Large deletions play a minor but essential role in congenital coagulation factor VII and X deficiencies

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Keywords
Factor VII deficiency, factor X deficiency, F7 gene, F10 gene, chromosome 13q34 deletion

Summary
Congenital factor VII (FVII) and factor X (FX) deficiencies belong to the group of rare bleeding disorders which may occur in separate or combined forms since both the F7 and F10 genes are located in close proximity on the distal long arm of chromosome 13 (13q34). We here present data of 192 consecutive index cases with FVII and/or FX deficiency. 10 novel and 53 recurrent sequence alterations were identified in the F7 gene and 5 novel as well as 11 recurrent in the F10 gene including one homozygous 4.35 kb deletion within F7 (c.64+430_131–6delins -TCGTAA) and three large heterozygous deletions involving both the F7 and F10 genes. One of the latter proved to be cytogenetically visible as a chromosome 13q34 deletion and associated with agenesis of the corpus callosum and psychomotor retardation.

Conclusions: Large deletions play a minor but essential role in the mutational spectrum of the F7 and F10 genes. Copy number analyses (e.g. MLPA) should be considered if sequencing cannot clarify the underlying reason of an observed coagulopathy. Of note, in cases of combined FVII/FX deficiency, a deletion of the two contiguous genes might be part of a larger chromosomal rearrangement.

Schlüsselwörter
FVII-Mangel, FX-Mangel, F7-Gen, F10-Gen, Chromosom-13q34-Deletion

Zusammenfassung

Coagulation factors VII (FVII) and X (FX) are vitamin K-dependent serine proteases that are essential for blood coagulation. The genes encoding FVII and FX, namely F7 and F10, are both located in close proximity on chromosome 13q34, 2.8 kb apart from each other.

* F7 is located proximal to F10, covers a region of about 14.8 kb and consists of nine coding exons.
* F10 spans 27 kb, comprises eight exons, each encoding a specific functional domain of the FX protein (1–4).

Both proteins share structural similarities and are synthesized in the liver and secreted to the plasma as inactive zymogenes.

So far, nearly 300 mutations in the F7 gene have been associated with FVII deficiency (OMIM #227500) [HGMD Professional 2014.4 product release; UMD F7
Mutation database update 7/01/14 (5, 6). Missense mutations caused by single nucleotide substitutions account for approximately 60% of published F7 mutations. The mutational spectrum of FX deficiency (OMIM #227600) is very similar: 129 F10 mutations are listed in the Human Gene Mutation Database, 77% of these are missense mutations [HGMD Professional 2014.4 product release].

Individuals carrying homozygous or compound heterozygous mutations in the F7 gene may develop severe bleeding symptoms such as gastrointestinal or intracranial hemorrhages while heterozygous carriers of pathogenic mutations are usually asymptomatic or present with mild mucocutaneous bleedings (7–9). Their detection often occurs incidentally for instance prior to elective surgery. A certain demarcation in FVII levels has also been observed between homozygotes / compound heterozygotes and heterozygotes. However, reliable genotype-phenotype correlations remain impossible in part due to the small number of homozygotes with identical mutations (9, 10). The rare FX deficiency is clearly more severe than FVII deficiency; for reference and comparison see (9, 10). The current data include

- 172 consecutive index cases with isolated FVII deficiency,
- 12 probands with isolated FX deficiency,
- 8 cases with combined deficiencies of FVII and FX.

While the Greifswald Registries of FVII and FX deficiencies have been international study groups (9, 10), the data presented here were obtained from molecular genetic screening of consecutive index cases in a diagnostic setting. Patients were referred to the department of human genetics in Greifswald from all over Germany between 2009 and 2014 based on low FVII and FX activities and bleeding symptoms if present.

In order to optimize genetic screening and to detect larger heterozygous deletions or duplications within or of the F7 and F10 genes, quantitative analyses (MLPA) were performed in addition to Sanger sequencing. Both heterozygous digenic F7 and F10 point mutations as well as large heterozygous deletions encompassing the contiguous F7 and F10 genes have been identified.

**Patients, material, methods**

**Mutation analyses**

With informed consent according to the German Gene Diagnostics Act and approval of the local ethics committee of the university medicine Greifswald, genomic DNA was isolated from peripheral blood using standard techniques. The nine coding exons of the F7 gene and the eight coding exons of the F10 gene including their flanking splice sites were analysed by direct sequencing. Primer pairs for genomic amplification were generated according to the GenBank Refseq NM_000131.4 and NM_000504.3, respectively. PCR products were purified using ExoSAP-IT (Affymetrix). DNA sequencing was performed on both strands, using a commercial kit (BigDye Terminator Cycle Sequencing v3.1 kit, Applied Biosystems) and an automated DNA sequencer (model ABI 3130XL, Applied Biosystems). SeqPilot analysis software (JSI medical systems GmbH) was used for final sequence read-out and mutation documentation. DNA mutation and amino acid numbering as well as nomenclature are used according to the international recommendations for the description of sequence variants of the Human Genome Variation Society (www.HGVS.org; update December 2014) with exon 1 being the first coding exon of the transcript and the A of the ATG translation initiation codon being nucleotide +1.

**Deletion screening**

Multiplex ligation-dependent probe amplification (MLPA) for detection of large, exon-covering deletions was performed in all eight index cases with combined deficiencies of FVII and FX, in 35 of the 172 cases with isolated FVII deficiency and in 5 of the 12 FX-deficient probands for two reasons: First, all alterations observed by direct sequence analyses were found in homozygous state suggesting the possibility of a large exon-spanning deletion on the second allele. Secondly, Sanger sequencing alone could not explain the patients’ factor deficiency. MLPA was done according to the manufacturer’s recommendations using SALSA MLPA Kit P207-C2 for the F7 gene and SALSA MLPA Kit P440-A1 for the F10 gene (MRC-Holland, Amsterdam; www.MRC-Holland.com).

Amplification products were run on an ABI 310 DNA Sequencer with the GeneScan ROX 500 size standard and analysed by Genemapper Software v4.0 (Applied Biosystems). Dosage analysis based on a comparison between patient and control DNA samples were performed using SeqPilot analysis software (JSI medical systems GmbH). A control/test bar ratio of 1.0 (variance: 0.7–1.3) corresponded to normal (100%) gene dosage. Long-range PCR analysis was applied to fine-map the breakpoints using a

- forward primer in the 5’-UTR (LR-FP 5’CTTAAACACCTGCACGGCTG-3’),
- reverse primer in intron 5 (LR-RP 5’CTAGACCATTTCTCAACTGGT-3’).

Direct sequencing of PCR products was performed with primers located in intron 1 and intron 3, respectively:

- LR-FP-Seq 5’CAGCATCCAGGCAC-AGGAGG-3’,
- LR-RP-Seq 5’CCAGCTTCTCAGCACGTCCCC-3’.

**Results**

**Mutation profile of isolated FVII deficiency**

Disease-causing mutations were found in 120 of 172 index cases with isolated FVII deficiency. Four individuals were homozygotes and 34 harboured at least two different mutations. Their biallelic occurrence could often not be determined owing to lack of further family members. 82 of the individuals analysed carried only one heterozygous mutation in the F7 gene. In the remaining index cases, no pathogenic mutation could be detected in the F7 coding, splice site and promoter regions analysed.

Direct sequencing revealed 63 different mutations in the F7 gene of which 10 have not been published in the literature or listed in mutation databases (HGMD, UMD) so far (Tab. 1). Only c.2T>C and c.808G>A are registered in the Exome
Aggregation Consortium (ExAC) Browser with a minor allele frequency of less than 0.01% ([http://exac.broadinstitute.org](http://exac.broadinstitute.org) [01/2015 accessed]). FVII coagulant activities in plasma of patients carrying these variants as well as results of in silico analyses (MutationTaster, PolyPhen-2, SIFT (11–13)) indicate their functional relevance (Tab. 1). Note, c.64+1G>A and c.379T>C were compound heterozygous point mutations found in a 6-month-old boy diagnosed to have severe FVII deficiency with FVII levels below 1.7% shortly after birth. He presented with intracranial bleedings and haematoma requiring FVII replacement therapy. Each of the non-consanguineous Chinese parents was heterozygous for one of the two mutations.

Using MLPA only one large genomic deletion within the F7 gene was found. Breakpoint analyses of this homozygous deletion on exon 2 which had been detected by PCR (data not shown) and MLPA (Fig. 1A) identified a 4.35 kb deletion spanning large parts of the adjacent intron 1 and almost the entire intron 2. In addition, a 6-bp-insertion was detected (c.64+430_131–6delinsTCGTAA) (Fig. 1B). In silico analyses (Human Splicing Finder, NetGene2 and BDGP) predicted either skipping of exon 3 or the usage of a cryptic splice site 14 bp downstream of the intron 2/exon 3 boundary. Both alternatives would result in a frameshift and probably in nonsense-mediated decay. Analyses of F7 mRNA from whole blood samples of both heterozygous parents did not reveal specific alternatively spliced transcripts (data not shown) which may also in part be due to the already low illegitimate transcription rate of F7 in human lymphocytes (14). F7 mRNA could not be analysed in the index case because no further blood sample of the 6-month-old boy, forth child of a consanguineous Syrian couple, was available. Nevertheless, the relatively moderate FVII deficiency (<4%) and mild bleeding symptoms (bloody stool during infectious disease) suggest that usage of the original splice site might be highly reduced but sufficient for maintenance of a minimal residual FVII protein level.

**Mutation profile of isolated FX deficiency**

Isolated FX deficiency was analysed in 12 index cases. Three individuals carried homozygous mutations and seven were heterozygous carriers of only one mutation. Together with sequence alterations in cases with combined FVII and FX deficiencies 16 different variants in the F10 gene were identified of which five have not been described in the literature or mutation databases so far. Only c.1097G>A is registered in the Exome Aggregation Consortium (ExAC) Browser with a minor allele frequency of less than 0.01%. In silico analyses suggest that they should be classified as probably disease-causing (Tab. 1).

The mutation spectrum of the F10 gene was comparable to the F7 gene’s spectrum. The majority of detected variants were nonsense mutations mostly located in the catalytic domain. Only two small deletions were identified which result in a frameshift. Large, exon-covering deletions could not be found in cases of isolated FX deficiency.

<table>
<thead>
<tr>
<th>gene</th>
<th>nucleotide exchange</th>
<th>predicted amino acid change</th>
<th>functional domain</th>
<th>mutation taster (score)</th>
<th>PolyPhen-2 (score)</th>
<th>SIFT (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F7</td>
<td>c.2T&gt;C</td>
<td>p.Met1?</td>
<td>pre-pro-peptide</td>
<td>disease causing (0,99)</td>
<td>possibly damaging (0,89)</td>
<td>damaging (0)</td>
</tr>
<tr>
<td></td>
<td>c.46C&gt;T</td>
<td>p.(Gln16*)</td>
<td>pre-pro-peptide</td>
<td>disease causing (1)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>c.64+1G&gt;A</td>
<td>p.?</td>
<td>n.a.</td>
<td>disease causing (0,99)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>c.379T&gt;C</td>
<td>p.(Ser127Pro)</td>
<td>EGF-1</td>
<td>disease causing (0,99)</td>
<td>probably damaging (0,97)</td>
<td>damaging (0,03)</td>
</tr>
<tr>
<td></td>
<td>c.740G&gt;T</td>
<td>p.(Trp247Leu)</td>
<td>activation peptide</td>
<td>disease causing (0,99)</td>
<td>probably damaging (1)</td>
<td>damaging (0,03)</td>
</tr>
<tr>
<td></td>
<td>c.808G&gt;A</td>
<td>p.(Glu270Lys)</td>
<td>catalytic domain</td>
<td>disease causing (0,99)</td>
<td>probably damaging (0,98)</td>
<td>damaging (0,03)</td>
</tr>
<tr>
<td></td>
<td>c.1166G&gt;A</td>
<td>p.(Cys389Tyr)</td>
<td>catalytic domain</td>
<td>disease causing (0,99)</td>
<td>probably damaging (1)</td>
<td>damaging (0)</td>
</tr>
<tr>
<td></td>
<td>c.1202dupA</td>
<td>p.(Asp403Glyfs*123)</td>
<td>catalytic domain</td>
<td>disease causing (1)</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>c.1235A&gt;G</td>
<td>p.(Tyr412Cys)</td>
<td>catalytic domain</td>
<td>disease causing (0,99)</td>
<td>probably damaging (1)</td>
<td>damaging (0)</td>
</tr>
<tr>
<td></td>
<td>c.64+430_131–6delinsTCGTAA</td>
<td>p.?</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

n.a.: not available
Fig. 1
A) Multiplex ligation-dependent probe amplification (MLPA) gene dosage assay. The F7 exon 2 peak in the peak area histogram is only present in the controls (blue bars) and missing in the patient (green bars) indicating a homozygous deletion. Exon 3 is not included in the MLPA kit applied.
B) Long-range PCR analysis and sequencing using a forward sequencing primer in intron 1 and a reverse primer in intron 3 could fine-map the breakpoints. The homozygous deletion comprises 4350 bp starting 430 bp downstream of exon 1 and ending 5 bp upstream of exon 3. Additionally, a 6-bp-insertion is evident at the 3'-breakpoint of the deletion.

Tab. 2 Laboratory parameters, bleeding histories and results of genetic analyses of index cases with combined FVII/FX deficiency

<table>
<thead>
<tr>
<th>index cases</th>
<th>patient</th>
<th>FVII : C (%)</th>
<th>FX : C (%)</th>
<th>bleeding symptoms</th>
<th>ethnicity</th>
<th>F7</th>
<th>F10</th>
</tr>
</thead>
<tbody>
<tr>
<td>large deletions within 13q34</td>
<td>P1</td>
<td>35</td>
<td>51</td>
<td>asymptomatic</td>
<td>German</td>
<td>deletion of exons 1–9</td>
<td>heterozygous</td>
</tr>
<tr>
<td>P2*</td>
<td>48</td>
<td>&lt;1</td>
<td>severe periumbilical bleeding and cutaneous bruises in postnatal period</td>
<td>Caucasian</td>
<td>deletion of exons 1–9</td>
<td>heterozygous</td>
<td>deletion of exons 1–9</td>
</tr>
<tr>
<td>P3</td>
<td>33</td>
<td>48</td>
<td>easy bruises</td>
<td>German</td>
<td>deletion of exons 1–9</td>
<td>heterozygous</td>
<td>deletion of exons 1–8</td>
</tr>
<tr>
<td>with and without sequence changes in F7 and F10 genes</td>
<td>P4</td>
<td>63</td>
<td>53</td>
<td>unknown</td>
<td>German</td>
<td>WT</td>
<td>c.[424G&gt;A];[=]</td>
</tr>
<tr>
<td>P6</td>
<td>40</td>
<td>58</td>
<td>asymptomatic</td>
<td>Turkish</td>
<td>WT</td>
<td>c.[1021G&gt;T];[=]</td>
<td>heterozygous</td>
</tr>
<tr>
<td>P7</td>
<td>32</td>
<td>50</td>
<td>asymptomatic</td>
<td>German</td>
<td>c.[143A&gt;T];[=]</td>
<td>heterozygous</td>
<td>WT</td>
</tr>
<tr>
<td>P8</td>
<td>42</td>
<td>70</td>
<td>epistaxis and easy bruises</td>
<td>German</td>
<td>c.[143A&gt;T];[=]</td>
<td>heterozygous</td>
<td>WT</td>
</tr>
</tbody>
</table>

*retrospective MLPA analysis of a previously published case refining the deletion within the F10 gene (15); † variant of unknown clinical significance; Mutation Taster: polymorphism (0,99), PolyPhen-2: probably damaging (0,98), SIFT: damaging (0,04), ExAc: not listed.
Combined FVII and FX deficiencies based on different mechanisms

Sequencing and MLPA results as well as activity levels and bleeding symptoms of all eight probands with a reported combination of FVII and FX deficiencies are listed (▶Tab. 2). Coincidental occurrence of digenic heterozygous point mutations occurred in two asymptomatic individuals with mild FVII and FX deficiencies (P5, P7). A variant of unknown clinical significance in the F7 gene was identified in P8 (c.143A>T). Several known pathogenic mutations have been located in close proximity to this previously not described variant which might indicate its deleterious effect. However, in-silico analyses are inconclusive.

In three individuals with combined FVII and FX deficiencies (P1–3), a large heterozygous deletion was detected comprising the F7 as well as the F10 gene. One of these (P2) was compound heterozygous for a four base pair deletion within F10 and presented with a severe bleeding history (15). Another (P1) turned out to be part of an interstitial chromosomal deletion 13q34.

The interstitial chromosomal deletion 13q34 was found in an 8-year-old female index, first child of a non-consanguineous German couple. Prior to an elective tonsillectomy, abnormal PT (39–54%) as well as FVII (35% measured twice) and FX (46 and 51%) levels were found, surgery postponed and molecular genetic analyses initiated. Sanger sequencing of the F7 gene only detected FVII reducing polymorphisms in apparently homozygous state. No point mutation could be found in F10. MLPA analysis revealed a complete heterozygous deletion of both F7 and F10 genes (▶Fig. 2A, B). Since the girl also displayed psychomotor retardation and agenesis of the corpus callosum, she was assumed to have a contiguous gene syndrome. Conventional karyotyping, fluorescence in situ hybridization of the index patient using probe RP11–17E4 revealed an interstitial deletion in 13q34.
We describe novel mutations combined FVII/FX deficiency with a dele-
tion in the $F7$ gene. We present data of systematic genetic analyses
confirmed a 3.6 Mb interstitial deletion of 13q34 (Fig. 2C, D). The
karyotype of the index patient is 46,XX,del(13)(q34).ish
13q34q34(RP11–17E4x1)[25]. arr[hg19]
13q34(111,138,603–114,781,854)x1. This
deletion was also found in the proband's 8-month-old sister who was also diagnosed
to have agenesis of the corpus callosum. Their mother was shown to carry the deletion
in 12% of her peripheral blood cells. Although the causative gene remains unknown,
the report of a ten-year-old boy with a submicroscopic deletion of chromosome 13q34 and corpus callosum agenesis
as the sole major malformation (16) further suggests that this deletion may be responsible not only for the girl's coagulation factor deficiencies but also for her CNS malformation.

Discussion

Hereditary isolated deficiencies of FVII or FX are rare genetic diseases. In the cohort presented here, eight probands were reported to have combined FVII and FX deficiencies based on coagulant activities among 180 index cases with FVII and 20 with FX deficiency. Large genomic deletions were only found in four out of 192 index cases. These included three large heterozygous deletions spanning the entire $F7$ and at least part of the $F10$ gene in index cases with combined FVII and FX deficiencies and one partial deletion within the $F7$ gene in a patient with moderately reduced FVII coagulant activity.

These results are in accordance with published data. Recently, four large heterozygous deletions spanning the entire $F7$ and $F10$ genes were detected in 10 individuals selected for combined FVII and FX deficiencies (17). Consistent with the published observations, our index cases with large heterozygous $F7$ and $F10$ deletions (P1 and P3) were asymptomatic or presented with minor bleeding symptoms (Tab. 2). A severe bleeding history was only observed in an individual harbouring a truncating mutation on the second $F10$ allele (Tab. 2, P2). As for isolated FVII deficiency, two large rearrangements of the $F7$ gene have been reported in a series of 43 individuals (18). Previous reports on deletions in FX deficiency were mainly single case reports (15, 19, 20) most likely due to the fact that a commercial $F10$ MLPA kit has only become available in 2014.

Although large deletions appear to play a minor role in $F7$ and $F10$ molecular genetics, MLPA analyses should be considered as part of a step-by-step diagnostic approach if sequencing alone cannot clarify the underlying reason of an observed coagulopathy or if polymorphisms and point mutations are exclusively found in homozygous state. The case of a girl with combined FVII and FX deficiencies, agenesis of the corpus callosum and psychomotor retardation known to be associated with an interstitial chromosome 13q34 deletion highlights this importance. Sequencing alone did not detect pathogenic mutations that would explain the coagulation factor deficiencies. Subsequent MLPA analyses detected a heterozygous deletion of both $F7$ and $F10$ genes. Knowledge of their close proximity on chromosome 13q34 and their association with ad-
ditional phenotypes due to deletions of other genes within this region (21–24) was the decisive factor for a critical re-evaluation of the girl’s and her sister’s medical history and phenotype.

Conclusions

Molecular genetic diagnostics of the $F7$ and $F10$ genes is essential to discriminate between inherited and acquired forms of the respective bleeding disorders and should be considered as part of the diagnostic work-up. While differential diagnosis may need to be reconsidered in mildly affected cases mutation-negative for $F7$ and/or $F10$ such as P4, P6 and P8 (Tab. 2), one has to take into consideration that routine Sanger sequencing and MLPA analyses do not detect disease-causing mutations in most non-coding regions of the genes. In more frequent bleeding disorders such as hemophilia A, disease-causing intronic mutations that create novel splice sites have been published (25–27). Particularly in individuals affected with a mild phenotype, next generation sequencing most recently revealed potentially pathogenic deep intronic variants in the $F8$ gene (28). Furthermore, a homozygous substitution deep within intron 8 of the $F5$ gene has been reported to result in inclusion of a pseudo-exon containing an in-frame stop codon into the mature $F5$ mRNA (29).

Future studies using ultra-deep sequencing techniques for FVII and FX deficient individuals thus far mutation-negative for $F7$ and $F10$ might show whether mutations in non-coding regions or somatic mosaicism represent disease-causing mechanisms leading to FVII and FX deficiencies.

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Conflict of interest

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

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