Molecular Genetic Studies of Haemophilia A in Maltese Patients

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INTRODUCTION

Haemophilia A is an X-linked bleeding disorder characterised by lack of Factor VIII. Classified as: mild (5-50%), moderate (1-5%) and severe (<1%) Symptoms depend on severity but include: prolonged bleeding after surgery, bleeding in joints, spontaneous bleeding and intracranial haemorrhage. F8 is one of the largest genes, located in the most distal band (Xq28) of the long arm of the X chromosome. Contains 26 exons. Exons 14 and 26 contain 3106 and 1958 bp, respectively.

There are currently over 1300 unique variants listed in the Haemophilia A database. Exon 14, having 10 times as much coding sequence as the other exons, carries a higher risk of mutations. 97% of nonsense mutations result in severe disease. Also, carries a much higher risk of inhibitor development compared with missense mutations. Of the known stop mutations associated with the generation of anti-FVIII antibodies, none are found in exons 1-7.

METHODS

18 individuals took part in this study. Following Ethics approval, blood was collected from all these individuals and have their DNA extracted. The patients were classified as mild, moderate or severe by making use of their clinical history. Inversion testing to detect intron 22 inversion was performed in severe patients by Long-distance PCR. A method adopted from Liu Qiang et al (1998) was used. Primers P, Q, A and B were used to differentiate wild-type, inversion and carrier (fig 1)

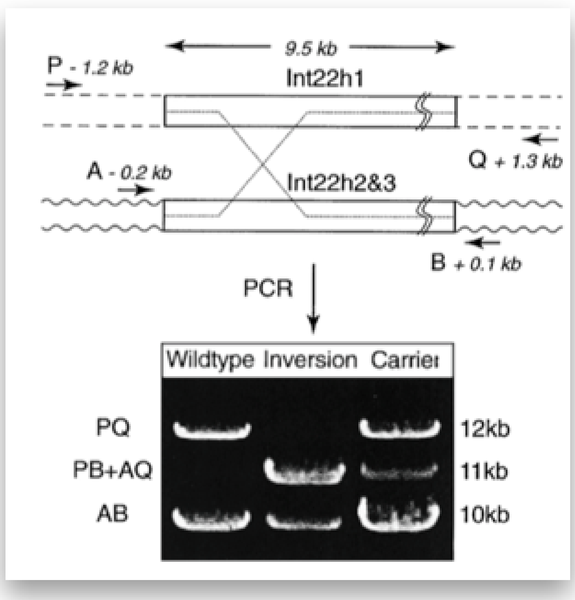

Fig 1: Schematic of the PCR assay. The four primers (P, Q, A, and B) are represented by arrows and their positions are indicated. The upper box represents *int22h1,* and the dashed lines indicate flanking sequences. The lower box represents *int22h2* and*int22h3,* and the wavy lines indicate the flanking sequences. Deleterious inversions can occur by recombination between*int22h1* and either *int22h2* or *int22h3* (dotted lines). Amplified products in male patients with the wild-type and the inverted factor VIII genes and a carrier female. PCR was performed in 25 µL with 250 ng of genomic DNA, a mixture containing 50 mmol/L Tris.HCl, pH 9.2, 2.25 mmol/L $MgCl_2$, 7.5% DMSO, 16 mmol/L $(NH_4)_2SO_4$, 250 mmol/L each of dGTP and deaza-dGTP, 500 mmol/L of the other dNTPs, and 3.3 U of Expand Long Template DNA polymerases (Boehringer Mannheim, Mannheim, Germany).

DNA sequencing was performed for accurate genotyping via amplification for all 26 exons. Multiplex Ligation-dependent Probe Amplification (MLPA) was carried out for deletion testing. Intron 1 inversion testing was carried out if patients with severe disease were negative for intron 22 inversion or any other mutation. Whole Exome Sequencing was performed on all samples apart from inversion positive samples, using Illumina TruSight One Sequencing Panel Kit.

RESULTS

4 subjects were Inversion 22 positive (1 female carrier and 3 male hemizygotes) A further 9 patients had 5 different DNA mutations that were classified as follow; 2 missense mutations (c.5399G>A p.Arg1800His [fig 2] and c.3780C>G p.Asp1260Glu), a frameshift duplication mutation (c.4825dupA [p.Thr1609Asnfs*4]), a novel indel mutation (c.5815+3_5815+5delinsTTGG [fig 3]) and a novel nonsense mutation (c.6181C>T p.Gln2061Ter [fig 4])

RESULTS and FIGURES

Multiplex ligation-dependent Probe Amplification (MLPA) was performed on 6 patients where 2 large deletions were found. In two of the patients, a large deletion involving exon 5 and 6 was found and spans approximately 520 bp (fig 5). The other large deletion was found in one patient, this time affecting exon 26 (fig 6). It is one of the largest exons and the deletion spans approximately 1,963bp.

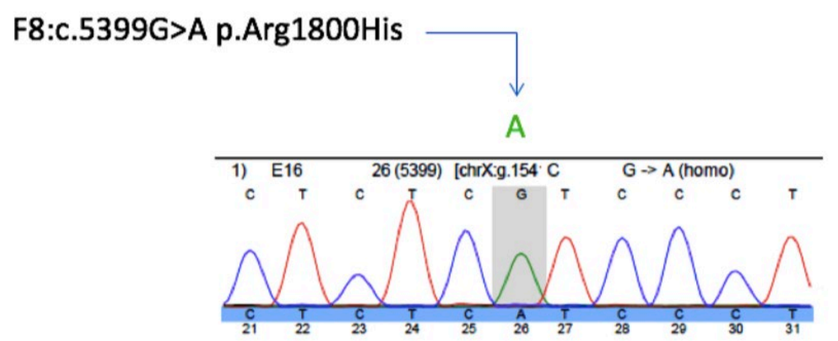

Fig 2: DNA Sequence chromatogram for patient carrying missense mutation in amino acid position 1800 of the F8 gene

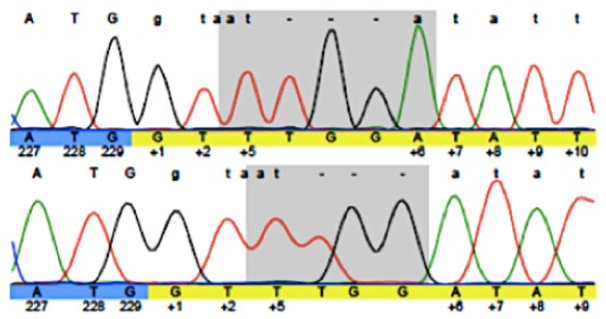

Fig 3: DNA Sequence chromatogram for patient carrying a novel indel mutation in the F8 gene affecting a splice site

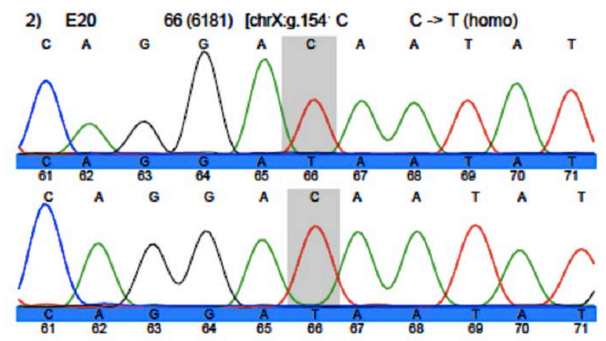

Fig 4: DNA Sequence chromatogram for patient carrying a novel nonsense mutation in the F8 gene that leads to a truncated protein

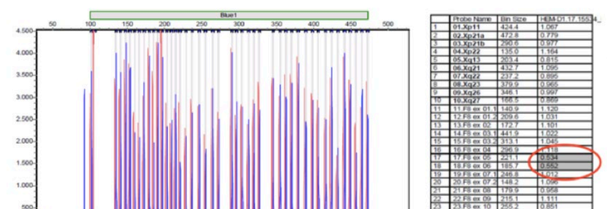

Fig 5: MLPA output showing the deletion of probes in exons 5 & 6

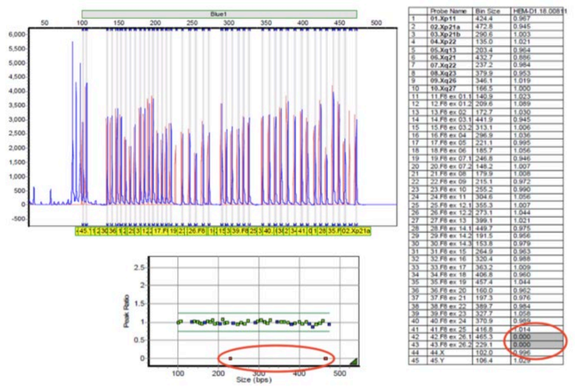

Fig 6: MLPA output showing a large deletion of probes in exon 26

CONCLUSION

Detection of F8 gene mutations is critical for genetic counselling and disease prevention in affected families. The detection of the F8 gene mutations among these patients enabled the very first molecular testing for affected subjects in Malta. This study will directly lead to further carrier testing using direct mutation analysis in potential female carrying variants in the F8 gene of the disease, including prenatal diagnoses when necessary.