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Identification of Ten Novel Mutations in Factor VIII Gene: A Study of A Cohort of 52 Haemophilia A Patients

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Abstract

Introduction: Haemophilia A (HA) is the most common X-linked recessive genetic disease caused by mutations in the gene coding for coagulation factor VIII (FVIII) resulting in spontaneous bleeding.

Aim: The aim of our study is to provide additional information about the genetic causes of HA describing the correlation between the observed mutations and the clinical phenotype in a cohort of 52 patients suffering from HA to different degrees.

Methods: First we performed a search of inversion 22 (IVS22) and of the intron 1 (IVS1) for severe HA patients; inversions negative and moderate/mild HA patients were screened by direct sequencing of the coding regions and exon-intron junctions of FVIII gene. Where no PCR amplification was observed we used the Multiplex Ligation-dependent Probe Amplification (MLPA) and, to confirm a large deletion, we performed an array-comparative Genomic Hybridization (array CGH).

Results: 42% of severe HA patients had IVS22 while the remainder had missense mutations, large deletions and small insertions. All but one moderate/mild HA patients had missense mutations. Only two patients did not show good correlation between the genotype and the clinical phenotype reported to us by the respective centers of haemophilia. We also identified 10 novel mutations not previously described in the most common mutation database.

Conclusion: We studied a group of 52 HA patients and we found 26 mutations in the FVIII gene of which 10 are new mutations. These results confirm the great heterogeneity of the molecular defects gene responsible for the deficiency of FVIII.

Keywords

Haemophilia A, FVIII gene, Molecular analysis, Genotype-Phenotype correlation, Novel mutations, Mutation database

Introduction

Haemophilia A (HA) is a recessive inherited X-linked blood coagulation disorder, due to mutations in procoagulant factor VIII (FVIII) that affects 1 in 6000 males. The FVIII gene maps on the distal end of the long arm of the X-chromosome (Xq28), spans 186 kb and comprises 26 exons [1]. HA is usually diagnosed during the first year of life in affected males although there are reports of female carriers with mild clinical manifestations of the HA disease. The severity of the disease is determined by activity levels of circulating factor VIII (FVIII:C) allowing a classification into 3 forms: severe (FVIII:C<1% of normal), moderate (FVIII:C 1-5% of normal) and mild (FVIII:C >5% - <40% of normal) HA disease [2]. In the HAMSTeRS factor VIII mutation database [3] are reported 2107 unique mutations, the majority of which (938) are missense mutations which, as is clear from the literature data, are reported in 37% of severe, 72% of moderate and 88% of mild haemophilia A patients [4]. The FVIII intron 22 inversion is the causative mutation in 45% of severe HA patients [5] while FVIII intron 1 inversion has a frequency of 1.8% [6] in severe HA population. The remaining patients negative for intron 22/1 inversion and missense mutations present small deletions, nonsense mutations, splice-site mutations, small insertions, large deletions and others mutations involving different mechanisms [4].

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Table 1: *Patients with de novo mutations (sporadic HA); §Patients FB19 and FB20 are brothers, M2-M3 and C1-C2 are relatives; °Patient P1 suffers from moderate haemophilia A (FVIII:C 2%) with presence of inhibitors. Mutations in bold are new mutations not described in HGMD, HAMSTeRS and CHAMP database; for those underlined have already been reported other mutations at the same site.

Patient ID	Clinical phenotype	Region	Nucleotide change	Codon change (HGVSnomenclature)	Domain	Mutation type
FB1	severe	exon 7	**c.943G>C**	**p.A315P**	A1	missense
FB2	severe	exon 3	**c.277_281insCAGGT**	**p.Pro93GlnfsX70**	A1	small insertion
FB3	mild	exon 11	c.1700T>C	p.I567T	A2	missense
FB4	mild	exon 11	c.1700T>C	p.I567T	A2	missense
FB5	mild	exon 3	**c.341C>A**	**p.P114H**	A1	missense
FB6	severe	exon 11	**c.1631A>G**	**p.D544G**	A2	missense
FB7	severe	intron 22	IVS22	/	/	inversion
FB8	severe	intron 22	IVS22	/	/	inversion
FB9	severe	intron 22	IVS22	/	/	inversion
FB10*	severe	exon 4	**c.530A>C**	**p.Y177S**	A1	missense
FB11	severe	exon 16	c.5392G>A	p.A1798T	A3	missense
FB12	mild	exon 11	c.1700T>C	p.I567T	A2	missense
FB13	moderate	exon 16	c.5398C>T	p.R1800C	A3	missense
FB14	severe	exon 14	c.3637dupA	p.I1213NfsX28	B	small duplication
FB15*	severe	exon 14	**c.4128C>G**	**p.Y1376X**	B	nonsense
FB16	severe	intron 22	IVS22	/	/	inversion
FB17	severe	intron 22	IVS22	/	/	inversion
FB18	severe	exon 18	**c.5830_5840delATAATGGATAC**	**p.Ile1944ThrfsX23**	A3	small deletion
FB19§	severe	exons 1-22	deletion ex1-22	/	/	large deletion
FB20§	severe	exons 1-22	deletion ex1-22	/	/	large deletion
M1	mild	exon 11	c.1636C>T	p.R546W	A2	missense
M2§	mild	exon 9	c.1372C>T	p.R458C	A2	missense
M3§	mild	exon 9	c.1372C>T	p.R458C	A2	missense
M4	mild	exon 14	**c.3465dupA**	**p.Ser1156IlefsX10**	B	small duplication
M5	mild	exon 11	c.1636C>T	p.R546W	A2	missense
M6	mild	exon 11	c.1700T>C	p.I567T	A2	missense
M7	mild	exon 23	c.6506G>A	p.R2169H	C1	missense
M8	severe	intron 22	IVS22	/	/	inversion
Patient ID	**Clinical phenotype**	**Region**	**Nucleotide change**	**Codon change (HGVSnomenclature)**	Patient #	**Mutation type**
M9	moderate	exon 24	c.6638C>G	p.S2213C	C2	missense
M10	mild	exon 11	c.1636C>T	p.R546W	A2	missense
M11	mild	exon 11	c.1636C>T	p.R546W	A2	missense
M12	mild	exon 23	c.6506G>A	p.R2169H	C1	missense
M13	mild	exon 11	c.1648C>T	p.R550C	A2	missense
M14	severe	intron 22	IVS22	/	/	inversion
M15	mild	exon 11	c.1636C>T	p.R546W	A2	missense
M16	mild	exon 16	c.5405A>G	p.Y1802C	A3	missense
M17	severe	intron 22	IVS22	/	/	inversion
M18	mild	exon 22	**c.6304G>A**	**p.G2102S**	C1	missense
M19	severe	intron 22	IVS22	/	/	inversion
M20	severe	exon 19	**c.6045G>A**	**p.W2015X**	A3	nonsense
M21	mild	exon 12	c.1834C>T	p.R612C	A2	missense
M22	mild	exon 12	c.1834C>T	p.R612C	A2	missense
M23	severe	exons 1-22	deletion ex1-22	/	/	large deletion
M24	severe	intron 22	IVS22	/	/	inversion
C1§	severe	exons 10-11	deletion ex10-11	/	/	large deletion
C2§	severe	exons 10-11	deletion ex10-11	/	/	large deletion
C3	severe	exon 3	c.331G>A	p.A111T	A1	missense
C4	severe	intron 22	IVS22	/	/	inversion
P1°	moderate	exon 18	c.5883G>A	p.W1961X	A3	nonsense
P2	mild	exon 11	c.1700T>C	p.I567T	A2	missense
P3	mild	exon 11	c.1700T>C	p.I567T	A2	missense
P4	mild	exon 11	c.1700T>C	p.I567T	A2	missense

We investigated FVIII gene abnormalities in 52 haemophilia A patients from different areas of Italy and from other countries, and we found 26 mutations, 16 of which are already described in the HAMSTeRS database and/or in the Human Gene Mutations Database (URL: http://www.hgmd.org) and/or CHAMP F8 Mutation List (http://www.cdc.gov/ncbddd/hemophilia/champs.html) that compared to the previous two database considers additional publications; the other 10 mutations are not reported in these database.

Materials and Methods

Informed consent was obtained from the patients after approval of the local Human Ethics Committees. The studies were carried out according to the Principles of the Declaration of Helsinki.

Patients and DNA sample

We collected blood samples, 5ml in sodium citrate, from 52 HA patients, from different Italian haemophilia treatment centers (Macerata, Bari, Pescara and Foggia). These 52 patients comprised 26 (50%) severe, 3 (5,8%) moderate and 23 (44,2%) mild cases based on FVIII:C.

Genomic DNA was purified from 200 µl of whole blood sample type using QIAamp DNA Blood Mini Kit (QIAGEN®).

Molecular analysis

The molecular analysis of FVIII gene was carried out depending on the severity of haemophilia A as this influences the diagnostic

strategy used [7]. First of all we screened severe HA patients for the FVIII intron 22 (IVS22) and later for intron 1 (IVS1) inversion mutation. The IVS22 was analysed by Long PCR method [8] and IVS1 by a multiplex PCR [9]. Severe HA patients negative for inversion mutations together with moderate and mild HA patients were analysed for the exons and exon-intron junctions of the entire FVIII gene by direct sequencing using the BigDye® TerminatorTM method on an ABI PRISM 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. The sequence data results from the analysis of sequencing were compared with the reference NM_000132 sequence of the FVIII gene deposited in NCBI database. The detected mutations, reported in accordance to the Human Genome Variation Society (HGVS http://www.hgvs.org/mutnomen/), were confirmed by repeating the amplification and sequencing reaction of interested FVIII gene fragments. Patients in which PCR amplification of FVIII gene exons failed were analysed by Multiplex Ligation-dependent Probe Amplification (MLPA) using SALSA MLPA probemix P178-B2 F8 Lot B2-0312 (MRC-Holland, Amsterdam, and the Netherlands). The reaction products were detected on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). To assess the presence of large deletions, in some patients, an array-comparative Genomic Hybridization (array CGH) analysis was subsequently performed.

Bioinformatic analysis

For the interpretation of new mutations found, we used the Alamut® Visual software (available online at www.interactive-biosoftware.com/) that allows interpretation of variant pathogenicity. Alamut is a decision support application developed by Interactive Biosoftware, which is widely used in medical genetics that works with the aid of integrated functions such as: data integration from public data sources, conservation information, polymorphisms, published mutations and protein annotations.

Results

IVS22 and IVS1 mutations

11 (42%) of the 26 patients with severe haemophilia A had the IVS22 mutation while none had the IVS1 (Table 1). This results confirms the high percentage (45-50%) of IVS22 found in severe HA population [5]. Two severe HA brothers (FB19-FB20) showed a strange IVS22 band pattern with the only AB (10Kb) control band amplified using the Long PCR protocol. The mother of the two patients showed, however, the presence of the two bands AB and PQ of 10 and 12 Kb respectively. This result led to think of a possible deletion including the intragenic homologous copy int22h-1 amplified by the primer pair PQ.

Missense and nonsense mutations

We found 15 missense mutations (Table 1). Of these five are novel mutations: c.943G>C (p.A315P), c.341C>A (p.P114H), c.1631A>G (p.D544G), c.530A>C (p.Y177S), c.6304G>A (p.G2102S); the nomenclature used in this work meets the HGVS instructions starting the nucleotide numbering from the A of the ATG start codon. Among four nonsense mutations two are not listed in the available database: c.4128C>G (p.Y1376X) and c.6045G>A (p.W2015X).

Small insertions and duplications

We also identified two novel insertions. The first one is the c.277_281insCAGGT (p.Pro93GlnfsX70), in exon 3, in a sporadic case of severe HA (FB2). The insertion of these five nucleotides leads to truncated protein due to the creation of a stop codon after 70 codons. The second one is an adenine duplication, c.3465dupA (p.Ser1156IlefsX10) in exon 14, which is not so far been reported in the aforementioned database and, like the previous one, create one stop site but only after 9 amino acids.

Deletions

A new deletion of 11 base pair c.5830_5840delATAATGGATAC was found in a severe HA patient. This caused a frameshift from codon 1944 in exon 18 with the consequent formation of a stop codon after 23 codons (p.Ile1944ThrfsX23). The other two deletions are described as large deletions detected by means of MLPA in five severe HA patients: g.EX1_EX22del [10] in FB19, FB20 (confirmed in their mother) and M23 patients. In FB19 and FB20 patients the array CGH revealed a 144 Kb deletion (arr[hg19] Xq28 (154,109,681-154,253,535)). The second deletion was g.EX10_EX11del [11] present in C1 and C2 patients.

Discussion

Haemophilia A is an X-linked disorder due to mutations in the FVIII gene, which is 186 kb long and consists of 26 exons. FVIII comprises a peptide leader of 19 amino acids and a mature protein of 2332 amino acids.

The present study aims to bring the spectrum of mutations that affect a heterogeneous population of patients with haemophilia A from various Italian haemophilia centers. We carried out the molecular analysis of the FVIII gene for all patients following a strategy of analysis depending on the clinical phenotype reported to us by the various haemophilia centers.

In our cohort of 52 HA patients we found 26 mutations including IVS22, missense mutations, nonsense mutations, large deletions, small insertions and small deletions. Of these 26 mutations 10 are novel mutations that have not been reported in the HAMSTeRS, HGMD and CHAMP database: five missense, two nonsense, two small insertions (one of which is duplication) and one small deletion (Table 1). The mutations not described were included in the online software Alamut® Visual to assess their pathogenicity (Figure 1).

Two of the novel mutations were found in patients (FB10-FB15) with sporadic severe haemophilia A.

In most cases the mutation found is able to explain the clinical phenotype of the patient, showing a good correlation between the genotype and HA phenotype. Our study presents cases (M4 and P1) that do not confirm this correlation.

The majority of frameshift mutations that create a premature stop codon have been reported in patients with severe hemophilia A. Despite this consideration in the literature are described such frameshift mutations, in exon 14 of FVIII gene, in moderate haemophilia A patients [12]. This is the case of patient M4, who suffers from mild haemophilia A, in which we found the mutation c.3465dupA, p.Ser1156fsX9 that creates a premature termination codon resulting in truncations in the B domain of the FVIII protein; thus the genotype and the clinical phenotype are discordant.

Patient P1 suffers from moderate haemophilia A with the presence of inhibitors; he showed c.5883G>A, p.W1961X mutation already reported in HAMSTeRS database but with different nucleotide transition (c.5882G>A) and only in severe HA patients and so the mutation found in patient P1 does not reflect his clinical phenotype.

The gross deletion g.EX1_EX22del was first found in FB19-FB20 brothers later the evidence of the anomalous INV22 band pattern. Subsequently MLPA assay was performed to assess the presence of an eventual deletion that has been confirmed by array CGH. Later, for patient M23 was used the same procedure for molecular diagnosis without making the array CGH.

In conclusion this study aims to validate the strategy of molecular analysis for the detection of mutations causative FVIII deficiency, confirming the great variability of this gene. We report 10 novel mutations spanning the entire FVIII gene that, in combination with all of that already given and those that will be identified, will benefit the genetic counseling and treatment of haemophilia A patients.

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Contributions of the authors:

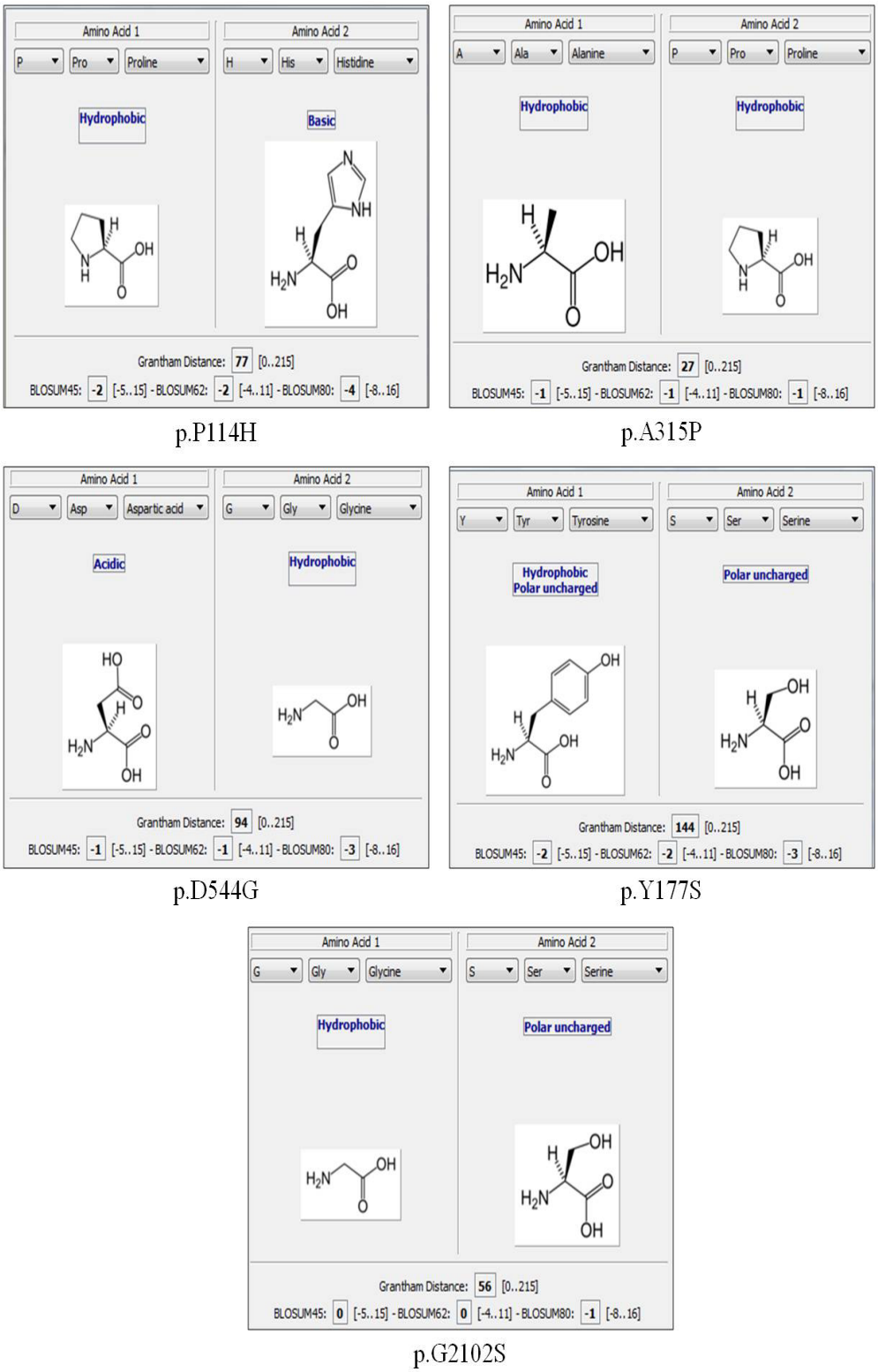

Figure 1: Results of Alamut software describing the functional changes of the five mutations not previously described.

• R. Santacroce, A. Leccese performed the research.

• M. Margaglione designed the research study.

• R.Trunzo, G. Lassandro, P. Giordano, C. Ettorre, S. Antoncecchi, I. Cantori, A. Dragani, R. Salviato, D. Belvini contributed essential reagents or tools.

• R. Santacroce, A. Leccese wrote the paper.

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