Quantitative and qualitative mRNA analysis in a severe Haemophilia A patient with high *F8* expression and no active FVIII protein

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Introduction

Hemophilia A (HA) is a common, X-linked, recessive disorder caused by deficiency of coagulant factor VIII *(*FVIII). The gene encoding this factor is large and complex [186 kb and 26 exons] and is situated at about 1 Mb from the telomere at the Xq28[1]. Various types of mutations in *F8* are responsible for the bleeding disorder. Despite tremendous improvements in mutation screening methods, in about 2% of HA patients no DNA change could be found[2]. Therefore, mutations or rearrangements in non-coding areas of *F8* introns, 3´- and 5´-untranslated regions (UTR) should be considered to be responsible for the HA phenotype in such patients.

Material and Methods

In this study we investigated the molecular mechanisms causing a severe HA phenotype (FVIII:C <1%) in a patient where no mutation was found in the coding sequence (cDNA) of *F8*. For mutation screening direct sequencing of all exonic sequences and exon/intron boundaries was done. Furthermore Multiple Ligation-dependent Probe Amplification (MLPA) was performed to detect copy number variations. These results were verified using a comparative genome hybridization (CGH) array. FISH analysis was performed to investigate possible translocation of *F8*. For RNA analysis, blood was collected in PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and total RNA was extracted using the PAXgene Blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure. Total RNA was used for semi-quantitative RT-PCR and Taqman based quantitative real time PCR to analyze splicing pattern and *F8* mRNA expression levels, respectively.

Results

The index patient was negative for intron 1 and 22 inversions. Complete cDNA sequencing revealed no deleterious mutations. The vWF 2N mutations were excluded as well. In contrast, MLPA assay for *F8* identified an increased copy number of exon 1–25 (duplication of exon 1-4 and 23-25 and triplication of exon 5-22) in the patient compared to control DNA (Fig. 1). Exon 26 of *F8* is not affected in this patient. Both carrier daughters show the same mutation (data not shown). CGH analysis confirmed these results and showed that the span of the duplication/triplication (Dup/Trp) is carried over the intronic regions of *F8* (Fig. 2A). Analysis was followed by FISH analysis to exclude intrachromosomal translocation. Our data shows normal hybridization site of the probe at Xq28 (Fig. 3). In addition, quantitative mRNA analysis revealed an unexpected high *F8* mRNA expression level in comparison to healthy individuals (Fig. 4). We hypothesize that the Dup/Trp is partially or completely in tandem inserted in the body of *F8* gene and it is not disturbing the transcription of *F8* but the translation or the transport of a misfolded non-functional protein to blood. Based on our linkage analysis results (data not shown) and due to lack of heterozygoticity in the SNPs located within the Dup/Trp regions we can conclude that genomic gains originate from intra-chromosomal rearrangement.

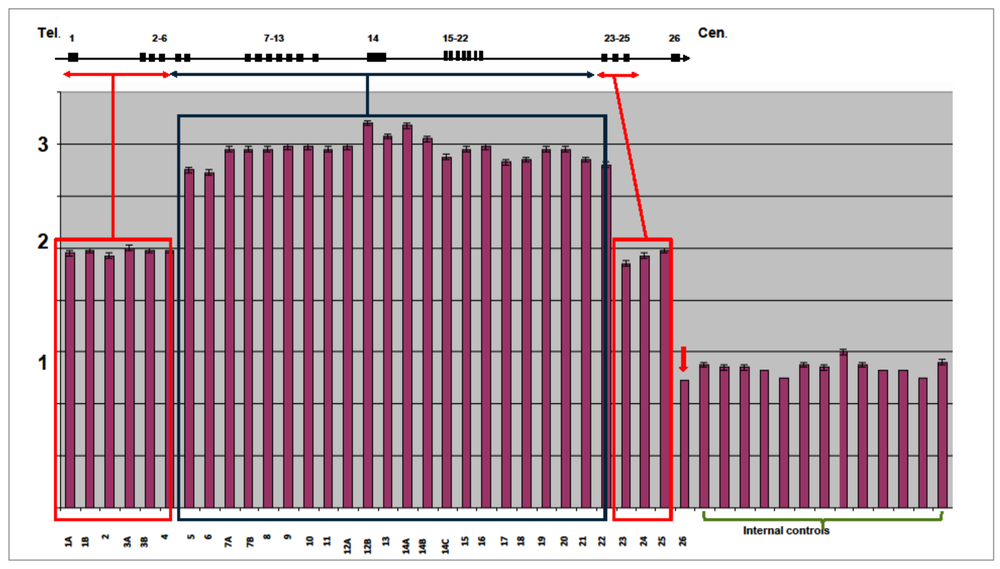

Figure 1. MLPA analysis of *F8*. Results show a duplication pattern of exons 1-4 & 23-25 and triplication of exons 5-22 in patient. The red arrow indicates exon 26 where no increased probe signal was observed.

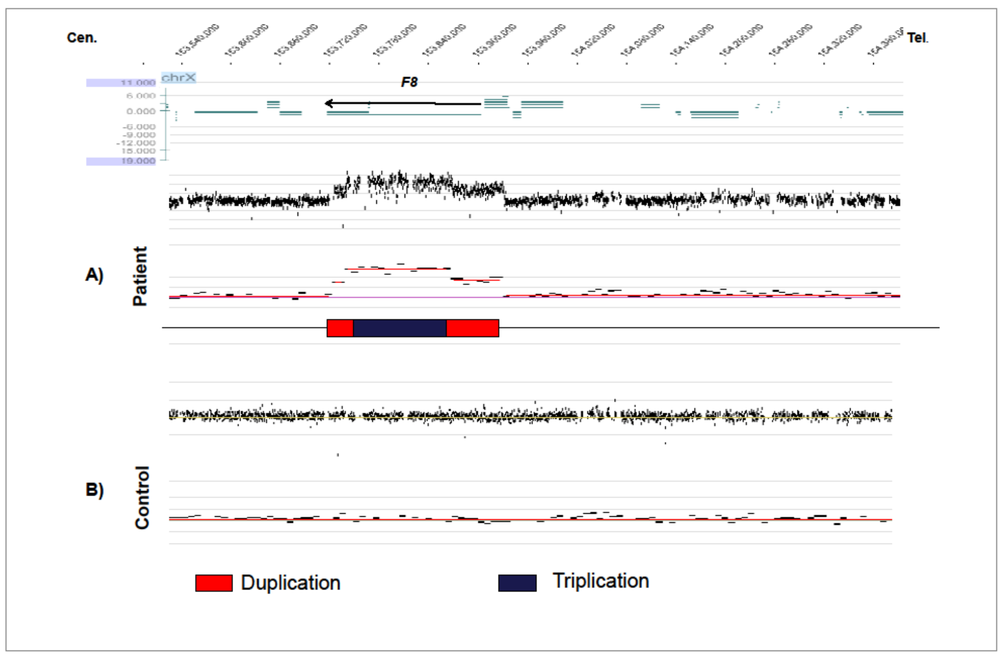

Figure 2. Genomic region harboring alterations at Xq28. The upper panel shows the position of *F8* on X chromosome (hg:18, NCBI build 36). The arrow indicates the direction of *F8* transcription. Results from CGH array show a large duplication and triplication of genomic region of *F8* in patient (A) comparing to B) pool of male controls. The Dup/Trp spans the complete genomic region of *F8* from exon 1-25 including the intronic regions (Cen: Centromere, Tel: Telomere).

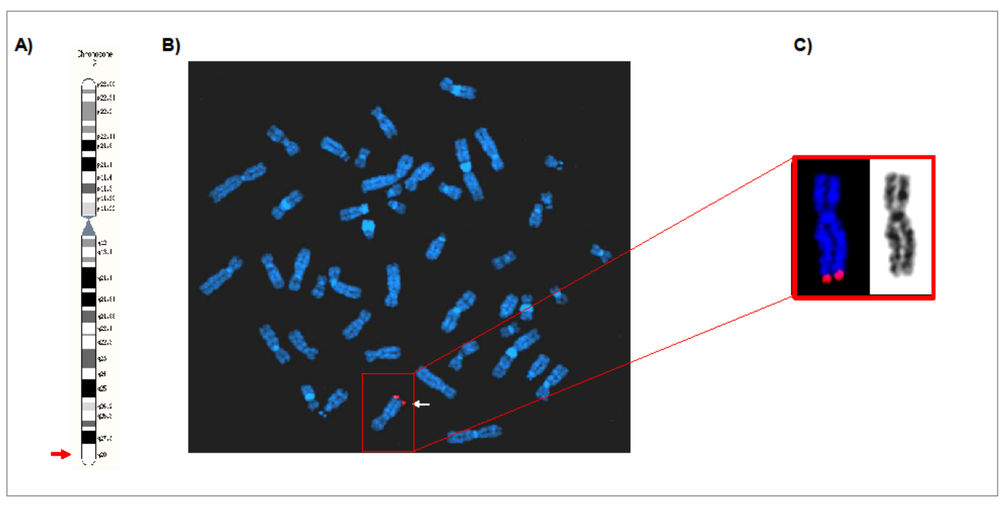

Figure 3. FISH using a *F8* Probe. A) Location of *F8* at Xq28. The red arrow indicates the site of hybridization. B) Normal location of the probe. At least 20 metaphases were checked. No additional hybridization sites were detected. C) X-chromosome cut-outs of the patient with and without signal.

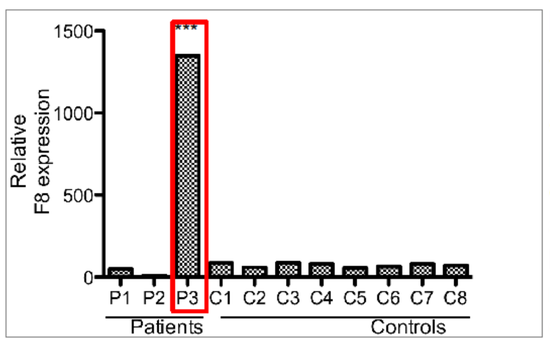

Figure 4. Quantitative mRNA analysis of *F8* expression in peripheral blood. An increased expression was observed in the index patient (P3) compared to other hemophilic patients (P1-P2) and healthy individuals (C1-C8). Results are presented as a ratio relative to expression of the internal control *PBGD* (***P < 0.001).

Conclusion

Our analysis revealed a so far not described duplication/triplication pattern comprising the whole genomic region of *F8* locus leading to a severe HA phenotype, where despite high *F8* mRNA expression levels, FVIII protein is lacking. Our results suggest that patients where no mutations are found in the cDNA should be considered for intensive analysis of the genomic *F8* locus.

References

1) Oldenburg J and El-Maarri O. New insight into the molecular basis of hemophilia A. Int J Hematol. 2006 Feb;83(2):96-10
2) El-Maarri O. et al. Analysis of mRNA in hemophilia A patients with undetectable mutations reveals normal splicing in the factor VIII gene. J Thromb Haemost. 2005 Feb;3(2):332-9

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