive bleeding in individuals asymptomatic until that moment. Our data show that the assay is specific (0.02% false positive rate), easy to interpret, reproducible (100% correlation with the results generated by DNA sequencing), and works well within a wide range of starting amounts of DNA (we obtained reliable results even forcing PCR conditions to 0.25 ng or 250 ng of genomic DNA, *data not shown*).

The genotyping showed that both type II and type III mutations are present in the Italian population, although at a low frequency (allele frequency 0.00064 and 0.00051, respectively). Among consecutively diagnosed FXI-deficient patients, the type II mutation was found in 14 (30.4%) of 46 mutant alleles (3 homozygotes, 4 heterozygotes and 4 compound heterozygotes) while the type III mutation was found in only one patient, a double heterozygote for both type III and type II mutations. These findings contrast with those in Ashkenazi Jews, in whom the frequencies of the two mutations are similar (0.0217 and 0.0254, respectively).[14] This difference can be explained on the one hand by the limited number of FXI-deficient patients analyzed in our study and on the other hand by a milder phenotype associated with type III mutation. In fact, the mean FXI levels in patients with II/II, II/III and III/III genotypes were 0-1.5 U/dL, 0-6 U/dL and 3-20 U/dL, respectively,[27] figures that were substantially confirmed by a more recent and larger study.[28] It is, therefore, more probable that FXI-deficient patients who come to clinical attention for bleeding problems bear the type II mutation.

Given that the bleeding tendency in individuals with FXI deficiency is highly variable, and that spontaneous bleeding, as well as bleeding after hemorrhagic challenge, occur rarely,[28] it is possible that some patients never come to clinical attention and that the prevalence of both mutations is underestimated. In fact, the prevalence of FXI deficiency itself is difficult to estimate correctly without large-scale population screening.

From the allele frequencies obtained in this study for the type II (0.00064) and the type III (0.00051) mutations, the prevalence of heterozygotes in the Italian population can be calculated to be 12.8:10,000 (type II) and 10.2:10,000 (type III). Consequently, the expected number of individuals with severe FXI deficiency due to type II/II, II/III, or III/III genotypes among 58,000,000 Italians (the population census data for 2005) should be 23.8, 15.1, and 18.9, respectively; i.e., approximately 58 individuals or $1:10^6$ inhabitants. Naturally, the prevalence of these FXI mutations in the Jewish communities in Italy would be much higher. Oddoux *et al.*[29] found that in Roman Jews, who are the descendents of the ancient community that has lived in Rome for more than 2000 years, the type III mutation was present in three of 86 individuals (3.5%). However, the vast majority of Jews currently living in Italy are of Ashkenazi or Sephardic origins in whom the frequency of type II and type III mutations would be similar to the frequencies described in these ethnic groups in Israel.[12]

Haplotype analysis demonstrated the Jewish origin of both the type II and type III mutations identified in this study. Given that none of the analyzed individuals reported a Jewish ancestry, we hypothesize that gene flow was responsible for the diffusion of these mutations among Italians as already proposed for other European populations.[21,22] Moreover, gene flow was previously suggested by Peretz and colleagues[14] to explain the transfer of the type II mutation from Middle Eastern Jews to Palestinian Arabs after the settlement of Arabs in Israel in the seventh century after Christ.

Table 3. Haplotypes associated with type II and III mutations in the Italian population.

Marker	Location	Haplotype	
		Type II mutation	Type III mutation
-231C>T[#]	intron A	**C**[a]	**T**[b]
-138A>C[#]	intron A	A	A
(CA)n	intron B	**11**[a]	**10**[b]
403G>T (type-II)*	exon 5	C	G
429T>C*	exon 5	T	C
-431G>A (HhaI)	intron E	**G**[a]	**G**[b]
-361C>T[#]	intron E	C	C
801A>G+	exon 8	A	A
901T>C (type III)*	exon 9	T	C
1191T>C*	exon 11	C	n.d.
(AT)n	intron M	**9**[a]	**9**[b]
1812G>T*	exon 15	G	n.d.
1839G>A*	exon 15	G	n.d.

*[#]Nucleotide positions of the intronic polymorphisms have been numbered starting from the nearest splicing junction. *Numbering refers to the cDNA sequence (GenBank, accession number NM000128), starting from the first nucleotide of the ATG start codon. Alleles belonging to Jewish haplotypes associated with type II and type III mutations are shown in bold. n.d.: not determined.*

In summary, this study describes the presence of type II and type III mutations in the Italian population and confirms the Jewish origin of both genetic defects. The high frequency of the type II mutation found among 31 patients with FXI deficiency suggests that testing for the presence of type II mutation should be the first genetic screening carried out in FXI-deficient patients. Moreover, we describe the first application of a four-color multiplex real-time PCR assay for the simultaneous detection of type II and type III mutations. The assay, which is specific and reproducible, is a useful method for the molecular diagnosis of FXI deficiency, especially in those populations in which the disease is frequent and type II and type III mutations are largely prevalent.

Authorship and Disclosures

All the authors participated in the conception and design of the present study, in the analysis and interpretation of data, and in revising the manuscript. GZ was responsible for Taqman assays, PCR amplifications, sequence analysis, interpretation of the results and drafting of the manuscript. RA participated in the conception of the study, the interpretation of results, and the statistical analyses. MLT, US, and PMM were involved in the study design, in the discussion of the results, and in reviewing the manuscript. GC and PMM were responsible for the clinical management of the patients. SD participated in writing the manuscript and supervised the entire study. SD is also a recipient of a Bayer Hemophilia Early Career Investigator Award 2006. The other authors reported no potential conflicts of interest.

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