MOLECULAR ANALYSIS OF MILD HAEMOPHILIA PATIENTS:THE EXPERIENCE OF A SINGLE CENTRE

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INTRODUCTION

According to the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH), mild haemophilia A or B is defined as a reduction of clotting factor VIII (FVIII) or factor IX (FIX) levels to between >0.05 and 0.40 IU Ml^{-1}

Despite 50% of affected haemophilia patients have a mild phenotype, published data on these patients are nowadays insufficient and mutations are described occasionally. In our study we characterised mild haemophilia patients followed at Hub Haemophilia Centre of Parma: 75 patients (44 index cases) with haemophilia A and 7 (5 index cases) with haemophilia B.

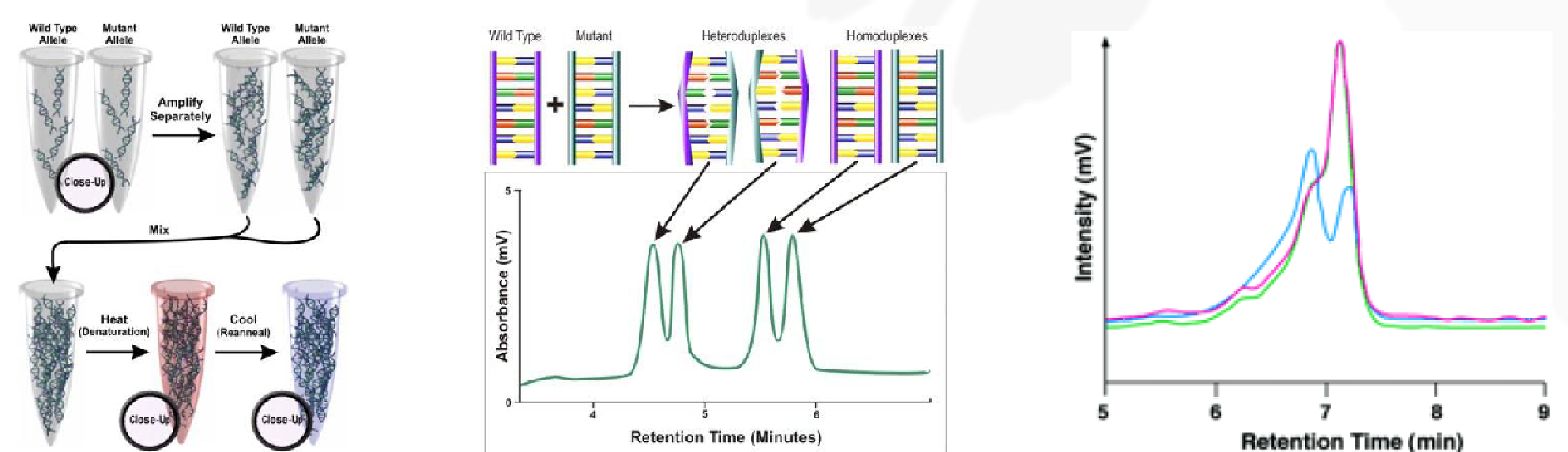
METHODS

We performed molecular analysis on the DNA extracted from EDTA peripheral blood samples; polymerase chain reaction (PCR) was performed on all 26 F8 exons including intron/exon boundaries as well as 5' and 3' regulatory gene regions. Amplicons analysis was performed with Denaturing High Performance Liquid Chromatography (DHPLC) and then with direct sequencing on samples with a DHPLC profile different from that of a normal control. One hundred wild-type chromosomes from healthy control subjects, without bleeding defects but from the same ethnic background, were investigated to exclude that a new variant could represent a common polymorphism. Exon 13 duplication was performed according to Acquila (Haematologica 2004). Mutations were classified as suggested by Oldenburg (Haemophilia 2002).

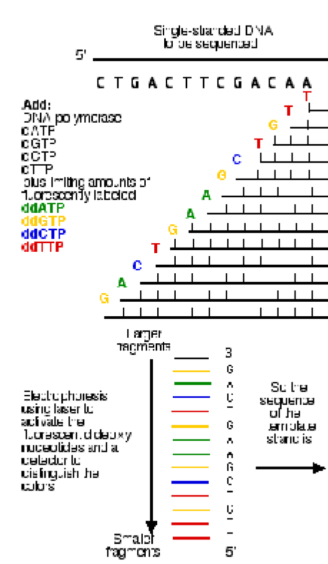
Nucleotidic Change	Exon No.	Predicted Effect (HGVS nomenclature)	Preditected Effect (Hamsters nomenclature)	Inhibitors (Yes/No)
c.409A>G	4	p.Thr137Ala	p.Thr118Ala	N
c.446C>G	4	p.Pro149Arg	p.Pro130Arg	N
c.545A>G	4	p.Asp182Ala	p.Asp163Ala	N
c.1587A>C	10	p.Gly498Arg	p.Gly479Arg	N
c.1587A>C	11	p.Lys529Asn	p.Lys510Asn	N
c.1648C>T	11	p.Arg550Cys	p.Arg531Cys	Y
c.1700T>C	11	p.Ile567Thr	p.Ile548Thr	N
c.1834C>T	12	p.Arg612Cys	p.Arg593Cys	Y
c.5123G>A	14	p.Arg1708His	p.Arg1689His	N
c.5270T>G	15	p.Phe1757Cys	p.Phe1738Cys	N
c.5405A>G	16	p.Tyr1802Cys	p.Tyr1783Cys	N
c.5957A>T	18	p.Lys1986Ile	p.Lys1967Ile	N
c.5976G>A	18	p.Met1992Ile	p.Met1973Ile	N
c.6506G>A	23	p.Arg2169His	p.Arg2150His	Y
c.6532C>T	23	p.Arg2178Cys	p.Arg2159Cys	N
c.6437T>C	23	p.Phe2146Ser	p.Phe2127ser	N
c.6557T>A	23	p.Met2186Lys	p.Met2167Lys	N
c.6752T>C	25	p.Val2251Ala	p.Val2232Ala	Y
c.7054T>C	26	p.X2352RextX*34	p.X2333RextX*34	N
c.1569G>T	11	p.Leu523Leu	p.Leu504Leu	N

Table 1.
missense mutations , nucleotidic change in the termination codon, suspected criptic splice site

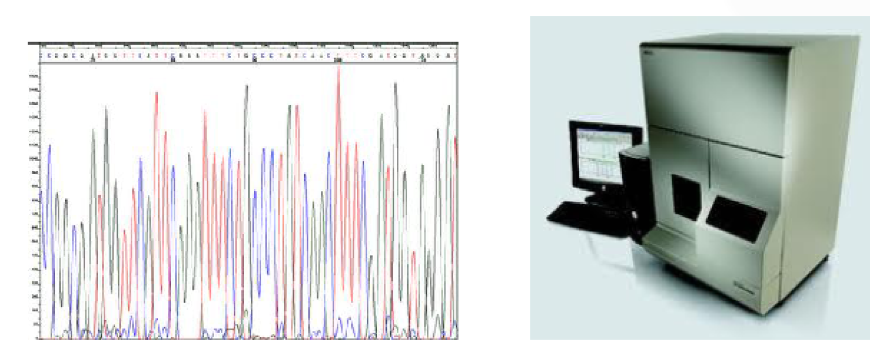
DHPLC principle



Denaturing High Performance Liquid Chromatography (DHPLC) is a chromatographic technique for the separation and analysis of DNA fragments with different length and/or base composition, allowing for mutation detection and SNP discovery.
The principle of fragment separation lies in the ability of the separation cartridge to bind double stranded DNA and release it as the helix of the molecule is unwound. The DNA is eluted from the column as an increasing concentration of acetonitrile flows across the matrix. Heteroduplexes formed in heterozygous samples will elute before homoduplexes, appearing ideally as two or more peaks in electropherograms. The mutant sample contains a double peak due to the presence of heteroduplexes that release from the column earlier than homoduplexes.



Direct sequencing



RESULTS AND CONCLUSIONS

Causative mutation was identified in 64 patients (35 index cases). The screening was terminated in 8 patients (6 index cases) but no mutation was detected. In 3 patients the analysis was not concluded yet. The detection rate was 85%. We found out 18 missense mutations, one nucleotidic change in the termination codon(table 1), a mutation in promoter region (Riccardi, JTH 2009) , exon 13 duplication and one suspected criptic splice site. Some of them are involved in inhibitor development (R550C, R612C, R2169H and V2251A). All 9 patients, with mutation in the promoter region show DDAVP resistance.

In conclusion, genetic screening is a useful tool for clinical overview of mild haemophilia patients.

Acquila 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 Jun;89(6):758-9
Riccardi F, Rivolta GF, Franchini M, Pattacini C, Neri TM, Tagliaferri A. Characterization of a novel mutation in the F8 promoter region associated with mild hemophilia A and resistance to DDAVP therapy.J Thromb Haemost. 2009 Jul;7(7):1234-5

WFH2012
126--Mo
Laboratory Sciences
federica riccardi
DOI: 10.3252/pso.eu.WFH2012.2012

Poster presented at:

PosterSessionOnline.com