Case Report

Targeted Next-Generation Resequencing of $F_5$ Gene Identifies Novel Multiple Variants Pattern in Severe Hereditary Factor V Deficiency

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The present study investigated the genetic defects underlying severe Factor V deficiency in a 26-year-old Columbian (South America) female and her immediate family (both parents and newborn child) by next generation sequencing (NGS) of the entire $F_5$ gene locus. Five mutations in the coding sequence of $F_5$, including three missense single-nucleotide variants (R2102H, R513K, D107H) and two synonymous variants (A135A, S184S), were identified and confirmed by the Sanger sequencing in the investigated proband (homozygote for all detected mutations), her parents, and her newborn child (all heterozygotic carriers for identified mutations). Each of the three missense variants was previously associated with separate phenotypes, including Factor V deficiency (R2102H), thrombosis (R513K) and frequent miscarriages (D107H). In addition, at least 75 additional single-nucleotide variants (including six novels) were identified in untranslated region of $F_5$.

1. Introduction

Coagulation Factor V is a large 330-kD glycoprotein which consists of 2224 amino acid residues including a 28-residue leader peptide, which is structurally and functionally homologous to coagulation Factor VIII [1, 2]. The human Factor V gene (official name $F_5$) maps to chromosome 1q23 and contains 25 exons (8). Factor V deficiency is a rare autosomal recessive disorder (incidence < 1 in 1 million), characterized by low levels of antigen and activity [3]. At present, more than one hundred deficiency-causing mutations in the $F_5$ locus have been described, and although most of them are private, a few are common, being found in several individuals of both European, Middle-Eastern and Asiatic descents. In the present study, we used for the first time DNA next-generation sequence (NGS) analysis to detect mutation pattern in the entire $F_5$ locus of a 26-year-old Hispanic parturient with severe Factor V deficiency, as well as in her asymptomatic parents and newborn baby. The relationship between combinations of mutations and clinical phenotypes was evaluated.

2. Case Presentation

The study protocol was approved by IRB at PSU Hershey Medical College. It was performed in adherence to the tenets of the declaration of Helsinki. Written informed consent was obtained from all participants. The investigated patient was 26-year-old Hispanic (born in Columbia, South America) parturient (G4P1) with several bleeding episodes before and during present pregnancy, multiple fresh frozen plasma (FFP) transfusions, and a history of three miscarriages in the past. The patient was previously diagnosed clinically to have severe Factor V deficiency on the basis of several previous bleeding episodes and laboratory studies demonstrating coagulopathy with moderate to severe decrease in Factor V activity. The remaining past medical history was unremarkable. The family history revealed that both biological parents had no history of bleeding or other coagulation symptoms and had reported normal Factor V activity. In addition, she has two siblings, apparently without any clinical signs of coagulation disorders. In the course of the current pregnancy the patient delivered
the healthy male newborn, who has not displayed, at the time of this analysis, any signs of coagulation abnormalities, besides decreased (36%) level of Factor V. For the purpose of this investigation, we collected the samples of blood (proband and newborn son) and saliva (both biological parents and proband's husband) for DNA analysis (Figure 1).

3. Mutation Analysis

Genomic DNA (gDNA) was extracted from venous EDTA-whole blood sample (proband) or cord blood (newborn child) employing membrane ultrafiltration method (FujiFilm Life Sciences distributed by Autogene, Holliston, MA, USA), according to the manufacturer recommendations. The saliva samples were collected from both parents and proband/s husband into Oragene container (DNA Genotek, Canada) and extracted according to the manufacturer recommendations. Subsequently two gDNA samples from the proband were submitted to Otogenetics Corporation (Norcross, GA, USA) for target capture and sequencing. Briefly, gDNA was subjected to agarose gel and optical density ratio tests to confirm the purity and concentration prior to Covaris (Covaris, Inc., Woburn, MA, USA) fragmentation. Fragmented gDNAs were tested for size distribution and concentration using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and Nanodrop (Thermo Fisher Scientific, Wilmington DE, USA). Illumina libraries were made from qualified fragmented gDNA using NEBNext reagents (New England Biolabs, Ipswich, MA, USA) and the resulting libraries were subjected to exome enrichment using custom probes targeting 75 kb target on chromosome 1 (169, 481, 192–169, 555, 469 by GRCh 37, Hg19). The resultant libraries were tested for enrichment by qPCR and for size distribution and concentration by an Agilent Bioanalyzer 2100. The samples were then sequenced on an Illumina HiSeq2000 (Illumina, San Diego, CA, USA) which generated paired-end reads of 90 or 100 nucleotides. Data was analyzed for data quality, exome coverage, and exome-wide SNP/InDel using the platform provided by DNAnexus (DNAnexus, Inc, Mountain View, CA, USA). The detected polymorphisms in the coding sequence of the F5 locus were subsequently verified using classical Sanger sequencing using exon primers described by van Wijk et al. [4]. This analysis was performed by direct DNA sequencing using ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

By NGS, we generated about 549 million bases of sequence as pair-end 90 or 100 nucleotide reads, 86% of which was able to align to human reference sequence. A total of 23% of these sequences mapped to the targeted region corresponding to 75 kb sequence of the F5 locus (NM_0001304.4), with 540-fold mean coverage (Table 1). At this depth of coverage, more than 95% of the target bases were covered to pass quality control filtering based on the PHRED score threshold of calling variants (PHRED > 30). Eighty high-confidence variants were annotated in the targeted region (3 nonsynonymous single-nucleotide variants and 2 synonymous single-nucleotide variants in the coding region of the target (Table 2), as well as 75 single-nucleotide variants in the noncoding sequence of the target (Table 3). The missense variants were located in the exon 3 (D107H), 10 (R513K), and 23 (R2102H) of the F5 locus (Figure 1), and the investigated patient was a homozygous carrier for all these variants. These variants were additionally confirmed by the Sanger sequencing and showed perfect match in two duplicate samples. The additional verification of the DNA samples from the proband's newborn son and our patient's biological parents performed using the Sanger sequencing revealed that all of them were heterozygous carriers for all 3 missense variants (Figure 2). No presence of these variants was shown in the husband of the patient (i.e., biological father of the newborn child).

4. Discussion

The current study presents several novel findings: (i) it employs NGS approach for molecular diagnosis of FV deficiency, including sequencing of both translated and untranslated regions of F5 locus; (ii) it diagnoses the inherited FV deficiency in the S. American Caucasian family of Hispanic ethnicity; and (iii) it depicts detailed genetic evidence for multiple, co-inherited mutations in the F5 locus which may be responsible for opposite phenotype characteristics (i.e., increasing and decreasing thrombosis process).

The results of the current study indicate that the investigated proband was a homozygotic carrier of three separate missense mutations in the coding sequence of F5. Surprisingly, however, only one of the 3 detected missense mutations (R2102H) was described previously (and called at that time, R2074H, based on different amino acid number order) by Schijver et al. (2002) in the context of the deficiency of...
the Factor V [5]. This substitution results in the replacement of an arginine (R) by a histidine (H) in amino acid position 2102 located in the C2-domain of Factor V and several lines of evidence reported previously support the notion that this sequence variant is causative for Factor V deficiency phenotype. Interestingly the remaining two detected missense mutations were reported to be associated mostly with either thrombosis [6–8] or increased risk of preterm delivery (D107H) [9]. It is noteworthy that two missense point mutations in the coding region of Factor V function remains unknown.

In addition, we established that the investigated proband was a homozygous carrier for two synonymous single-nucleotide variants: A135A (rs6029) and S184S (rs6022) previously reported in the online SNP Medline database. There is no information about the potential phenotypic significance of these mutations. In addition to the previously described single-point mutations in the coding region of F5 gene, the analysis of the NGS data from the proband established a presence of additional 75 polymorphisms in the untranslated (5'-UTR part of the gene) (Table 3). Six polymorphisms were found in the 5'-UTR part, 71 in the intronic and 4 in the 3'-UTR part. The phenotypic significance of these polymorphisms for the Factor V deficiency phenotype remains unknown.

### Table 1: Summary of coverage for analyzed samples by next generation sequencing method.

| Sample                  | Average target coverage | Total bases | Total reads | Mapped reads | Bottleneck score |
|-------------------------|-------------------------|-------------|-------------|--------------|-----------------|
| Original proband DNA sample | 547                     | 583,280,200 | 5,832,802   | 4,964,887    | 31.81           |
| Duplicate               | 534                     | 549,426,400 | 5,494,264   | 4,720,781    | 32.81           |

Figure 2: Amino acid sequence of human factor V deduced from the F5 DNA sequence of proband. Red fonts indicate missense mutations, and blue fonts indicate synonymous mutations. The sequence of first 28 amino acid fragments is given in italics, and the consecutive 25 exons of causative mutation in Caucasians, it is to our knowledge, the first detailed description of causative mutation in F5 locus in a family from South America and/or Hispanic ethnicity.
Table 2: Mutations observed in coding sequences (CDS) of F5 gene in the investigated proband and her blood relatives.

| Patient          | Bleeding phenotype | FV activity (%) | Variants in CDS of F5 gene | Mature FV protein variants | SNPs (ID) |
|------------------|--------------------|----------------|-----------------------------|---------------------------|-----------|
| **Female proband** | Epistaxis multiple miscarriages | 0–4% | Homozygote for nonsynonymous variants: |
|                  |                    |               | 169486641 G > C | 202 R/H (exon 23) | No dbSNP ID |
|                  |                    |               | 169519112 C > T | 513 R/K (exon 10) | rs6020    |
|                  |                    |               | 169541513 C > G | 107 D/H (exon 3) | rs6019    |
|                  |                    |               | Homozygote for synonymous variants: |
|                  |                    |               | 169529973 C > T | 135 A/A (exon 4) | rs6029    |
|                  |                    |               | 169529826 C > A | 184 S/S (exon 4) | rs6022    |
| **Newborn son of proband** | No bleeding | 36% | Heterozygote for nonsynonymous variants: |
|                  |                    |               | 169486641 G > C | 202 R/H (exon 23) | No dbSNP ID |
|                  |                    |               | 169519112 C > T | 513 R/K (exon 10) | rs6020    |
|                  |                    |               | 169541513 C > G | 107 D/H (exon 3) | rs6019    |
|                  |                    |               | Heterozygote for synonymous variants: |
|                  |                    |               | 169529973 C > T | 135 A/A (exon 4) | rs6029    |
|                  |                    |               | 169529826 C > A | 184 S/S (exon 4) | rs6022    |
| **Mother of proband** | No bleeding | Reported normal | Heterozygote for nonsynonymous variants: |
|                  |                    |               | 169486641 G > C | 202 R/H (exon 23) | No dbSNP ID |
|                  |                    |               | 169519112 C > T | 513 R/K (exon 10) | rs6020    |
|                  |                    |               | 169541513 C > G | 107 D/H (exon 3) | rs6019    |
|                  |                    |               | Heterozygote for synonymous variants: |
|                  |                    |               | 169529973 C > T | 135 A/A (exon 4) | rs6029    |
|                  |                    |               | 169529826 C > A | 184 S/S (exon 4) | rs6022    |
| **Father of proband** | No bleeding | Reported normal | Heterozygote for nonsynonymous variants: |
|                  |                    |               | 169486641 G > C | 202 R/H (exon 23) | No dbSNP ID |
|                  |                    |               | 169519112 C > T | 513 R/K (exon 10) | rs6020    |
|                  |                    |               | 169541513 C > G | 107 D/H (exon 3) | rs6019    |
|                  |                    |               | Heterozygote for synonymous variants: |
|                  |                    |               | 169529973 C > T | 135 A/A (exon 4) | rs6029    |
|                  |                    |               | 169529826 C > A | 184 S/S (exon 4) | rs6022    |
| **Proband’s husband** | No bleeding | Reported normal | No above CDS variants detected in F5 | — | — |

The most recently (Oct 2012) accessed Human Genome Mutation database (www.hgmd.org) lists 145 missense mutations in the F5 locus associated with altered function of Factor V. From this list, 94 mutations represent single-point mutations, from which approximately 80 have been strongly associated with Factor V deficiency. In this respect, the present study does not add new mutations to this list but confirms the fact of previously described coinheritance of several separate mutations in the F5 locus [10, 11], and more importantly provides an example of co-inheritance of F5 mutations with presumably opposite phenotypic coagulation characteristics. Similar situation (i.e., coinheritance of polymorphic variants from which one is associated with decreased Factor V activity and other with increased thrombosis) was described previously for much more frequent prothrombotic Leiden mutations, or more recently, promoter −426 G/A polymorphism [12, 13]. Our finding confirms that other prothrombotic mutations in the F5 gene locus may be independently inherited in one patient.

The present study exemplifies the use of NGS approach for detailed diagnosis of the specific clinical pathology and identification of causative mutations for rare genetic disorder. This approach has been recently advocated for both diagnosis and therapy [14]. The obtained genetic data for this cases were successfully used clinically for the peripartum anesthetic management of the previously described patient.
Table 3: Summary of observed variants in noncoding sequences of F5 gene in investigated proband.

| Type of variant | Type of carrier | Position-R | Position-L | Reference base | Variant base | SNPs (ID) |
|-----------------|-----------------|------------|------------|----------------|--------------|-----------|
| Variants in 5' - UTR | | | | | | |
| SNP             | Hom             | 169556050  | 169556051  | c              | t            | rs2269648 |
| SNP             | Hom             | 169556152  | 169556153  | g              | t            | New variant |
| INS             | Het             | 169556812  | 169556812  | a              |             | rs58897818 |
| Intronic variants | | | | | | |
| SNP             | Hom             | 169486641  | 169486642  | g              | c            | rs9332666 |
| SNP             | Hom             | 169490392  | 169490393  | g              | c            | rs2420370 |
| SNP             | Hom             | 169491555  | 169491556  | g              | a            | rs2420371 |
| SNP             | Hom             | 169496536  | 169496537  | g              | c            | New variant |
| SNP             | Hom             | 169498056  | 169498057  | a              | g            | rs2420372 |
| SNP             | Hom             | 169516507  | 169516508  | g              | a            | rs12046953 |
| SNP             | Hom             | 169517159  | 169517160  | c              | t            | rs12026997 |
| SNP             | Hom             | 169517385  | 169517386  | a              | g            | rs12044669 |
| SNP             | Hom             | 169517904  | 169517905  | g              | a            | rs2420374 |
| SNP             | Hom             | 169518703  | 169518704  | t              | c            | rs58875232 |
| SNP             | Het             | 169518885  | 169518886  | a              |             | rs55717622 |
| SNP             | Hom             | 169519417  | 169519418  | g              | a            | rs7537742 |
| SNP             | Hom             | 169519765  | 169519766  | a              | g            | rs1306331 |
| SNP             | Hom             | 169520098  | 169520099  | a              | g            | rs10800456 |
| SNP             | Hom             | 169521553  | 169521554  | g              | a            | rs2213868 |
| SNP             | Hom             | 169521733  | 169521734  | c              | t            | rs9332582 |
| SNP             | Hom             | 169523346  | 169523347  | a              | g            | rs7555832 |
| SNP             | Hom             | 169523389  | 169523390  | t              | c            | rs1577059 |
| SNP             | Het             | 169523995  | 169523996  | a              | t            | New variant |
| SNP             | Het             | 169523996  | 169523997  | a              | g            | New variant |
| SNP             | Het             | 169523998  | 169523999  | t              |             | rs16132528 |
| INS             | Het             | 169524860  | 169524864  | cac            |             | New variant |
| SNP             | Het             | 169524865  | 169524866  | a              | g            | New variant |
| SNP             | Hom             | 169525312  | 169525313  | c              | t            | rs9332579 |
| INS             | Het             | 169525558  | 169525558  | cctggc         |             | rs16684 |
| SNP             | Het             | 169525680  | 169525681  | c              | a            | rs15199761 |
| SNP             | Hom             | 169525766  | 169525767  | t              | c            | rs2239853 |
| INS             | Hom             | 169526266  | 169526266  | a              |             | rs9332577 |
| SNP             | Hom             | 169526300  | 169526301  | c              | t            | rs4656688 |
| SNP             | Hom             | 169526367  | 169526368  | a              | g            | rs4656689 |
| SNP             | Hom             | 169526425  | 169526426  | g              | t            | rs4656188 |
| SNP             | Hom             | 169526601  | 169526602  | g              | c            | rs894697 |
| SNP             | Hom             | 169526646  | 169526647  | a              | g            | rs894698 |
| SNP             | Hom             | 169526950  | 169526951  | c              | g            | rs894699 |
| SNP             | Hom             | 169527226  | 169527227  | a              | g            | rs981891 |
| DEL             | Hom             | 169527470  | 169527471  | a              |             | rs3835454 |
| SNP             | Hom             | 169528075  | 169528076  | c              | g            | rs9332570 |
| INS             | Het             | 169528255  | 169528255  | aaa            |             | rs58738850 |
| SNP             | Hom             | 169528580  | 169528581  | c              | t            | rs6012 |
| SNP             | Hom             | 169528722  | 169528723  | c              | t            | rs6427201 |
| SNP             | Hom             | 169528830  | 169528831  | c              | t            | rs6427202 |
| SNP             | Hom             | 169529031  | 169529032  | a              | c            | rs6427203 |
| SNP             | Hom             | 169529132  | 169529133  | c              | t            | rs6699691 |
| SNP             | Hom             | 169529138  | 169529139  | c              | t            | rs5893047 |
| SNP             | Hom             | 169530070  | 169530071  | a              | c            | rs7545236 |
| SNP             | Hom             | 169530077  | 169530078  | g              | t            | rs7523043 |
Table 3: Continued.

| Type of variant | Type of carrier | Position-R | Position-L | Reference base | Variant base | SNPs (ID) |
|-----------------|-----------------|------------|------------|----------------|--------------|-----------|
| SNP             | Hom             | 169530093  | 169530094  | t              | c            | rs7534848 |
| SNP             | Hom             | 169530176  | 169530177  | c              | g            | rs7522982 |
| INS             | Hom             | 169530532  | 169530532  | a              | rs5778621; rs7719210I |
| SNP             | Hom             | 169530586  | 169530587  | t              | c            | rs1894701 |
| SNP             | Hom             | 169531442  | 169531443  | t              | c            | rs7540556 |
| SNP             | Hom             | 169531571  | 169531572  | t              | c            | rs4656690 |
| SNP             | Hom             | 169533266  | 169533267  | g              | a            | rs6678795 |
| SNP             | Hom             | 169533744  | 169533745  | t              | a            | rs724509  |
| SNP             | Hom             | 169534028  | 169534029  | t              | c            | rs724507  |
| SNP             | Hom             | 169534966  | 169534967  | t              | a            | rs2040443 |
| SNP             | Hom             | 169535353  | 169535354  | c              | g            | rs6685578 |
| SNP             | Hom             | 169535367  | 169535368  | t              | c            | rs2213869 |
| SNP             | Hom             | 169536650  | 169536651  | g              | a            | rs2187955 |
| DEL             | Het             | 169536796  | 169536798  | tt             | t            | New variant |
| SNP             | Hom             | 169537678  | 169537679  | a              | g            | rs6670678 |
| SNP             | Hom             | 169538466  | 169538467  | g              | a            | rs9287095 |
| SNP             | Hom             | 169538544  | 169538545  | c              | t            | rs2298908 |
| SNP             | Hom             | 169538603  | 169538604  | t              | g            | rs2298906 |
| SNP             | Hom             | 169539348  | 169539349  | t              | g            | rs6663533 |
| SNP             | Hom             | 169543263  | 169543264  | c              | a            | rs10800457 |
| SNP             | Hom             | 169545413  | 169545414  | a              | g            | rs6677374 |
| SNP             | Hom             | 169549775  | 169549776  | t              | c            | rs10753787 |
| INS             | Het             | 169551560  | 169551560  | a              | rs56901113  |
| SNP             | Hom             | 169554058  | 169554059  | c              | g            | rs3753305 |

Variants in 3' -UTR

| SNP         | Hom                          | 169479974 | 169479975 | c         | t         | rs970740 |

Hom: homozygote, Het: heterozygote, SNP: single-nucleotide polymorphism, Del: deletion, and Ins: insertion

[15]. Comprehensive genetic analysis through NGS based approaches will increasingly be helpful in establishing the diagnosis of Factor V deficiency (or other genetic coagulation disorders) and thereby improve patient management.

**Conflict of Interests**

The authors declare that there is no conflict of interests.

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