Haemophilia
The Official Journal of the World Federation of Hemophilia, European Association for Haemophilia and Allied Disorders and the Hemostasis & Thrombosis Research Society

Haemophilia (2015), **21**, 392–397 DOI: 10.1111/hae.12606

ORIGINAL ARTICLE *Genetics*

Novel characterization of a breakpoint in *F8*: an individualized approach to gene analysis when PCR and MLPA results contradict

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Summary. Haemophilia A is an X-linked bleeding disorder caused by heterogeneous mutations in the *F8* gene. Two inversion hotspots in intron 22 and intron 1, as well as point mutations, small insertions and deletions in the *F8* gene account for causal mutations leading to severe haemophilia A. Rarely, novel molecular mechanisms lead to a haemophilia A phenotype which cannot be completely characterized by routine molecular diagnostic methods. Here, we characterized the molecular abnormality in a boy with a severe haemophilia A phenotype. On investigation by PCR and DNA sequencing, exon 18 of *F8* repeatedly failed to amplify. However, analysis by multiplex ligation-dependent probe amplification demonstrated the presence of exon 18 sequence, suggesting a more complex rearrangement than a single exon deletion. The analysis of exon 18 and its flanking regions by inverse PCR revealed a complex mutation comprising insertions of extragenic sequences from Xq28 along with a partial duplication of exon 18. Based on the successful analysis and characterization of the familial breakpoint, we developed a PCR-based diagnostic approach to detect this defect in family members in whom no diagnostic test could be offered until this time.

Keywords: breakpoint, gene rearrangement, haemophilia A, inverse PCR, Multiplex Ligation-Dependent Probe Amplification

Introduction

To date, a wide range of mutations, scattered throughout the coding and non-coding sequence of *F8*, have been reported that lead to a qualitative or quantitative defect of the FVIII protein. The most common *F8* mutations leading to a severe phenotype are inversions [1–3]. Other mutations leading to a severe phenotype are mainly point mutations (missense, nonsense and splice site mutations) and, less frequently, deletions, insertions and duplications [4,5]. Most of these mutations are identified using routine PCR-based analysis methods [6] and multiplex ligation-dependent probe amplification (MLPA) [7,8]. Rarely, novel molecular mechanisms lead to a haemophilia A phenotype which cannot be completely characterized by routine molecular diagnostic methods and require an individualized research approach to investigate fully.

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Accepted after revision 14 November 2014

Here, we present the molecular mechanism leading to severe haemophilia A in a patient resulting from a complex rearrangement within the genomic region of *F8*. Upon investigation by PCR and MLPA, apparently contradictory results for exon 18 of *F8* were obtained. Although the amplification of *F8* repeatedly failed, the analysis by MLPA demonstrated the presence of exon 18 sequence, suggesting a more complex mutational event than just a single exon deletion. The subsequent inverse PCR analysis of the non-amplifiable exon and its flanking regions revealed a complex mutation comprising insertions of sequences of large non-coding regions at Xq28 into the *F8* gene along with a small duplication of 99 bp of exon 18.

The breakpoint in the *F8* gene was fully characterized by this investigation, and a diagnostic PCR was established to analyse the carrier status of family members. In addition, we present a straightforward strategy to investigate inconsistent results of PCR and MLPA that facilitates characterization of the

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breakpoint junctions to establish a short range PCR for diagnostic purposes.

Materials and methods

Patient profile

The index patient is a 10-year-old boy with severe haemophilia A and no family history of haemophilia. Genomic DNA from the patient and potential carriers of haemophilia in the family were extracted from peripheral blood leucocytes using a standard salting-out precipitation procedure. The family gave written informed consent in accordance with the Declaration of Helsinki.

Mutation analysis

For the molecular diagnosis of the index patient, intron 1 and intron 22 inversions were initially excluded. All 26 exons and adjacent intronic regions of *F8* were then amplified using standard PCR conditions and MLPA was carried out using the *F8* MLPA kit P178 (MRC Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. Exon dosage was calculated using Coffalyser.Net software (MRC Holland). The PCR using the partial sequences of MLPA probe for verification of the MLPA results was carried out using the primers listed in Table S1, A. Long range (LR)-PCR was performed to amplify the region flanking exons 15–22 using the primers listed in Table S1, B as described earlier [9].

Inverse PCR amplification and cloning of the PCR products

To characterize the breakpoint junctions, an inverse PCR approach was designed and carried out as previously described [9] by digesting 1 μg of genomic DNA with *Hind*III (New England Biolabs, Frankfurt, Germany). Primers used for amplification are listed in Table S1; C. Sequences were assembled and analysed using Geneious Pro (Biomatters Ltd, Auckland, New Zealand). Generated contigs were annotated against assembly Build: hg19 NCBI.

Junction-specific diagnostic PCR

To establish the carrier status of family members, a simple diagnostic PCR was designed using a primer set amplifying across the junctions of the breakpoint (Table S1, D). PCR was performed using the following conditions: 15-min initial denaturation at 92°C, 35 cycles at 92°C for 30 s, 62°C for 30 s and 72°C for 1 min using HOT FIREPol® DNA Polymerase (Solis BioDyne, Tartu, Estonia).

Results

Identification of a breakpoint in exon 18 of the F8 gene

The patient was initially investigated and found to be negative for both the intron 1 and intron 22 *F8* gene inversions (data not shown). Subsequent amplification

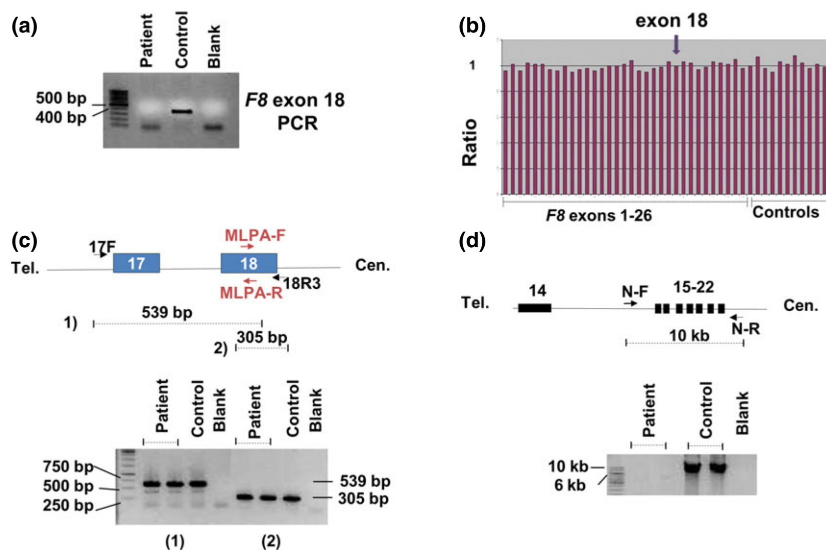

Fig. 1. Identification of a breakpoint in exon 18 of the *F8* gene. (a) PCR gel of amplification of exon 18. The amplification failed for the patient using primers located in intronic regions flanking exon 18 of *F8*. (b) Multiplex ligation-dependent probe amplification (MLPA) result showing normal copy number of all exons of the *F8* gene. The arrow indicates the presence of exon 18. (c) Upper panel: schematic presentation of the amplified region flanking exon 18 using the MLPA probe as primers (red arrows: MLPA-F/R). Lower panel: PCR gel of the successful amplification of exon 18 in two fragments. (d) Upper panel: schematic presentation of the amplified region showing the relative position of LR-PCR primers. Lower panel: PCR gel of LR-PCR across exon 18 using primers in intron 14 and intron 22 of *F8*. LR-PCR reveals no amplification for patient confirming the presence of a breakpoint (Cen., centromere; Tel., telomere).

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and complete sequencing of all other *F8* exons revealed no mutations but amplification of exon 18 of *F8* repeatedly failed (Fig. 1a), predicting the deletion of exon. However, subsequent MLPA analysis, carried out for verification of the deletion, showed normal copy number for all exons of *F8* including exon 18 (Fig. 1b). Based on this result, a more complex rearrangement than a single exon deletion was hypothesized.

To further investigate the causal mutation, we used the sequence information at the site of ligation of the probe (supplied in the MLPA kit). These sequences located within exon 18 were used to design primers for amplification of exon 18 in two fragments. Interestingly, this PCR approach resulted in successful amplification of exon 18 (Fig. 1c) and subsequent sequencing of the PCR fragments showed no changes in exon 18, suggesting that a breakpoint within exon 18 resulted from a large insertion or inversion. The next step for verification of the breakpoint involved LR-PCR using primers of a 10.611 bp region located between intron 14 and intron 22 (chrX: 154, 134, 911–154, 124, 301; hg19). The inability to amplify this region flanking exon 18 of *F8* in the patient confirmed the presence of a genomic breakpoint in this area (Fig. 1d).

Characterization of the breakpoint junctions reveals insertion of extragenic sequences originating from Xq28

To characterize the breakpoint junctions, an inverse PCR approach was designed to amplify the regions upstream and downstream of exon 18 of the *F8* gene (Fig. 2a). The genomic DNA of the index patient and a healthy male control was digested using the *Hind*III enzyme. Subsequently, the purified ligated products were amplified using primers located upstream and downstream of the breakpoint, and the PCR fragments were sequenced for both patient and the healthy control. As expected for the control DNA, both primer pairs are embedded in the same restriction domain and therefore they should give the same size products. In contrast for the patient, due to the rearrangement, additional restriction site(s), lying asymmetrically from the original two restriction sites, become available. Amplification of these new ligated products leads to different altered size of the products compared to that of the wild type.

The upstream inverse PCR revealed a smaller PCR product than the control (1771 bp compared to the 2725 bp, Fig. 2b, upper panel). Sequencing of the patient's amplicon revealed a break within exon 18 of

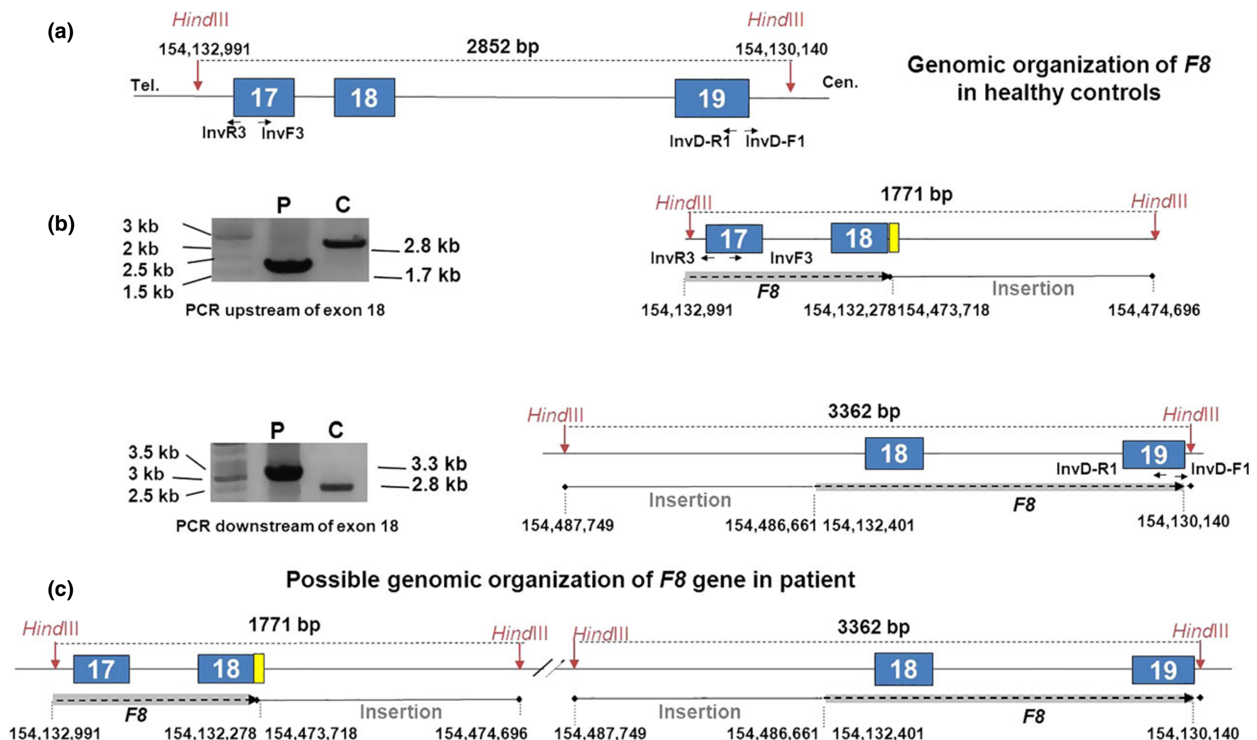

Fig. 2. Inverse PCR approach for characterization of the breakpoint. (a) Relative positions of the primers in the upstream and downstream inverse PCRs together with the surrounding *Hind*III cutting sites. PCR gels of the upstream and downstream inverse PCRs are shown in (b) upper and lower panel respectively; additionally, the maps of breakpoints are shown in the lower panels. (c) Possible genomic organization of *F8* gene after insertion within exon 18. All genomic positions are according to hg19 (Yellow Box: L1 repeat insertion; Cen., centromere; Tel., telomere).

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the *F8* gene (chrX: 154, 132, 278, c.5933; hg19) joined with a short repetitive element of the L1 family of repeats (chrX: 154, 474, 460–154, 474, 686; hg19) followed by a non-coding sequence of Xq28 located distal to *F8* genomic region (chrX: 154, 473, 718–154, 474, 696; hg19) (Fig. 2b, upper panel; Fig. S1a). On the other hand, the downstream inverse PCR revealed a fragment of 3362 bp, which is larger in size compared to the product obtained from a healthy control (2819 bp) (Fig. 2b, lower panel). Sequencing of the aberrant PCR product revealed an insertion of non-coding sequences located at Xq28 region (chrX: 154, 487, 749–154, 486, 661; hg19) in intron 17 of *F8* at c.5998-38 (Fig. 2b; lower panel, Fig. S1b).

The sequence information obtained from the inverse PCR clarified both the reason for the failure of the amplification of exon 18 as well as the reason for the positive MLPA. Firstly, the routine primers (located in intron 17 and intron 18 of *F8*) cannot amplify due to the insertions within exon 18 of *F8*. Secondly, the results of sequencing the downstream PCR demonstrate the presence of an intact exon 18, explaining the positive MLPA (Fig. 2c, Fig. S1c), and as the duplication consists of only part of the probe hybridising sequence of exon 18, the MLPA probe is unable to detect this duplication (breakpoint is at c. 5933 and MPLA probe starts at c.5897 and ends at c.5964) and will give a normal result. These findings confirm disruption of the *F8* gene within intron 17 and are consistent with the severe haemophilia A phenotype. Moreover, the two breakpoint junctions within *F8* were characterized, but the exact arrangement of the inserted regions on the X chromosome and the molecular mechanisms leading to this mutational event remain elusive.

Establishing a multiplex PCR for detection of the rearrangement

Using the sequence information revealed after characterization of the breakpoints, a simple multiplex short PCR was designed to provide a diagnostic test for detection of the breakpoint in other family members (Fig. 3a). The amplification was done using a forward primer in intron 17 of *F8* (MPX-F8-F) with two reverse primers, one amplifying the wild-type *F8* sequence located in intron 18 of *F8* (MPX-F8-R) and one located in the inserted fragment in the genomic region of *F8* (MPX-R2) (Fig. 3b). The PCR products of wild type and mutant regions differ in size; 450 and 603 bp respectively. All the available potential carrier members of the family (Fig. 3c) were tested and their carrier status was determined using the multiplex PCR (Fig. 3d).

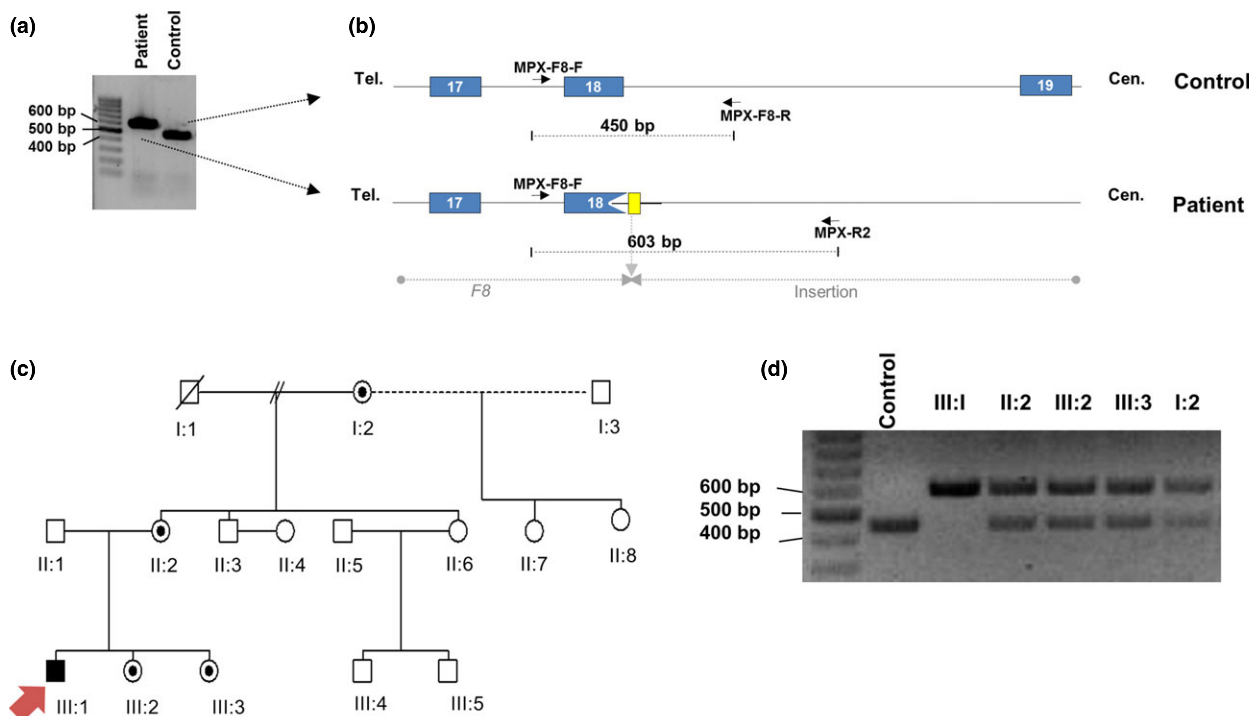

Fig. 3. Diagnostic multiplex PCR for detection of the breakpoint. (a) PCR gel of the diagnostic PCR with (b) schematic representation of the amplification with relative positions of the primers (Yellow Box: L1 repeat insertion; Cen., centromere; Tel., telomere). (c) Pedigree of the patient with (d) the picture of the diagnostic PCR carried out for all the carriers of the family (red arrow indicates the index patient).

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Discussion and conclusions

In majority of patients suffering from a severe haemophilia A phenotype, the causative mutation is detectable after applying the haemophilia A diagnostic flow chart [6]. Rarely, novel molecular mechanisms lead to a haemophilia A phenotype which cannot be completely characterized by routine molecular diagnostic methods. Among others, mutations deep in introns [10,11], insertions associated with deletions [12–14] and deletions arising from both unequal homologous recombination between Alu-derived sequences [15,16] and non-homologous recombination [17] have been reported.

In the present case, a simple deletion of exon 18 was initially thought to be the causal *F8* mutation leading to haemophilia A. However, contradictory results were obtained following MLPA analysis, and therefore an individualized research approach was required to elucidate the exact genetic cause. The results shown in this work highlight an important aspect that needs to be taken into consideration when performing genetic analysis of haemophilia A, namely, the importance of implementing complementary methods in a routine diagnostic workflow to confirm the deletion of one or more exons in cases of unsuccessful PCR amplification.

This report demonstrates the limitations of both PCR and MLPA applications resulting in inconsistent results. In the present case, the failure of the amplification is easily explicable by the *F8* gene insertion. However, the finding of the positive MLPA result has led to two important developments. Firstly, the implementation of MLPA for the verification of the PCR failure led to the design of an individualized diagnostic approach, which could then be used for diagnosis of carrier status. Secondly, our results show that partial duplication of exons can be easily missed depending on the position of the probe, for definite exclusion of duplications and to a lesser extent deletions, the availability of several probes for each exon could avoid false-positive and false-negative results. In the future, application of new technologies such as next-generation sequencing would advance the genetic analysis in haemophilia A and could facilitate faster and easier detection of such duplications and could provide simultaneous characterization of the breakpoint junctions.

In summary, we have identified and characterized a novel breakpoint in *F8* gene leading to a severe haemophilia A phenotype. Moreover, a specific PCR test was established for family carrier detection and prenatal diagnosis of the rearrangement. We provide a straight forward protocol for cases where inconsistent results are obtained from PCR and MLPA; comprising a simple general strategy using inverse PCR for characterization of breakpoint and a multiplex PCR for simple diagnostic analysis purposes.

Acknowledgements

This work was supported, in part, by funding from Baxter Germany GmbH (GGER 8260, to JO and OE) and the Deutsche Forschungsgemeinschaft (DFG: EL499/2-1, to OE and JO). We gratefully acknowledge the technical assistance of Nicole Nuesgen.

Disclosures

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

Author contributions

BP and BDMT designed the study and performed research; BP and OE analysed the data; AMG, OE, MDW, JO revised the manuscript; BP wrote the manuscript.

References

1 Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood* 2002; **99**: 168–74.

2 Liu Q, Nozari G, Sommer SS. Single-tube polymerase chain reaction for rapid diagnosis of the inversion hotspot of mutation in hemophilia A. *Blood* 1998; **92**: 1458–9.

3 Bagnall RD, Ayres KL, Green PM, Giannelli F. Gene conversion and evolution of Xq28 duplicons involved in recurring inversions causing severe hemophilia A. *Genome Res* 2005; **15**: 214–23.

4 Keeney S, Mitchell M, Goodeve A. The molecular analysis of haemophilia A: a guideline from the UK haemophilia centre doctors' organization haemophilia genetics laboratory network. *Haemophilia* 2005; **11**: 387–97.

5 Oldenburg J, Ananyeva NM, Saenko EL. Molecular basis of haemophilia A. *Haemophilia* 2004; **10**(Suppl. 4): 133–9.

6 Pezeshkpoor B, Pavlova A, Oldenburg J, El-Maarri O. F8 genetic anylsis strategies when standard approaches fail. *Haemostaseologie* 2014; **34**(2): 167–173.

7 Acquila M, Pasino M, Di Duca M, Bottini F, Molinari AC, Bicocchi MP. MLPA assay in F8 gene mutation screening. *Haemophilia* 2008; **14**: 625–7.

8 Rost S, Loffler S, Pavlova A, Muller CR, Oldenburg J. Detection of large duplications within the factor VIII gene by MLPA. *J Thromb Haemost* 2008; **6**: 1996–9.

9 Pezeshkpoor B, Rost S, Oldenburg J, El-Maarri O. Identification of a third rearrangement at Xq28 that causes severe hemophilia A as a result of homologous recombination between inverted repeats. *J Thromb Haemost* 2012; **10**: 1600–8.

10 Castaman G, Giacomelli SH, Mancuso ME *et al.* Deep intronic variations may cause mild hemophilia A. *J Thromb Haemost* 2011; **9**: 1541–8.

11 Pezeshkpoor B, Zimmer N, Marquardt N *et al.* Deep intronic 'mutations' cause hemophilia A: application of next generation sequencing in patients without detectable mutation in F8 cDNA. *J Thromb Haemost* 2013; **11**: 1679–87.

12 Van de Water N, Williams R, Ockelford P, Browett P. A 20.7 kb deletion within the factor VIII gene associated with LINE-1 element insertion. *Thromb Haemost* 1998; **79**: 938–42.

13 Chen JM, Chuzhanova N, Stenson PD, Ferec C, Cooper DN. Complex gene rearrangements caused by serial replication slippage. *Hum Mutat* 2005; **26**: 125–34.

14 Sheen CR, Jewell UR, Morris CM *et al.* Double complex mutations involving F8

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and FUNDC2 caused by distinct break-induced replication. *Hum Mutat* 2007; **28**: 1198–206.

15 Rossetti LC, Goodeve A, Larripa IB, De Brasi CD. Homeologous recombination between AluSx-sequences as a cause of hemophilia. *Hum Mutat* 2004; **24**: 440.

16 Vidal F, Farssac E, Tusell J, Puig L, Gallardo D. First molecular characterization of an unequal homologous alu-mediated recombination event responsible for hemophilia. *Thromb Haemost* 2002; **88**: 12–6.

17 Woods-Samuels P, Kazazian HH Jr, Antonarakis SE. Nonhomologous recombination in the human genome: deletions in the human factor VIII gene. *Genomics* 1991; **10**: 94–101.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sequencing results of the upstream and downstream inverse PCR products of the patient. (a) Sequence alignment of exon 18 of *F8* boxed in blue in *the* genomic sequence and patient's sequence. The inserted regions in patient's genomic DNA starts with the L1M1 repeat insertion (yellow box) followed by the inserted region from Xq28 (chrX: 154, 473, 718; hg19) in red box. (b) Sequence alignment of the downstream PCR showing the intact exon 18 of *F8* boxed in blue in *F8* genomic sequence and patient's sequence. The breakpoint is located in intron 17 at position chrX: 154, 132, 401; hg19. (c) Sequence information of genomic region of exon 18 and its flanking intronic sequences. Exons and introns are in capital and lower case letters respectively. The MLPA probes are marked in green and red for the LPO and RPO (5′ and the 3′ half of the MLPA probe) respectively. The breakpoint within exon 18 is shown as bold and italic letters. The yellow marked sequences are the diagnostic primers for amplification of exon 18.

Table S1. Primers used for characterization of the breakpoint.

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