The Italian AICE-Genetics hemophilia A database: results and correlation with clinical phenotype

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ABSTRACT

Background

The high mutational heterogeneity of hemophilia A is a challenge for the provision of genetic services. We plan to identify the mutation in patients with hemophilia A in order to create a confidential national database of mutations for the optimization of genetic services in Italy.

Design and Methods

The factor VIII gene (*F8*) was analyzed in 1296 unrelated patients with hemophilia A using screening methods for intron 22 and 1 inversions and rare mutations (denaturing high performance liquid chromatography, conformation sensitive gel electrophoresis) and/or direct sequencing.

Results

F8 mutations were identified in 874 (89%), 146 (89%), and 133 (94%) families with severe, moderate, or mild hemophilia A, respectively. Mutations predicting a null allele were responsible for 80%, 15%, and less than 1% of cases of severe, moderate, or mild hemophilia A, respectively. About 40% of missense and nonsense mutations occurred at a CpG site, arginines being most frequently affected. Of the small deletions or insertions, 29% occurred at one of two stretches of adenines, codons 1191–1194 (8As) and 1439–1441 (9As). Overall, these "hotspots" accounted for 31% of the point mutations in the patients with hemophilia A. Inhibitors developed in 22% of the patients with severe hemophilia A, 8% of those with moderate disease and in 4% of patients with mild hemophilia A. Patients who had severe hemophilia A and mutations predicting a null allele developed inhibitors more frequently (22 to 67%) than patients with missense mutations (5%).

Conclusions

We report a wide spectrum of mutations in a large national database. The type of mutation was a strong predictor of the clinical phenotype. This database is expected to considerably improve the genetic counselling and medical care of families with hemophilia A in Italy.

Key words: hemophilia A, *F8* gene, mutations, hotspots, phenotype.

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The online version of this article contains a supplementary appendix.

Introduction

Hemophilia A (HA) is an X-linked bleeding disorder caused by heterogeneous mutations in the factor VIII gene (*F8*). Its incidence is estimated to be between 1:5,000 and 1:10,000 in men.[1] *F8* maps to the distal end of the long arm of the X-chromosome (Xq28) and spans 186 kb of genomic DNA. It consists of 26 exons and encodes a mature protein of 2332 amino acids.[1] Over the last decades, rapidly increasing numbers of causative gene alterations have been described in different ethnic groups. At present, more than 900 mutations within the *F8* coding and untranslated regions have been identified and listed in the *F8* HAMSTeRS mutation database [*URL: http://europium.csc.mrc.ac.uk*].[2] These findings support the concept that HA has a very high mutational heterogeneity and represents a challenge for the provision of genetic services because carrier and prenatal diagnosis cannot be based on the screening of a limited number of common mutations.[3] The exponential discovery rate of new genomic alterations leading to HA, as well as the need for comparative studies of mutation frequencies in different populations make it important to record the population-wide spectrum of mutation in databases. These databases are likely to facilitate genetic counselling of patients' families and can help to optimize national DNA diagnostic services also by enhancing awareness among clinicians, patients, and the general public. Finally, mutation analysis in a given population is useful for further understanding of the structural and functional aspects of the mutant protein and the correlation between genotype and phenotype.

The Italian Association of Hemophilia Centers (AICE), therefore, decided to set up a national database aiming at characterizing *F8* mutations to elucidate the molecular basis of hemophilia A in Italy and to provide insights into protein structure-function relationships, unravelling the correlation (if any) between genotype and clinical phenotype. In the present study, we report the phenotypic and genotypic data from 1153 independent families with HA, comprising about 89% of Italian HA patients.

Design and Methods

This study was carried out according to the Principles of the Declaration of Helsinki; informed consent was obtained from all participants. All the patients involved in this study are registered and regularly followed at one of 49 hemophilia treatment centers (see Appendix) spread all over the country and belonging to the Italian Association of Hemophilia Centers (*AICE; URL: http://www.aiceonline.it*), an organization that was founded with the aim of organizing national clinical and research activities in inherited bleeding disorders. Patients were diagnosed as having hemophilia A according to the international consensus criteria of the 2001 International Society on Thrombosis and Haemostasis (ISTH) Factor VIII and Factor IX Subcommittee.[4] For each patient clinical and laboratory data (including FVIII clotting activity and inhibitor) were recorded.

DNA collection

A blood sample (5-10 mL) was collected in EDTA or sodium citrate from each patient and stored at -20°C until it was sent to one of the nine laboratories that performed the genetic analyses. DNA was isolated from leukocytes using standard procedures.[5]

Polymerase chain reaction (PCR) detection of intron 1 and 22 inversions

Intron 1 inversions were detected by PCR, as reported earlier.[6] Long PCR for detecting the intron 22 inversion was performed as previously described.[7]

Amplification of F8

PCR was carried out according to standard procedures.[8] A total of 14 kb of the *F8* gene, including the entire coding sequence, exon-intron junctions and part of the 5' and 3' untranslated regions, were amplified by PCR. Most of the primers and amplification conditions have been previously reported; primer sequences, annealing temperatures and the size of PCR fragments are available on request.

Mutation detection screening

Mutational analysis was performed through all of the coding exons and the exon/intron boundaries of *F8* using denaturing high performance liquid chromatography (DHPLC) analysis in four of the nine laboratories (Florence, Genoa, Parma, and Vicenza), whereas another one (Castelfranco Veneto) used conformational sensitive gel electrophoresis (CSGE). The success rate ranged from 80 to 95%. All amplification products that showed an abnormal pattern were then analyzed by direct sequencing. The remaining four laboratories (Foggia, Milan, Naples, and Padua) did not employ any screening method and samples were subjected to direct gene sequencing.

DNA sequencing

Amplified DNA fragments were purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All the nucleotide changes identified were confirmed by repeating the PCR and sequencing reactions. To address whether new mutations identified were polymorphisms, 100 control subjects from the same ethnic background were investigated. Following recommendations for the description of sequence changes given on the HGVS website (*www.hgvs.org/mutnomen*), the mutation nomenclature used is that of the *F8* HAMSTeRS mutation database (*http://europium.csc.mrc.ac.uk*).

Splice site prediction

Missense mutations and mutations at or near the splice junction consensus sequences were analyzed by the *Splice Site Prediction program* (*http://www.fruitfly.org/*

seq_tools/splice.html) to predict the changes in RNA splicing.

Results

Overall population of HA patients

In this study, *F8* mutations were identified in 1153 of 1296 unrelated patients with a history of HA, representing a mutation detection rate of 89%. Among these unrelated patients, a causal *F8* mutation was identified in 874 (89%), 146 (84%), and 133 (94%) patients with severe, moderate, or mild HA, respectively. The relative frequency of different *F8* mutation types according to the clinical phenotype is shown in Figure 1. Overall, in addition to intron 22 and 1 inversions, 380 different mutations were recorded, including 193 missense mutations, 64 small deletions, 49 nonsense mutations, 32 splice site mutations, 26 small insertions, 11 large deletions (7 spanning more than one domain), and 5 involving different mechanisms (exon 15, p.Val 1727delins22bp; exon 24, p.Gln2189Argfs + p.Thr 2191LeufsX32; exon 1, c.-43 C>T; exon 1, c.-25 A>G, and duplication of the exon 13).[9] Twenty-eight point mutations (7%) were found twice or more and occurred in unrelated patients presenting with different clinical phenotypes (*Online Supplementary Table S1*). The complete list of mutations identified is given in *Online Supplementary Table S1*.

The intron 22 inversion was found in 52% of patients with severe HA (n=451) with an identified mutation, while intron 1 was present in 2% of them (n=19).

Overall, mutations that are likely to give rise to a null allele (inversions of an intron, deletions, insertions, and nonsense mutations) were found in 80%, 15%, and less than 1% of patients with severe, moderate or mild HA, respectively. On the other hand, a missense mutation was identified in 16%, 68%, and 80% of patients with severe, moderate or mild HA, respectively. Mutations predicted to affect *F8* mRNA splicing occurred in 4% of patients with severe HA, 16% of those with moderate HA and in 5% of patients with mild HA.

Exon 14 accounts for approximately 43% (3106 out of 7227 bp) of the coding region. In keeping with this, after having excluded patients with inversion of intron 22 or intron 1, one fourth of point mutations occurring in the coding region (n=142/585) were found within exon 14. Most of mutations predicted to lead to a null allele were found in exon 14 (121/237; 51%). In detail, 50/94 (53%) of small deletions, 43/58 (74%) of small insertions, and 28/85 (33%) of nonsense mutations were identified within exon 14. However, missense mutations in exon 14 occurred in only 21/348 patients (6%). Among different missense mutations recorded within exon 14, only pQ1128H and pT1353A occurred in the region coding for the B domain. These estimates were similar to those calculated using available data (n=2426) recorded in the HAMSTeRS database for patients carrying point mutations in the coding region (Table 1). In both settings, the rate of mutation per nucleotide was not higher than the mean rate (0.7×10^{-4} in AICE and 0.6×10^{-4} in HAMSTeRS).

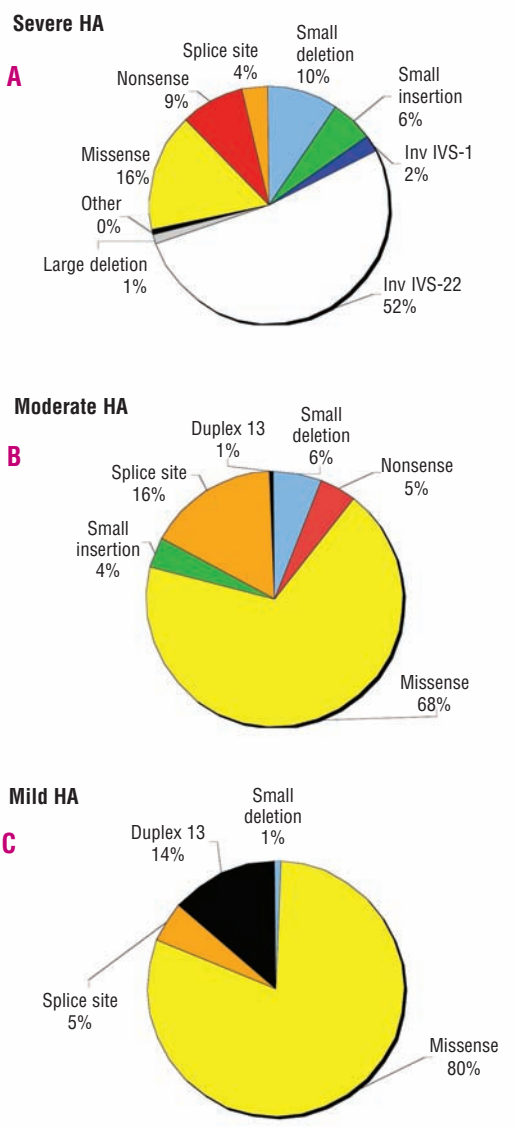

Figure 1. Distribution of the different *F8* mutation types in unrelated hemophilia A patients according to the clinical phenotype.

Common mutations and hot spot consensus sequences

Two recurrent mutations, duplication of the exon 13 and the intron 10 splice site mutation c.1538-18 G>A, were identified in 19 (18 with mild and 1 with moderate HA) and 17 (16 with moderate and 1 with mild HA) unrelated patients, respectively. In both cases, all patients shared a common haplotype, suggesting that both mutations likely occurred in single ancestors.

Taking into account all hemophiliacs, 193 different missense and 49 nonsense mutations were found and 39 of them (16%) occurred at a CpG site (*Online Supplementary Table S2*). In fact, 348 patients (142 with severe, 99 with moderate, and 107 with mild HA) carried a missense mutation and 85 (78 with severe and 7 with moderate HA) carried a nonsense mutation. Among these, 163 (38%) had a mutation at a CpG site

(OR: 29.8; 95% CI: 23.1-38.5). Taking into account only those patients with severe HA and giving the 12-fold higher rate of mutability of CpG dinucleotides,[10] the number of mutations identified at a CpG site (n=80) was similar to that expected in this survey (n=65).[11] As expected, most recurrent mutations at a CpG site involved arginines (*Online Supplementary Table S2*). Overall, about 25% of missense mutations (83/348) occurred at positions 372, 531, 1781, 1997, 2150, 2163, 2209, 2304, and 2307 and some of them were identified in patients presenting with different HA phenotypes (*Online Supplementary Table S2*). The most frequently reported missense mutation, pArg1997Trp, was found in 19 unrelated patients (13 with severe, 5 with moderate, and 1 with mild HA). Similarly, 37% of nonsense mutations (31/85) involved arginines at positions 427, 583, 1696, 1966, 2116, and 2209. The most frequently reported nonsense mutation, pArg1696Stop, was found in seven unrelated patients with severe HA.

Fifty-six of 94 small deletions (60%) identified in HA patients are associated with major motifs: short direct repeats (n=21; 22%) and homonucleotide tracts (n=35; 37%). Among unrelated hemophiliacs presenting with a small insertion, 34 of 58 (59%) had a mutation in a homonucleotide tract. A couple of point mutations were frequently found in two A runs occurring in exon 14. Fifteen patients (11 with severe and 4 with moderate HA) had a deletion of one nucleotide in a run of 9 A at position 3629-3637 (codons 1191-1194) and six (all with severe HA) had an insertion of an adenine residue in the same A run. Six patients with severe HA had a deletion of one nucleotide in a series of 8 A at codons 1439-1441 (c.4372-4379), whereas 17 patients (16 with severe and 1 with moderate HA) had an insertion of an adenine residue in the same A run. Overall, about 29% of small deletions or insertions (44/153) occurred at these two sites. The remaining point mutations do not appear to fit into any of these categories.

Gene mutations and inhibitor development

Information about the development of inhibitors (yes/no) was available for 870 of the 874 patients with severe HA (99%): an inhibitor was detected in 193/870 patients, with a prevalence of 22%. The distribution of inhibitor prevalence according to the type of mutation is reported in Figure 2. The risk of developing inhibitors was higher in patients carrying large deletions of *F8* (67%) (ranging from 60% in single-domain mutations to 70% in multi-domain deletions), nonsense mutations (40%), inversion of intron 1 (29%), inversion of intron 22 (25%), and splicing mutations (22%). A lower prevalence was found in patients with small deletions (17%), small insertions (15%), and missense mutations (5%). When the prevalence of inhibitors was analyzed according to the occurrence of the causative mutation in a hot spot consensus sequence, patients with small deletions or insertions had a lower prevalence (11% and 8%, respectively) than patients without these types of mutations (24% and 25%, respectively). Overall, the likelihood of developing inhibitors was higher in HA patients with a mutation not occurring at a hot spot consensus sequence than in those in whom the mutation was in such a sequence (OR: 2.9; 95%-CI: 1.2-7.0). In patients carrying a nonsense mutation, the likelihood of inhibitor development was higher when the mutation occured in the light chain (53%) than in the heavy chain (39%) or in the B-domain (18%) (OR: 2.6; 95%-CI: 1.0-6.4). Information about the development of inhibitors was available for 142 patients with moderate HA (97%) and 131 with mild HA (98%) in whom a mutation was found. The presence of inhibitors was recorded in 12 patients with moderate HA (8%) and in 5 with mild HA (4%) (*Online Supplementary Table S3*). None of the patients carrying a small deletion or insertion, or with exon 13 duplication had inhibitors.

Table 1. Proportion of hemophilia A patients carrying a *F8* gene mutation occurring in exon 14 according to different types of point mutations.

	Italy *146/585 (25%)*		*HAMSTeRS* *526/2426 (22%)*	
Nonsense mutations	28/85	(33%)	86/360	(24%)
Small deletions	50/94	(53%)	181/341	(53%)
Small insertions	43/58	(74%)	111/158	(70%)
Missense mutations	25/348	(7%)	148/1567	(9%)

Discussion

We report the largest series of HA patients analyzed for *F8* mutations in Italy. The present study includes the vast majority of Italian HA families, especially those with severe disease, and it may be considered to be representative for the purpose of a population-based study of mutational heterogeneity. We found 382 different mutations among 1296 unrelated patients, demonstrating the high degree of heterogeneity of *F8* mutations besides the common inversions of introns 22 and 1. In this large series the causative mutation within the *F8* gene was identified in 89% of the families, while the causative genetic event remained undetected in 107, 17 and 9 patients with severe, moderate, and mild HA, respectively. Thus, the percentage of unidentified mutations was higher than reported in other studies, in which no genetic disorder could be found in the coding region of *F8* in 2-5% of patients with severe HA.[12-14] Screening methods (denaturing high performance liquid chromatography and denaturing gradient gel electrophoresis) employed in most laboratories have success rates ranging from 80 to 95%. It is likely that a re-analysis of patients in whom the causative mutation went undetected using a different approach, i.e. direct *F8* gene sequencing, would lead to detection of the mutation in most of them. On the other hand, rearrangements in untranslated regions other than intron 1 and intron 22, which could go undetected by current PCR-based analysis of genomic DNA, may be the cause of disease. In addition, the genetic abnormality may be located outside the analyzed regions of *F8*, i.e. the abnormality could be intronic sequence changes that might affect transcription or translation, or lie in

modifier genes important for processing FVIII expression and/or activity.

Our study confirms the well-known correlation between the type of mutation and the severity of HA. The types of mutations found were in agreement with results reported in other settings (Table 2).[15-24] As expected, most of patients with severe HA carry a molecular defect predicting a null allele (large deletion, inversion, nonsense, and insertion/deletion mutations), whereas missense mutations have been found in the majority of patients with moderate (68%) and mild HA (80%).

Accurate splicing requires canonical sequences at the 3'- and 5'-splice sites at the exon-intron borders as well as the weakly conserved branch point sequence, located 18 to 40 bp upstream of the 3' splice site.[25,26] Most of the splicing defects associated with human diseases, estimated to account for about 10% to 15% of disease-causing mutations, are point mutations within the 5' donor and 3' acceptor splice sites.[27] Most of the mutations predicted to affect *F8* mRNA splicing occurred in families with moderate or mild HA, suggesting that low levels of normal transcripts may escape aberrant splicing.

A variety of mutation hot spot consensus sequences have been reported in literature.[28] DNA methylation is considered responsible for base pair substitutions at CpG sites.[29,30] The higher risk of mutation calculated by us confirms that an average CpG dinucleotide (~80% are methylated)[31] in the human genome is subject to a 12-fold higher frequency of mutation than any other dinucleotide.[10] About 40% of missense and nonsense mutations occurred at one of the 70 CpG sites within the *F8* gene, although these represent only 2% of the coding sequence.[11] Arginine is the most frequent amino acid encoded by codons containing CpG sites (36/70) and is the most commonly mutated amino acid, being affected in about one-fourth of all missense and nonsense mutations (114/433; 26%). Homonucleotide tracts show a high mutability that increases with the number of nucleotides within the stretch.[28,32] About 29% of all small deletions or insertions were found within exon 14 at one of two stretches of adenines: codons 1191–1194 (8A) and 1439–1441 (9A).[33] Overall, hot spots account for more than one-third of all point mutations in unrelated patients with HA (253/585; 43%).

Exon 14 represents about one half of the coding region and encodes for the FVIII B domain, a region lacking procoagulant activity that is spliced out from the mature protein. This finding explains the occurrence of very few missense mutations in the central portion of exon 14 in our series and in other settings, (see the HAMSTeRS mutation database).

The pathogenesis of inhibitor formation is only partly understood. Several factors have been suggested to modulate inhibitor formation. The incidence of inhibitors depends on both genetic factors (severity of hemophilia, type of mutation, ethnicity, family history

Table 2. ***F8*** **gene mutations identified in patients with severe hemophilia A.**

	Italy	*Germany*[15]	*Argentina*[16]	*Portugal*[17]	*India*[18]	[a]*Pooled smaller studies*[19-24]
Patients	874	753	80	119	84	222
Inversion IVS-22 n (%)	451 (52)	-- (45)	36 (45)	73 (61)	51 (61)	98 (44)
Missense n (%)	143 (16)	-- (15)	8 (10)	11 (9)	6 (7)	27 (12)
Small deletion n (%)	84 (10)	-- (16)[b]	13 (16)[b]	12 (10)	6 (7)	28 (13)
Nonsense n (%)	77 (9)	-- (13)	7 (9)	6 (5)	9 (11)	38 (17)
Small insertion n (%)	52 (6)	-----	-----	10 (9)	2 (2)	8 (4)
Splice sites n (%)	31 (4)	-- (4)	1 (2)	3 (3)	3 (4)	5 (2)
Inversion IVS-1 n (%)	19 (2)	-- (3)	1 (2)	2 (2)	2 (2)	12 (5)
Large deletion n (%)	13 (1)	-- (5)	10 (13)	1 (1)	4 (5)	6 (3)
Other n (%)	4 (0)	-	-	-	1 (1)	-

[a]Small series including fewer than 50 patients with severe HA, in which a mutation stratification was available. [b]small deletion plus small insertion.

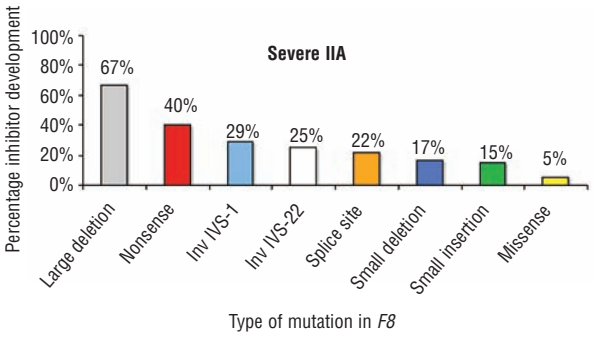

Figure 2. **Incidence of inhibitor occurrence in patients with different types of** ***F8*** **mutations.**

Table 3. **Proportions of patients with severe hemophilia A and different** ***F8*** **mutations who developed inhibitors.**

	Italy (n=870)	*Germany*[15] *(n=753)*	*HAMSTeRS (n=845)*
Large deletions	7/13 (67%)	41%	46%
Nonsense mutations	31/77 (40%)	31%	35%
IVS-1 inversions	6/19 (29%)	17% (26%)[a35]	n.a.
IVS-22 inversions	113/451 (25%)	21%	n.a.
Splicing-site mutations	7/3 (22%)	17%	8%
Small deletions	14/84 (17%)	16%[b]	17%
Small insertions	8/52 (15%)		19%
Missense mutations	7/143 (5%)	5%	10%

n.a.: not available. [a]1127 patients investigated; [b]small deletion plus small insertion.

of inhibitors, the HLA genotype) and non-genetic factors (age at first treatment, intensity of treatment, continuous infusion, and multiple product switches).[34] The type of mutation is one of the most important risk factors predisposing to inhibitor development. In keeping with previous results,[15,35] among patients with severe HA, a higher proportion of those with a mutation predicting a null allele developed inhibitors (24-71%), compared to those carrying a missense mutation (5%) (Table 3). Replacement therapy in patients completely lacking FVIII may lead to immunization with a foreign protein and cause the development of antibodies. These findings support the hypothesis that trace amounts of protein, although non-functional, which are synthesized are sufficient to induce immune tolerance in most of these patients. In addition, the present study suggests that mutations at specific regions of *F8* (hot spots, exons coding for the heavy chain) are associated with a lower risk of developing inhibitors.[15,36]

In conclusion, we report the finding of a large national database, in which a wide spectrum of mutations has been collected. The type of mutation was a strong predictor of the clinical phenotype. This database will be of great value for genetic counselling in HA and is expected to considerably improve the medical care of HA families in Italy.

Authorship and Disclosures

MM takes the direct responsibility for the manuscript; he gave substantial contributions to the conception and design of the study, acquisition, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content. GCC, MM, AR, ES, GT, ART, EZ, MPB, GC, FP, RS, FT and PMM gave substantial contributions to the acquisition, analysis and interpretation of data, and drafting the article or revising it critically for important intellectual content. All the authors approved the version to be published.

Appendix

The patients whose data are the subject of this study are cared for at the following hemophilia centers belonging to the Italian Association of Hemophilia Centers (AICE): Alessandria, Dr. L. Contino; Arezzo, Dr. A. Accorsi; Bari "Policlinico" I, Dr. A. Scaraggi; Bari "Policlinico" II, Dr. N. Ciavarella; Bologna, Dr. G. Rodorigo; Cagliari, Dr. R. Targhetta; Castelfranco Veneto, Dr. G. Tagariello, Dr. D. Belvini, Dr. R. Salviato; Catania, Dr. R. Musso; Catanzaro, Dr. G. Muleo; Cesena, Dr. C. Biasoli; Cosenza, Dr. V. Rossi; Cremona, Dr. S. Testa; Ferrara, Dr. G.L. Scapoli; Faenza, Dr. D. Vincenti; Florence, Dr. M. Morfini; Genoa, Dr. A.C. Molinari, Dr. M.P. Bicocchi; Ivrea, Dr. M. Girotto; L'Aquila, Prof. G. Mariani; Latina, Dr. C. Ciabatta; Macerata, Dr. MT. Carloni; Milan Policlinico, Prof. P.M. Mannucci, Dr. E. Santagostino, Prof. F. Peyvandi; Milan Niguarda, Dr. F. Baudo; Modena, Dr. M. Marietta; Naples "Ospedale Pediatrico Pausillipon", Dr. C. Perricone, Dr. M. Schiavulli; Naples "Ospedale San Giovanni Bosco", Dr. A. Rocino; Naples "Policlinico Universitario Federico II", Prof. G. Di Minno, Dr. A. Coppola; Orvieto, Dr. M. Berrettini; Padua, Dr. E. Zanon; Palermo "Ospedale G. di Cristina", Prof. G. Mancuso; Palermo "Policlinico Universitario", Prof. S. Siragusa; Parma, Dr. A. Tagliaferri, Dr. F. Rivolta; Pavia, Prof. G. Gamba; Perugia, Dr. A. Iorio; Pescara, Dr. A. Dragani; Piacenza, Dr. MC. Arbasi; Ravenna, Dr. A. Mancino; Reggio Calabria, Dr. V. Trapani Lombardo; Reggio Emilia, Dr. M. D'Incà; Rome "Ospedale Pediatrico Bambin Gesù", Prof. G. De Rossi; Rome "Policlinico Universitario A. Gemelli", Prof. R. Landolfi; Rome "Policlinico Universitario La Sapienza", Prof. G. Mazzucconi; Sassari, Dr. G. Piseddu; Torino "Policlinico Pediatrico", Dr. L. Perugini, Dr. M. Messina; Turin "Le Molinette", Dr. G. Tamponi, Dr. P. Schinco; Trento, Dr. G. Rossetti; Udine, Dr. G. Barillari; Vallo della Lucania (SA), Dr. A. Catalano, Dr. G. Feola; Verona, Dr. G. Gandini; Vicenza, Dr. G. Castaman, S. Giacomelli, R. Ghiotto.

References

1. Kazazian HH, Tuddenham EGD, Antonarakis SE. In Scriver CR, Beaudet AL, Sly WS, Valle D. (eds), The Metabolic and Molecular Bases of Inherited Disease, 7th Edn. McGraw-Hill, New York, 1995:pp. 3241-67.
2. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, et al. Characterization of the human factor VIII gene. Nature 1984;312:326-30.
3. Kemball-Cook G, Tuddenham EG, Wacey AI. The factor VIII structure and mutation resource site: HAMSTeRS version 4. Nucleic Acids Res 1998;26:216-9.
4. White GC 2nd, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev J, for the Factor VIII and Factor IX Subcommittee. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost 2001;85:560.
5. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988; 16:1215.
6. Bagnall RD, Waseem N, Green PM, Gianelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood 2002;99:168-74.
7. Liu Q, Nozari G, Sommer SS. Single-tube polymerase chain reaction for rapid diagnosis of the inversion hotspot of mutation in hemophilia A. Blood 1998;92:1458-9.
8. D'Andrea G, Colaizzo D, Vecchione G, Grandone E, Di Minno G, Margaglione M. Glanzmann's Thrombasthenia Italian Team (GLATIT). Glanzmann's thrombasthenia: identification of 19 new mutations in 30 patients. Thromb Haemost 2002; 87:1034-42.
9. Aquila M, Pasino M, Lanza T, Bottini F, Molinari AC, Bicocchi MP. Duplication of exon 13 causes one third of the cases of mild hemophilia A in northern Italy. Haematologica 2004;89:758-9.
10. Cooper DN, Krawczak M. The mutational spectrum of single base-pair substitutions causing human genetic disease: patterns and predictions. Hum Genet 1990;85:55-74.
11. Pattinson JK, Millar DS, McVey JH, Grundy JB, Wieland K, Mibashan RS, et al. The molecular genetic analysis of hemophilia A: a directed search strategy for the detection of point mutations in the human factor VIII gene. Blood 1990;76:2242-8.
12. Citron M, Godmilow L, Ganguly T, Ganguly A. High throughput mutation screening of the factor VIII gene (F8C) in hemophilia A: 37 novel mutations and genotype-phenotype correlation. Hum Mutat 2002;20: 267-74.
13. Oldenberg J, Ivaskevicius V, Rost S, Fregin A, White K, Holinski-Feder E, et al. Evaluation of DHPLC in the analysis of hemophilia A. J Biochem Biophys Methods 2001;47:39-51.
14. Ahmed RP, Ivaskevicius V, Kannan M, Seifried E, Oldenburg J, Saxena R. Identification of 32 novel mutations in the factor VIII gene in Indian patients with hemophilia A. Haematologica 2005;90:283-4.

15. Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factor VIII and IX. Haemophilia 2006;12 [Suppl 6]:15-22.
16. Rossetti LC, Radic CP, Candela M, Pérez Bianco R, de Tezanos Pinto M, Goodeve A, et al. Sixteen novel hemophilia A causative mutations in the first Argentinian series of severe molecular defects. Haematologica 2007;92:842-5.
17. Deszo D, Ventura C, Moreira I, Diniz MJ, Antunes M, Tavares A, et al. The spectrum of mutations and molecular pathogenesis of hemophilia A in 181 Portuguese patients. Haematologica 2006;91:840-3.
18. Jayandharan G, Shaji RV, Baidya S, Nair SC, Chandy M, Srivastava A. Identification of factor VIII gene mutations in 101 patients with haemophilia A: mutation analysis by inversion screening and multiplex PCR and CSGE and molecular modelling of 10 novel missense substitutions. Haemophilia 2005;11: 481-91.
19. Ivaskevicius V, Jurgutis R, Rost S, Müller A, Schmitt C, Wulff K, et al. Lithuanian haemophilia A and B registry comprising phenotypic and genotypic data. Br J Haematol 2001; 112:1062-70.
20. Hill M, Deam S, Gordon B, Dolan G. Mutation analysis in 51 patients with haemophilia A: report of 10 novel mutations and correlations between genotype and clinical phenotype. Haemophilia 2005;11:133-41.
21. Fernández-López O, García-Lozano J-R, Núñez-Vázquez R, Pérez-Garrido R, Núñez-Roldán A. The spectrum of mutations in Southern Spanish patients with hemophilia A and identification of 28 novel mutations. Haematologica 2005;90:707-10.
22. Boekhorst J, Verbruggen B, Lavergne JM, Costa JM, Schoormans SC, Brons PP, et al. Thirteen novel mutations in the factor VIII gene in the Nijmegen haemophilia A patient population? Br J Haematol 2005; 131:109-17.
23. Repessé Y, Slaoui M, Ferrandiz D, Gautier P, Costa C, Costa JM, et al. Factor VIII (FVIII) gene mutations in 120 patients with hemophilia A: detection of 26 novel mutations and correlation with FVIII inhibitor development. J Thromb Haemost 2007;5:1469-76.
24. Laurie AD, Sheen CR, Hanrahan V, Smith MP, George PM. The molecular aetiology of haemophilia A in a New Zealand patient group. Haemophilia 2007;13:420-7.
25. Nissim-Rafina M, Kerem B. Splicing regulation as a potential genetic modifier. Trends Genet 2002;18: 123-7.
26. Faustino NA, Cooper TA. Pre-mRNA splicing and human disease. Genes Dev 2003;17:419-37.
27. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, et al. Human Gene Mutation Database (HGMD): 2003 update. Hum Mutat 2003;21:577-81.
28. Krawczak M, Cooper DN. Gene deletions causing human genetic disease: mechanisms of mutagenesis and the role of the local DNA sequence environment. Hum Genet 1991;86:425-41.
29. Rodenhiser D, Chakraborty P, Andrews J, Ainsworth P, Mancini D, Lopes E, Singh S. Heterogenous point mutations in BRCA1 breast cancer susceptibility gene occur in high frequency at the site of homonucleotide tracts, short repeats and hypermethylatable CpG/CpNpG motifs. Oncogene 1996;12:2623-9.
30. Denissenko MF, Chen JX, Tang MS, Pfeifer GP. Cytosine methylation determines hotspots of DNA damage in the human P53 gene. Proc Natl Acad Sci USA 1997;94:3893-8.
31. Cooper DN. Eukaryotic DNA methylation. Hum Genet 1983;64: 315-33.
32. Darvasi A, Kerem B. Deletion and insertion mutations in short tandem repeats in the coding regions of human genes. Eur J Hum Genet 1995;3:14-20.
33. Oldenburg J, Ananyevan M, Saenko EL. Molecular basis of haemophilia A. Haemophilia 2004;10 [Suppl 4]:133-9.
34. Hay CRM. The epidemiology of factor VIII inhibitors. Haemophilia 2006; 12 [Suppl 6]:23-9.
35. SchrŒder J. El-Maari O, Schwaab R, Muller CR, Oldenburg J. Factor VIII intron-1 inversion: frequency and inhibitor prevalence. J Thromb Haemost 2006;4:1141-3.
36. Boekhorst J, Rastegar Lari G, d'Oiron R, Costa JM, Novakova IRO, Ala FA, et al. The risk of inhibitor development in 1104 patients with hemophilia A according to factor VIII genotype. J Thromb Haemost 2007;5 [Suppl 2]: O-S-064.