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Risk of erroneous results in carrier testing for haemophilia A without prior DNA analysis in male index patients

D. E. FRANSEN VAN DE PUTTE,* W. S. FRANKHUIZEN,* L. VIJFHUIZEN,* L. GROENEWEGEN,* R. Y. J. TAMMINGA,† K. BOUMAN,‡ A. J. VAN ESSEN,‡ A. C. J. GIJSBERS,* C. A. L. RUIVENKAMP* and E. M. J. BOON*

Department of Clinical Genetics, Leiden University Medical Center, Leiden; †University Medical Center Groningen, Beatrix Children's Hospital; and ‡Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Haemophilia A is a genetic bleeding disorder showing X-linked inheritance. Mutations in the *F8* gene on the X-chromosome result in inadequate production of clotting factor VIII (FVIII). Males carrying such a mutation will have clinical features of haemophilia A, varying in severity according to the specific mutation present [1,2]. Female mutation carriers have a normal *F8* gene on their other X-chromosome, and are therefore usually unaffected. Due to skewed X-inactivation, however, some female carriers have decreased FVIII levels and clinical features of mild haemophilia [3]. Moreover, in each pregnancy, female carriers of haemophilia have a 25% chance of having an affected son. For their own health, to enable them to make reproductive choices, and for optimal management during delivery, it is important to adequately identify haemophilia carriers. Usually, and ideally, female carriers are identified by DNA analysis after the familial mutation has been detected in a male index patient. In clinical practice, however, DNA of potential carriers is sometimes sent for analysis without prior identification of the familial mutation. In some of these situations, performing standard DNA analysis techniques (sequencing of the *F8* gene, inversion PCRs to detect frequent inversions of introns 1 and 22, and multiplex ligation-dependent probe amplification (MLPA) analysis to detect deletions/duplications if no abnormalities are found in the first three tests [4,5]) in a potential carrier will wrongly label her as a non-carrier or will misjudge the severity and potential impact of a *F8* abnormality. We describe three clinical cases to illustrate the importance of DNA analysis of the *F8* gene in male index patients with haemophilia A.

Case 1 and 2 are both young boys, born in 2010 and 2009 respectively. In case 1, a bleeding disorder was suspected at the age of 9 months after prolonged bleeding at neonatal heel-prick blood screening, haematoma development after vaccination and excessive bruising. His FVIII level is <1%. His mother is clinically a carrier of haemophilia, her FVIII level is 44%. Case 2 was diagnosed with severe haemophilia (FVIII <1%) at the age of 16 months after excessive bruising. A brother of his maternal grandmother was known to have haemophilia, but no DNA analysis had been performed. In both cases, we detected large insertions in exon 14 on routine sequencing of the *F8* gene: one insertion of at least 280 base pairs after position c.4485 leading to a premature stop (p.Asn1496fs) and one of 288 base pairs followed by an A-stretch after position c.2640, also leading to a premature stop (p.Lys811fs). However, when both mothers of the index patients were tested, no insertion could be detected (Fig. 1a). To assess the possibility of preferential amplification of the wild-type allele, specific PCR primers inside and outside the insertion were designed. Using these primers, heterozygosity for the familial insertions could be determined and carriership confirmed (Fig. 1b). This proved that in the obligate female carriers both insertions led to preferential amplification of the wild-type allele, thus explaining the sequence analysis results. The family-specific PCRs enabled carrier testing in other female relatives.

Case 3 is a man, born in 1946, with mild haemophilia (FVIII 16%). He has a history of easy bruising, epistaxis and excessive bleeding after tooth extractions and surgery. His sister has no bleeding problems, but three of her six sons are reported to have increased bleeding tendencies.

At the time of testing, the medical history of this patient was unknown and thus routine analysis of the *F8* gene was performed in parallel. An apparently hemizygous pathogenic missense mutation was detected in exon 12 of the *F8* gene (c.1834C>T, p.Arg612Cys), consistent with the diagnosis of mild haemophilia A (Fig. 2a). The inversion PCR for the common inversion in intron 22 [6], however, presented a pattern consistent with heterozygosity for this inversion (Fig. 2b). Because this was unexpected in a male patient, MLPA analysis was then performed to search for duplications in the *F8* gene [7]. It showed a

Correspondence: Elles M. J. Boon, PhD, Department of Clinical Genetics, Leiden University Medical Center, LDGA – building 2, Post zone S-06-P, PO Box 9600, 2300 RC Leiden, The Netherlands.
Tel.: +31 71 5269810; fax: +31 71 5268276;
e-mail: e.m.j.boon@lumc.nl

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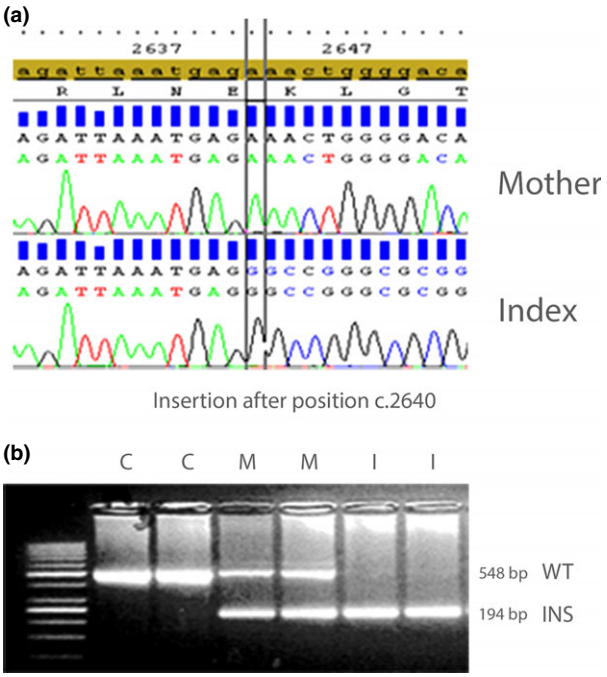

Fig. 1. Detection of a large insertion in exon 14 of the *F8* gene. (a) Example shown for case 2. Sequencing analysis of the *F8* gene showing an insertion after position c.2640 in the index patient and the wild-type sequence in the results for his mother. (b) A PCR designed to specifically detect the familial insertion for case 2, showing the insertion (194 bp band) for both the index patient and his mother. C, (negative) control sample; M, mother; I, index; WT, wild-type; INS, insertion.

duplication of exons 1–22 (Fig. 2c). Combining the sequencing results with the inversion PCR and the MLPA, we deduced that the patient has a homozygous missense mutation in exon 12 and an inverted duplication of exons 1–22. SNP-array analysis (Affymetrix GeneChip Human Mapping 250K Sty Array) showed an interstitial duplication on chromosome Xq28 of at least 827.1 kb (11 SNP probes), containing the *F8* gene and 14 other genes. These other genes do not appear to be associated with haemophilia or any other known phenotype. Fluorescence *in situ* hybridization (FISH) analysis with a specific DNA-probe for the duplicated Xq28 locus showed only one signal on the long arm of the X-chromosome, indicating that the duplication is intra-chromosomal and is not present elsewhere in the genome. Since the missense mutation was detected homozygously at sequence analysis, we assume that the duplication occurred after the mutation. Moreover, since the duplication is inverted, we hypothesize that this duplication is most likely not expressed and will thus have no clinical consequences. This is in line with findings by Lannoy *et al.* [8], who described specific duplications in *F8* involving intron 22 homologous copies. Moreover, it is consistent with the patient's mild haemophilia phenotype, which is probably solely caused by the missense mutation. In affected female relatives, however, the inversion PCR, which is part of the standard DNA analysis of the *F8* gene, will show a pattern consistent with heterozygosity for the inversion in intron 22. Since MLPA is not standard in routine diagnostics, the inversion PCR could be misinterpreted as the common intron 22 inversion associated with severe haemophilia, leading to a wrong conclusion about the severity of the disease within this family. Therefore, as suggested previously by the UK Haemophilia Centre Doctors' organisation, a positive result for an inversion in intron 22 in a female should always be confirmed by additional quantitative analysis, such as MLPA, to avoid false-positive results [9]. Alternatively, Rossetti and colleagues have described an inverse shifting PCR to characterize large rearrangements, which could be used for diagnostic testing [10].

The cases described in this report illustrate that not identifying the familial mutation in a male index patient can lead to erroneous conclusions when standard DNA

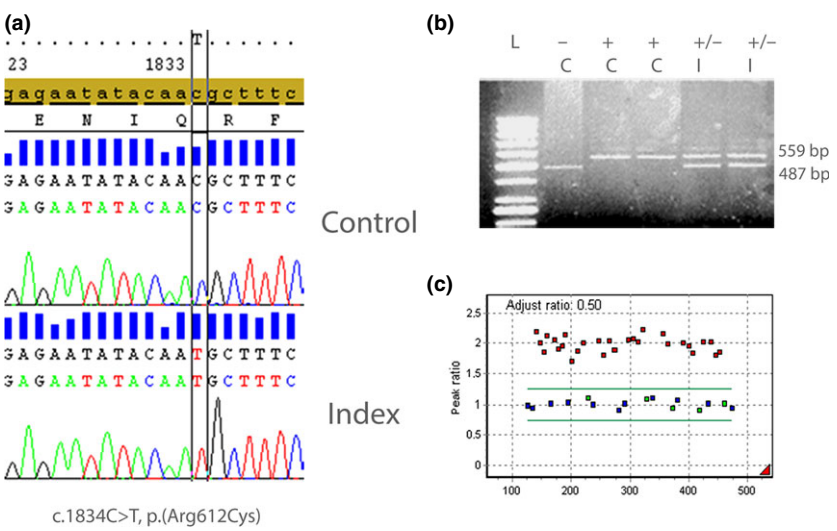

Fig. 2. Detection of a pathogenic missense mutation in the *F8* gene and an inverted duplication. (a) Sequencing analysis of the index patient in case 3 showing an apparently hemizygous pathogenic missense mutation in exon 12 of the *F8* gene (c.1834C>T, p.Arg612Cys). (b) The intron 22 inversion PCR for the index patient shows two bands consistent with heterozygosity for this inversion. C, control sample; I, index; –, wild-type band (487 bp); +, inversion band (559 bp); +/–, wild-type and inversion band. (c) Multiplex ligation-dependent probe amplification analysis showing a duplication of exons 1–22. Duplicated exons are indicated in red.

analysis is performed on potential female carriers of haemophilia A. Since most *F8* mutations will be easily detectable by routine laboratory techniques, this will only be true in a small proportion of cases (in our diagnostic laboratory, the patients described here were the only 'possibly problematic' cases detected in around 550 analyses, indicating an incidence of <1%). However, the impact of a wrong conclusion can be large. When a female relative is labelled a non-carrier, as could happen in cases 1 and 2, her FVIII levels will not be determined and mild haemophilia might remain undetected. This could have major clinical consequences in the event of severe trauma or surgery. Moreover, she will be unaware of her risk of having a son with haemophilia and will thus be denied the opportunity to make relevant reproductive choices and receive optimal obstetric care. In case 3, detection of an intron 22 inversion on routine DNA testing could have led to the conclusion that a female relative is a carrier of severe haemophilia, which could have a larger clinical impact and might have led to different reproductive choices than knowing that she is, in fact, a carrier of mild haemophilia. In this case, the clinical data of the affected male relative are of vital importance in determining the need for additional DNA testing and drawing the correct conclusion. It is very important to have extensive and up-to-date pedigree data in haemophilia treatment centres and/or DNA laboratories to enable identification of living male index patients, and to provide clinical information when requesting DNA analysis.

In some cases, DNA analysis in male index patients is not possible, for example because they are deceased. In those situations, DNA analysis in an obligate carrier, for example a daughter of an index patient, could be done to determine the familial mutation before other female relatives are tested. If this is not possible either, any normal DNA results in a potential female carrier should be interpreted with care. There will always remain a small chance that she is indeed a carrier of a mutation not detectable by routine diagnostic techniques. Her FVIII levels should be determined at least once to make sure she does not have an increased bleeding tendency, and the possibility of having a son with haemophilia despite her normal DNA test result should be discussed.

Based on the cases described here, we strongly recommend that DNA analysis of the *F8* gene in a male index patient with haemophilia A is always performed to determine the familial mutation, before DNA analysis in a potential female carrier is attempted. When no male index is tested, a positive result for the inversion PCR for intron 22 in females should be confirmed by further analysis, when not using a PCR test discriminating between different rearrangements. A family-specific PCR approach can be helpful for efficient carrier analysis of large insertions in the *F8* gene.

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Author contributions

D. E. Fransen van de Putte collected data and wrote the manuscript; E. M. J. Boon supervised and interpreted laboratory analyses, issued diagnostic reports for the patients and wrote the manuscript; R. Y. J. Tamminga, K. Bouman and A. J. van Essen provided clinical data; W. S. Frankhuizen, L. Vijfhuizen and L. Groenewegen performed laboratory analyses; A. Gijsbers and C. A. L. Ruivenkamp supervised and interpreted FISH and array analyses. All authors critically reviewed and approved the final version of the manuscript.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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