



Experience of a single Italian center in genetic counseling for hemophilia: from linkage analysis to molecular diagnosis

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ABSTRACT

Background and Objectives. We describe our three year experience in genetic counseling at the Castelfranco Veneto Hemophilia Center, Italy.

Design and Methods. A total of 258 individuals were involved in the study of 142 females. These formed 40 families with hemophilia A and 6 families with hemophilia B. Following pedigree analysis, the FVIII inversion was first examined in severe hemophilia A patients by polymerase chain reaction (PCR) analysis. DNA polymorphisms were used to track the affected gene through the remaining families. In uninformative cases, we initiated analysis of the FVIII or FIX gene coding region by conformation sensitive gel electrophoresis and DNA sequencing to identify the mutation responsible for the disease.

Results. The FVIII gene inversion was present in 16 of the 32 patients (50%) affected by severe hemophilia A and was informative for 44 females. For hemophilia A, 45 cases (55%) were informative by linkage analysis, however 37 (45%) were uninformative because of lack of key individuals, homozygosity, or sporadic disease. Information from extragenic linked polymorphisms alone was present in 9 cases (6%). For hemophilia B, linkage analysis was informative in only 50% of females (8 out of 16). To date, nine mutations have been identified in patients with hemophilia A and three in patients with hemophilia B. Six novel missense mutations in hemophilia A are discussed briefly.

Interpretation and Conclusions. Using this approach we are now able to offer accurate genetic analysis to virtually all families with hemophilia.

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Key words: hemophilia, genetic counseling, inhibitors, mutation analysis, CSGE

Genetic counseling still remains an important part of comprehensive hemophilia care, despite the improvement of quality of life of these patients now that safe replacement therapy is available. In Italy, genetic counseling for hemophilia is traditionally available at a few Hemophilia Centers (Genoa and Milan), but a large proportion of the country is still uncovered. In 1995, we began a program, in collaboration with the Division of Molecular and Genetic Medicine of the University of Sheffield (UK), to develop a service for genetic counseling in hemophilia in our Institution. Our center is located in the north east of Italy and follows nearly 500 hemophiliacs coming from all regions of Italy.¹

Hemophilia A and B are X-linked bleeding disorders caused by diverse mutations throughout the FVIII and FIX genes which result in reduced or absent functional proteins in the plasma. As the genes are on the X chromosome, only males are affected but their female relatives are at risk of being carriers, with the potential of passing the disorder on to their sons. On average, carriers of hemophilia A or B have about 50% of the normal levels of FVIII or FIX, but plasma procoagulant activity is not a precise method for determining carrier status. In the early 1980s, when the FVIII and FIX genes were identified and cloned,^{2,3} genetic diagnosis became available. Analysis of carrier state can be made indirectly by DNA linkage analysis or directly by identifying the mutation that leads to the disease.

Linkage analysis is now available world-wide, but has several limitations including those resulting from non-informative polymorphisms and, in sporadic cases, the possibility only of excluding the carrier state. For extragenic markers, a major risk is the possibility of crossover during meiosis. In severe cases of hemophilia A, the most common mutation, found in up to 50% of patients, is the inversion of a portion of the FVIII gene (exons 1 to 22) due to homologous intra-chromosomal recombination occurring between the three or more copies of a particular DNA sequence termed sequence int22h.^{4,6} With the exception of the inversion, nearly every family with hemophilia A has a different FVIII gene mutation,⁷ so the simplest way to assess the carrier status or to distinguish the two X chromosomes in an obligate carrier, is indirect detection using linked DNA polymorphisms. No prevalent mutation has been identified for the FIX gene and linkage analysis of carrier status is performed through use

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of DNA polymorphisms. Many mutation screening methods are available, but conformation sensitive gel electrophoresis (CSGE), a form of heteroduplex analysis,⁸ has been used successfully for screening for mutations in hemophilia A and B.^{9,10}

The main side effect of replacement therapy today is the development of antibodies to FVIII or FIX which can switch these patients into a severely affected group. The determinants of this problem are not yet fully clarified. However, it is emerging that genetic defects have a major responsibility. Severe molecular defects such as the FVIII inversion, large deletions and stop mutations have been demonstrated to produce a major risk of development of antibodies to FVIII in hemophilia A¹¹⁻¹³ or of anaphylaxis in hemophilia B.^{14,15} This particular aspect is also important in modern genetic counseling, including prenatal diagnosis, for families with hemophilia.

Design and Methods

Samples and DNA extraction

Blood samples were obtained from males with hemophilia, family members and normal unrelated females. DNA was extracted from 10 mL of whole citrated blood according to a previously described method.¹⁶

Analysis of the FVIII gene inversion

The FVIII inversion mutation was examined by Southern blotting in Sheffield as described¹⁶ or by a PCR method that is now available in Castelfranco Veneto.¹⁷ This method is a long polymerase chain reaction (PCR) in which the Taq polymerase is added during the denaturation step at the beginning of the cycling (hot start). Briefly: genomic DNA and four primers which are able to differentiate the wild-type and inversion in affected males and in female carriers are used. Two primers, P and Q, are located within the factor VIII gene flanking *int22h1*. Two further primers, A and B, flank *int22h2* and *int22h3*. DNA fragments PQ (12 kb) and AB (10 kb) are amplified in hemizygous wild-type males. Males with hemophilia due to the inversion produce PB (11 kb) and AQ segments (11 kb) along with the 10 kb AB from the non-recombined extragenic *int22h* homolog.

Table 1. FVIII gene inversion and linked polymorphism analysis in hemophilia A and B.

	Inversion		Polymorphism		ND	Tot.
	C	NC	C*	NC*		
HA Familial cases	11	7	19	10	7	54
Sporadic cases	12	14	4	12	30	72
HB Familial cases			6	1	3	10
Sporadic cases				1	5	6
Total	23	21	29	24	45	142

C: carriers; NC: not carriers; ND: not diagnosed; *in 9 cases of hemophilia A the diagnosis was carried out on the basis of an extragenic polymorphism only. In total 54 (9+45) out of 142 (38%) probands do not have accurate diagnosis of their carrier state.

Females carriers produce PQ, PB + AQ, and AB segments. In all cases, an AB segment serves as an internal control because at least one copy of *int22h2* or *int22h3* remains intact. PCR products are characterized by electrophoresis on an 0.6% agarose gel. This method is now in use not only for genetic counseling of families, but also for screening in all hemophiliacs followed at our center.

FVIII gene polymorphisms

The *Bcl I* restriction site in intron 18 was analyzed by the technique used by Gitschier *et al.*¹⁸ Intron 13 and 22 variable number CA repeats were analyzed using PCR methods as reported.^{19,20} Products were visualized by silver staining.²¹

Extragenic VNTR St14 (DXS52) was studied according to a reported method.²²

FIX gene polymorphisms

Hha I, *Mnl I*, *Mse I*, *Taq I*, *Xmn I* and *Dde I* polymorphisms were analyzed by methods already reported.²³⁻²⁶

Mutation detection by conformation sensitive gel electrophoresis (CSGE)

This is a procedure for distinguishing homoduplex from heteroduplex containing a mismatch by polyacrylamide gel electrophoresis in a mildly denaturing solvent system.⁸ The whole coding region of the FVIII or FIX genes were analyzed by PCR from genomic DNA in 34 and 9 fragments respectively for hemophilia A and B patients.^{9,10}

DNA sequencing was performed using a Thermo-sequenase cycle sequencing kit (Amersham) and the appropriate ³²P or fluorescein labeled primers.

Results

Hemophilia A

To date we have studied 126 females belonging to 40 families. FVIII inversion analysis revealed the presence of this mutation in 16 out of 32 (50%) unrelated patients with severe hemophilia A. Twenty-three out of 44 female relatives were found to carry the inversion (Table 1). Linkage analysis using the *Bcl I* RFLP, CA repeats in intron 13 and 22, and the St14 VNTR was carried out for all the other cases. Results are shown in Table 1. It was not possible to give a result in 37 cases (29%): 3 because of the unavailability of key relatives (2%), 3 because of evidence of genetic recombination using extragenic markers (2%), 29 (23%) because the disease was sporadic and 2 because of homozygosity for all markers examined (2%). In 9 cases only extragenic linkage analysis was informative (7%). In the last two years one out of the three prenatal diagnoses performed was carried out by CSGE because of the absence of other informative markers. All the limitations of the diagnostic strategy based on linkage analysis were related to the fact that the mutation itself was not identified.

We are now screening for mutations in all the non-informative cases and to date we have identified 9 mutations (8 novel and 1 previously reported) which are listed in Table 2. We have found 7 missense mutations, one inframe deletion of a single amino acid and one large deletion which spanned from exon 2 to 25.

Nineteen normal unrelated females (38 X chromo-

Table 2. Mutations in hemophilia A and B patients.

Type/Domain	Pt. ID	Severity	Exon	Nucleotide change ^o	Amino acid change ^o	Amino acid side chain	Hydrophobic/hydrophilic	Comments	
A	-	C 132	severe	2-25 del	-	-	-	Novel large gene deletion, all 3 patients have inhibitors	
A	A1	C 35	severe	1	T 103 A	Tyr 16 His	Aromatic→basic	H→h	Novel, tertiary structure changed
A	A1	C 209	moderate	7	T 883 C	Phe 276 Leu	Aromatic→aliphatic	H→H	Novel
A	A1	C 150	moderate	7	T 920 G	Ile 288 Ser	Aliphatic→Aliphatic hydroxyl	H→h	Novel, tertiary structure changed
A	A1	C 165	severe	7	958 to 960 del ATG	301 del Met	Sulphur contain	-	Novel
A	A3	C 334	moderate	17	A 5726 G	Tyr 1890 Cys	Aromatic→sulphur	H→h	Novel, tertiary structure changed, inhibitor
A	C1	C 254	moderate	20	G 6171 C	Ala 2039 Pro	Aliphatic→secondary amino group	H→H	Novel
A	C2	C 47	severe	24	G 6683 A [#]	Arg 2209 Gln	-	h→h	One previous report in severe case
A	C2	C 149	severe	26	T 6995 C	Trp 2313 Arg	Aromatic→basic	H→h	Novel, tertiary structure changed
B	-	C 116	severe	A	107 to 110 del	Frameshift	-	-	Novel
B	EGFD2	C 324	severe	E	G 17741 C [#]	Cys 109 Ser	-	-	One previous report in severe case
B	APD	C 227	severe	F	G 20519 A [#]	Arg 180 Gln	-	-	Decreased XIa activation, 23 previous reports

[#]Previous reported mutations; ^ofor reference number system see #27 and #28 of the main reference list. H = hydrophobic, h = hydrophilic. EGFD = epidermal growth factor domain; APD = activation peptide domain.

somes) were also screened for all novel missense mutations in exon 1, 7, 17, 20, 24 and 26 by CSGE. In none of them did we find any heteroduplex and these results confirm that these mutations are absent from a normal population.

Two patients, one with a large deletion (exons 2-25) and one with a missense mutation, had inhibitors to FVIII. A high risk of development of inhibitors has been described in patients with FVIII gene inversion^{11,12}) and we are now screening all severely affected patients for the inversion. To date we have looked for the inversion in 96 patients with severe disease and found it to be present in 38 (40%). Inhibitors were present in 45% of patients with the inversion compared to in 17% of patients without the inversion (Table 3).

Table 3. Patients tested for inversion and inhibitors. The percentage of patients carrying the inversion in our cohort is 39% of severely affected. In this group inhibitors developed in 45% compared with in 17% of patients carrying other mutations.

	Inversion	No inversion	Total
Inhibitor	17 (45%)	10 (17%)	27
No inhibitor	21 (55%)	48 (83%)	69
Total	38 (39%)	58 (61%)	96

Hemophilia B

Six families were studied, involving 10 patients and 16 family members (26 individuals in total). Table 1 reports the results of linkage analysis. For 8 female relatives (50%), we could not give a diagnosis (5 belonging to families with sporadic hemophilia and 3 due to homozygosity). To date, we have identified two missense mutations (both previously reported) and 1 novel frameshift (Table 2).

Discussion

Since 1996 we have been able to offer a genetic counseling service in our Hemophilia center in order to provide a comprehensive care for our patients and their families. The genetic approach consists of accurate pedigree analysis which allows us to establish whether the disease is familial or sporadic and, for each female, whether she is an obligate or possible carrier. For patients with severe hemophilia A, the inversion is sought by using a PCR method. This is rapid, does not require the use of radioisotopes and consists of a single-tube multiplex PCR directly from genomic DNA. The results of this method have fully confirmed those obtained previously with Southern blotting and it is now the only method in use in our laboratory. All hemophilia B and all non-inversion hemophilia A families were studied in the indirect way by linkage analysis. This method is straightforward, rapid and inexpensive to perform, but has some limitations. The presence of heterozygosity is a prerequisite which must be satisfied if genotypic studies are to

be effective. In one family with moderate familial hemophilia A, the study was uninformative because of recombination with extragenic markers. Linkage analysis is frequently uninformative when key individuals are unavailable for study. In families with sporadic cases of hemophilia A, since it is not possible to ascertain at which level of the pedigree the mutation has arisen and become linked with the allele found in the patient, polymorphism studies can only be used to exclude transmission of the mutation when the patient's allele is not found in females relatives.

To summarize our experience, in 45 out of 98 (46%) females analyzed by linkage analysis, we were not able to make an accurate diagnosis of whether they were carriers or not (Table 1). Table 1 also reports 12 cases in which carriership was excluded by linked polymorphisms, in three cases by intron 13 and 22 CA repeats, but in 9 cases by the St14 extragenic polymorphism. This is a very highly polymorphic marker, but the risk of recombination led us to include those cases into the not accurately diagnosed group.

All these limitations are due to the fact that linkage analysis does not identify the mutation that causes the defect. To address this point, we have now optimized the mutation detection technique conformation sensitive gel electrophoresis. To date we have been able to identify 9 novel mutations, 8 in hemophilia A and 1 in hemophilia B (Table 2). Four out of 6 novel missense mutations in the hemophilia A group result in amino acid side chain changes from hydrophobic to hydrophilic which are predicted to have substantial influence on the tertiary structure of FVIII, and may be responsible for the decreased protein activity. In patient C 254 the side chain for both Ala to Pro is hydrophobic, but the proline side chain is bound to both the amino group and the α -carbon forming a cyclic structure which could interfere with the conformation and, possibly, the function of the protein. Patient C 209 shows a novel mutation in the A1 domain from Phe to Leu which are both hydrophobic, but the change from aromatic, which is uncharged at physiological pH, to aliphatic, can affect the conformation and correlates well with the moderate disease in this patient. The mutation in patient C 47 has been reported several times previously, predominantly in severe disease. Human factor VIII gene was aligned with canine, porcine and murine factor VIII and all the novel missense mutations were in highly conservative regions.

In the case of hemophilia B, only one was previously unknown (frameshift in patient C 116); the mutation found in patient C 324 has already been reported in another severely affected patient, while that found in patient C 227 (Arg 180 Gln) in the activation peptide domain, at the site of the FXIa cleavage, has been reported previously several times.

Screening all patients with severe hemophilia A for FVIII gene mutations, including the inversion, gives us the possibility of predicting the risk of inhibitor development in severely affected patients. Previous reports have demonstrated the strong relationship between inversions, large deletions and stop muta-

tions as a major risk factor for the development of inhibitors. In our cohort, we found the presence of the inversion in 40% of patients with severe hemophilia A and 45% of these had a history of inhibitors. In the case of the large deletion spanning exon 2-25, interestingly all three affected males carrying this mutation have a high titer of inhibitors to FVIII. These results are in keeping with previous reports that patients with large deletion have a very high risk of inhibitor development.^{11,12} Information on this aspect could also be relevant in genetic counseling when prenatal diagnosis is requested. Clinicians dealing with patients may also be able to use this information to select patients to include in prophylaxis regimes or for a different follow up schedule, as well as in the selection of patients to enroll for gene therapy trials.

A serious side effect in hemophilia has been recently described due to antibodies to FIX which cause anaphylaxis. The risk of developing this severe reaction has been demonstrated in at least 26% of patients with a complete deletion of the FIX gene¹⁴ and, as this is a very severe reaction in a high percentage of patients, it could be very relevant in a careful follow-up, mainly in patients on prophylaxis or home treatment programs.

In conclusion, we think that genetic counseling by linkage analysis can be used successfully, as confirmed by our experience, in nearly 50% of females. When the mutation is known, this provides many additional benefits. The knowledge of a specific mutation can add information on the side effects of replacement therapy, such as the risk of inhibitor formation to select patients for different treatments or follow-ups. As methods for screening for hemophilia mutations are now available, genetic counselling should take this into consideration and provide improved support to patients, their families and clinicians, including the risk of some specific mutations for inhibitor development.

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GT: conception and design of study, analysis and interpretation of data, drafting and final version; DB, RS, AA: analysis and interpretation of data, critical revision and approval of the final version; EDB and PGD: conception of the study and interpretation of data, revision and approval of the final version; AG: conception and design of study, revision for important intellectual content, final approval of the version to be submitted.

The order of authorship was as follows: GT, PGD and AG: prevalent intellectual input, in decreasing order. DB,RS,AA and EDB: prevalent technical support, in decreasing order.

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Disclosures

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Potential implications for clinical practice

- ◆ Mutation analysis allows the most accurate genetic counseling.
- ◆ As the development of inhibitors is a major side effect of the replacement therapy in hemophilia, determination of the genetic risk may allow the prevention of severe complications.

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