Marker and real-time quantitative analyses to confirm hemophilia B carrier diagnosis of a complete deletion of the F9 gene

Approximately 3% of hemophilia B patients have major deletions in the F9 gene, half of which are complete. Marker and quantitative PCR analyses were employed for carrier diagnosis in a family of a mentally retarded hemophilia B patient with a total deletion of the F9 gene and neighbor genes. Both methodologies allowed the confirmation of carrier or non-carrier status.

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Large deletions (>50bp) occur in a minority of hemophilia B (HB) patients. From 2891 patients reported in the HB database to date (*www.kcl.ac.uk/ip/petergreen/haemBdatabase.html*) 91 are major deletions and approximately half of these are deletions encompassing all the exons of the F9 gene. Deletions in a HB patient can be detected by lack of amplification of specific exons or by non-inheritance of marker alleles of the family. However, if a complete deletion of F9 gene is detected, the definition of hemizygous females constitutes a challenge.

We present an isolated case of severe HB with an approximately 2 Mb genomic deletion with complete loss of the F9 gene and neighbor genes. The pedigree of the family is shown in Figure 1. The patient is a 22-year old male with severe HB. He developed low-response permanent inhibitors. At 5 months he presented seizures and at age 4 he was diagnosed with autism. At present he is mentally handicapped. His mother and two sisters requested carrier diagnosis. The marker analysis[1] (Figure 1) revealed the absence of the corresponding bands in the probandus II-1 and apparently, homozygozity in the mother I-2. His sister II-2 only inherited the paternal haplotype without markers of maternal origin. The sister II-3 was heterozygous for different markers demonstrating the inheritance of the paternal and maternal haplotypes. In the patient, attempts to amplify the exons of the F9 gene[2] for sequencing purposes proved unsuccessful. However, the patient did amplify one of the DXS52 alleles from the mother (Figure 1). In view of these results, the diagnosis of a big deletion encompassing the F9 gene was made. The employment of markers DXS1192 (situated 1cM proximal) and DXS1205 (situated 4.3 cM distal) flanking the F9 gene[3] yielded results identical to those of the intragenic markers, indicating that the deletion spanned beyond the F9 gene. It was assumed that the mother I-2 and the sister II-2 were most likely carriers of the whole deletion and that the sister II-3 was not a carrier. To confirm the foregoing, a further quantitative examination was performed. We designed an experiment to discriminate between one or two copies of exons 1 and 6 of the F9 gene employing a Real Time PCR. The exon 1 of the F9 gene was amplified by the forward 1-F GGGAGATGGACATTATTTCCC primer and the reverse 1-R GTGAAGAAGACAGCATCAGAT primer (262 bp). The exon 6 was amplified with the forward 6-F GCCAATGAGAAATATCAGG primer and the reverse 6-R CCAGTTTTGACACACCATC primer (279 bp). PCR was performed as previously described[4] with minor modifications (protocols available on request). We diluted to 7.5, 5, 2.5, and 1.25 ng/μL of DNA external standards that carry two *F9* copies each dilution corresponding to 3, 2, 1, and 0.5 fictive copies of *F9*. All standards were used to calculate the regression curve. Given that all exons of the F9 gene were deleted, we analysed exon 23 of the F8 gene[4] as an external reference to determine the ratios of the deleted versus non-deleted gene (Table 1). Sample concentrations were inferred from the regression curve in each experiment. All samples were measured twice by using independent DNA dilutions of 5 ng/μL.

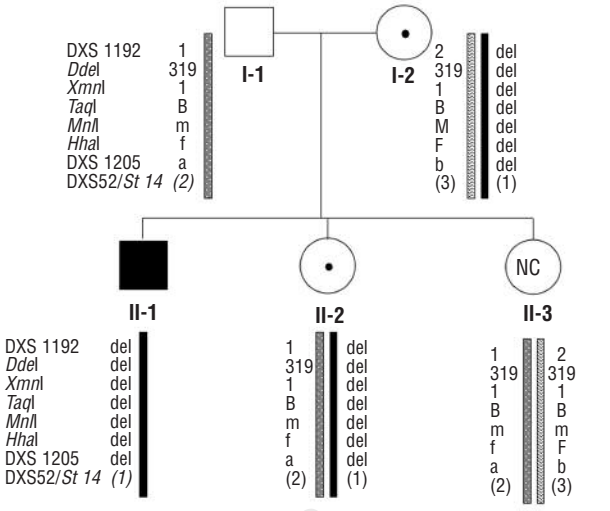

Figure 1. Pedigree of the family reported. NC: non carrier. The haplotypes were obtained from several microsatellite markers: *Dde*I in intron 1; *Xmn*I in intron 3, *Taq*I in intron 4, *Mnl*I in exon 6 and *Hha*I at the 3´ end of the gene. To localize the end point of the deletion of the F9 gene we also used *DXS1192* and *DXS1205* flanking the F9 gene. The *DXS52* locus (St14 marker) adjacent to the factor F8 gene in Xq28 was employed to determine the segregation of the X chromosome in the family.

Table 1. Average values ± SD of exon 1 and exon 6 copy number of F9 in individuals of family 1.

Individual	*Exon 1*	*Exon 6*	*Ex 23 F8*	*Ratio F9/F8**
I-1 father	0.97±0.02	0.94±0.06	1.05±0.06	0.92/0.89
I-2 mother	1.03±0.03	0.97±0.01	1.8±0.05	0.57/0.53
II-1 patient	0.02±0.007	0.01±0.003	0.92±0.02	0.02/0.01
II-2 sister	1.04±0.09	1.03±0.01	1.9±0.06	0.54/0.54
II-3 sister	1.82±0.02	1.78±0.06	1.8±0.05	1.01/0.98

**The first value of the column corresponds to the ratio exon 1 F9/exon 23 F8 and the second to the ratio exon 6 F9/exon 23 F8. Testing of 10 healthy males with this method showed mean values of 0.95 ± 0.05 corresponding to 1 copy of the exon 1 and 6, whereas 10 non-HB carriers showed values of 1.81± 0.107 corresponding to 2 copies of the exon 1 and 6. The coefficient of validation (CV) of the assay was 5.91% for the female group and 5.26% for the male group.*

Carrier diagnosis of gross deletions involving one or more exons along the F9 gene has been resolved by marker analysis to track the X-chromosome bearing the *F9* deletion. However, the occurrence of a complete deletion may constitute a limitation on this approach. Moreover, isolated cases, uninformative markers and unavailable clue family members may complicate the diagnostic scenario. Our patient lacked the 7 markers analysed whereas the mother was apparently homozygous for all of them. One of the sisters inherited only the paternal alleles and the other inherited both the paternal and maternal alleles. Therefore, marker analysis was

helpful to indicate that a complete deletion was present in the patient and that the mother and one of his sisters were carriers. The real time quantitative PCR approach by LightCycler methodology is a major tool to confirm carrier diagnostic situations[4,5] as in the family reported here. To study the complete deletion of the *F9* in our patient, we designed two primer sets (exon 1 and 6) to determine the exon copy number for comparison with an external standard curve. Furthermore, we chose the exon 23 of the F8 gene as an external reference exon[4] to corroborate the results. A second external reference of the X-chromosome, i.e. the dystrophin gene at the DMD locus[6] in Xp21 can be employed when *F9* markers are uninformative. The method was validated in HB with the analysis of controls and members of the family under study.

Our patient has a deletion encompassing at least 2Mbp between *F9* flanking markers DXS1192 and DXS1205. A number of genes such as *MCF2* (a transforming gene member of a GDP-GTP exchange factors), CDR1 (cerebellar degeneration-related autoantigen 1) and SOX3 (a transcription factor involved in neural development) are included in this deleted region. The absence of one or more of these genes may justify the mental impairment observed in our patient. Interestingly, a male patient with HB and mental retardation with a 1.5Mbp deletion, which included the F9 and SOX3 genes, has been reported.[7] Furthermore, *SOX3* has been involved in X-linked mental retardation with growth hormone deficiency.[8] On the other hand, HB patients with a deletion of the transforming gene MCF2 and with no evidence of a neoplastic process or other clinical manifestations have been described.[9,10]

In conclusion, we performed an accurate HB carrier diagnosis in this family using traditional markers and a rapid, simple and robust quantitative Real Time assay. Both methods are warranted to overcome the carrier diagnosis of deletions. Moreover, genetic counselling in the HB carriers of this family should also consider the high-risk occurrence for autism-mental retardation in the descendants.

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Erratum

In the article by Vande Broek I et al. entitled "Clinical significance of chemokine receptor (CCR1, CCR2 and CXCR4) expression in human myeloma cells: the association with disease activity and survival" which appeared on Haematologica 2006; 91:200-6, an author name was misspelled. The first author should be read as Vande Broek I, and not Van de Broek I.

Erratum

In the article by Menu E et al. entitled "The involvement of stromal derived factor 1alpha in homing and progression of multiple myeloma in the 5TMM model" which appeared on Haematologica 2006; 91:605-12, an author name was misspelled. The name of seventh author should be read as Vande Broek I, and not Van de Broek I.

Erratum

In the article by Giansily-Blaizot M et al. entitled "Analysis of biological phenotypes from 42 patients with inherited factor VII deficiency: can biological tests predict the bleeding risk?" which appeared on Haematologica 2004;89:704-9, an author name was misspelled. The name of seventh author should be read as Le Cam-Duchez V, and not LeCam-Duchez V.