Ninety novel F8 and F9 gene variants causing hemophilia A or B – report from the PedNet cohort

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April 28, 2020

Abstract

In hemophilia A and B, analysis of the F8 and F9 variants has become standard over recent decades, giving information on the severity of hemophilia, inhibitor formation and allowing counseling for the families. The PedNet Registry collects data on hemophilia in children and has more than 2000 children listed. Genetic reports are collected uniformly and re-evaluated following international guidelines. We report 90 novel variants in the F8 and F9 gene, respectively, causing hemophilia with detailed information on severity, factor level and inhibitor formation. This will lead to further guidance for genetic laboratories and the treating physician. These findings can be implemented in hemophilia variant databases. The study highlights the need to re-evaluate and update earlier genetic reports in hemophilia both locally but also in variant databases in the light of changed nomenclature, the use of in silico prediction and new sequencing techniques.

Introduction

Hemophilia A and B are X-linked recessive congenital bleeding disorders caused by pathogenic variants in, respectively, the *F8* or*F9* gene. Hemophilia A, caused by lack or dysfunction of the plasma protein factor VIII (FVIII), affects about 1/5000 males, while hemophilia B, caused by lack or dysfunction of factor IX (FIX), affects approximately 1/30,000 males (Mannucci and Tuddenham 2001). Depending on the residual clotting activity in plasma levels of FVIII or FIX, hemophilia is categorized as severe (< 1%), moderate (1–5%), or mild (6–40%). The cornerstone of hemophilia treatment is replacement therapy with FVIII/FIX concentrates and – recommended by the World Health Organization (WHO) – treatment with prophylaxis in moderate and severe hemophilia (Andersson*, et al* 2017, Manco-Johnson*, et al* 2007). The main complication of replacement therapy is the development of anti-FVIII/FIX antibodies (inhibitors), which are able to neutralize the clotting activity of therapeutic clotting factors. (Gouw*, et al* 2013).

Since the *F8/F9* mutation type is the main determinant of disease severity, the analysis of the *F8* or *F9* gene variant in hemophilia patients and their families has become standard in hemophilia treatment centers

in recent years. Knowledge of the variant allows genetic counseling and provides information on the risk of inhibitor development. Additionally, information on clotting assays discrepancies, and in mild hemophilia A the probability of a therapeutic response to DDAVP, can be retrieved (Goodeve and Peake 2003, Seary*, et al*2012). Sporadic cases, *i.e.* with no known family history of hemophilia, accounts for approximately 50% of all cases. If hemophilia is diagnosed for the first time in a patient, studies show that the new variants are found in around 70–80% of the mothers of these index cases (Ljung*, et al* 1991, Martensson*, et al* 2016).

Currently, direct gene sequencing either through Sanger or next-generation sequencing (NGS) methodologies is the predominant technique for the testing of single nucleotide variants (SNVs) and small insertions and deletions (Gomez and Chitlur 2013). Nowadays, copy number variant (CNV) analysis for large deletions and duplications is performed by NGS or complimentary technologies such as array comparative genomic hybridization (aCGH) and multiplex ligation-dependent probe amplification (MLPA). For the *F8* intron 22 inversion, Southern blot, long-range PCR, and inverse PCR protocols are used, while for the*F8* intron 1 inversion, a PCR-based method is the standard technique. The most common variant causing severe hemophilia A is intron 22 inversion in *F8* affecting roughly 40% of the patients but today a broad spectrum of more than 2000 mutations causing hemophilia A and more than 1000 mutations causing hemophilia B are described in FVIII or FIX variant databases, such as the American CDC Hemophilia Mutation Project databases CHAMPS/CHBMPS (*https://www.cdc.gov/ncbddd/hemophilia/champs.html*)or the European EAHAD Coagulation Factor Variant Databases (*http://www.eahad-db.org*), to which *F8 and F9* gene variants from all over the world are reported randomly (Li*, et al* 2013, McVey*, et al* 2020, Payne*, et al* 2013). The variant types in hemophilia cover a broad spectrum: in addition to the *F8* gene specific inversion 22 and inversion 1, SNVs, deletions, duplications, and complex mutations are found causing missense, nonsense, frameshift, deletion/insertion/duplication in frame, splice site mutations and promotor variants. Usually, new variants are crosschecked with the above-named hemophilia variant databases, such as the European Coagulation Factor Variant Databases from EAHAD, the CDC-based CHAMPS/CHBMPS or Human Gene Mutation database (HGMD), which collects large number of published gene alterations. In these databases, additional information about the number of patients with each reported variant and clinical information on severity of the disease, factor levels and inhibitor development on every reported patient may be available (Li*, et al* 2013, Payne*, et al* 2013).

The clinical interpretation of a new or an unpublished genetic variant in the *F8* or *F9* genes as well as other genes should be based on guidelines published by American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards 2015). International *F8/F9* gene variant databases assist effective variant classification especially when possible to combine with phenotypic and pedigree information. Various *in silico*prediction programs developed for missense or splice site variants may be helpful but the provided information should be interpreted by caution as vast majority of predicted pathogenic missense variant are not disease causing. Recently, a guideline specific for genetic analysis in bleeding disorders has been published (Gomez*, et al* 2019). When predicting the pathogenicity of a gene variant it is recommended to combine several prediction programs (Richards*, et al* 2015).

The PedNet Registry cohort contains prospective data on children < 18 years with hemophilia A or B born since 1 January 2000 who are followed up regularly in 31 hemophilia centers in 18 countries in Europe, Canada and Israel. More than 2100 patients were included in 2019 and the *F8/F9* gene mutation is known in 85% of the cases (Fischer*, et al* 2014). The purpose of the Registry is to promote and facilitate research and development of care in this large unselected patient population. The aim of this paper is to report all new variants, not previously published or known in databases, of *F8* and*F9* found in the PedNet cohort and predict the pathogenicity.

Methods

The PedNet Cohort

Data were retrieved from the 'PedNet Registry Cohort' which is owned and administered by the 'PedNet Haemophilia Research Foundation', consisting of 31 international hemophilia treatment centers in 18 coun-

tries and registered at ClinicalTrials.gov at NCT02979119.A complete list of PedNet members is added in the appendix. Approval for data collection was obtained from each center's ethical review board, and written informed consent was obtained from the parents or guardians of all participants, in accordance with the Declaration of Helsinki.

Subjects

All patients with either hemophilia A or B, registered in the PedNet registry by 1 January 2018 (n=1967) were included. Data on patients' demographics, type and severity of hemophilia, and family history of hemophilia were collected. Reports on genotyping collected from each single center were then classified uniformly by a central genetic laboratory according to the recommendations of the Human Genome Variation Society (HGVS). Mutation nomenclature was based on the following transcripts: NM_000132.3 (F8) and NM_-000133.3 (F9) and GRCh38 genome build. All mutations were cross-checked with the CDC-based databases CHAMPS and CHBMPS, the EAHAD database and the HGMD (Human Genome Mutation database) and a literature search on 1 January 2020, and only mutations not described in these databases or published in a scientific journal searchable on Medline were included in this manuscript; these are referred to as 'new variants'.

Phenotype of hemophilia

The PedNet Registry follows the international classification for hemophilia valid when the Registry was initiated (*i.e* .: severe form FVIII/FIX <1%, moderate 1–5% and mild with 6–25%) and not the present classification where mild form is defined as 6–40% (Blanchette*, et al* 2014). FVIII/FIX levels were measured at each participating center according to local standards. Both chromogenic and one-stage assay methods were accepted.

Inhibitors

All patients in the PedNet Registry are followed regarding development of inhibitors in regular annual follow-ups. Inhibitors are divided into low- and high-titer inhibitors according to international guidelines (Blanchette*, et al* 2014). In this study, inhibitors are reported for the new variants in order to support clinical information on this specific variant. Following international standards, low-titer inhibitors were defined as [?] 5 BU (Bethesda Units) and high responder as > 5 BU.

Classification of reports on genotypes

In similarity with the established databases of CHAMPS, CHBMPS and EAHAD, we used the following classifications:

The type of mutation in *F8* was classified as inversion 22, inversion 1, point mutation, deletion, duplication, insertion, polymorphism or complex mutation.

The type of mutation in *F9* was classified as point mutation, deletion, duplication, insertion, polymorphism or complex mutation.

The effect of mutation was reported in both *F8* and *F9* as missense, nonsense, frameshift, large deletion/insertion/duplication (>50 base pairs), small deletion/insertion/duplication (<50 base pairs) in frame, silent mutation, splice site mutation and promotor mutation.

In silico analyses

The deleterious effects of missense variants were assessed with ALAMUT VISUAL (http://www.interactive-biosoftware.com/alamut-visual/), a web-based tool, which allows simultaneous analysis by POLYPHEN-2 (http://genetics.bwh.harvard.edu/pph2) SIFT (*http://sift.bii.a-star.edu.sg*), MutationTaster (*http://www.mutationtaster.org* and GVGD (*http://agvgd.iarc.fr/index.php*).

The effects of variants at splice junctions were evaluated with ALAMUT VISUAL v.2.8.1 (http://www.interactive-biosoftware.com/alamut-visual/), which allows a simultaneous analysis with the programs SPLICE SITE

FINDER-LIKE, MAXENT SCAN, NEURAL NETWORK SPLICE SITE, GENESPLICER, and HUMAN SPLICING FINDER (http://www.cbs.dtu.dk/services/NetGene2/). These tools were used together in accordance with guidelines for using prediction methods (Niroula and Vihinen 2016). Missense variants close to splice sites underwent splice site prediction, too. If three or more of four prediction programs predicted that the mutation under consideration was causing disease, it was accepted as causing hemophilia. For splice site mutations, four out of five prediction programs had to be significant (defined how).

Results

Overall, 1967 patients were included in the study. Of those, 1681 patients had a report on genotyping in the Registry (85.5%). Out of 1681 patients with hemophilia A or B, with all severities, 106 patients had 97 new variants, of which 90 were likely to cause hemophilia: 73 hemophilia A and 17 hemophilia B; five variants in *F8* and two variants in *F9* gene were classified as non-disease causing after*in silico* analysis (table 1 and table 8). Of the 90 new disease-causing variants, 82 represent new unique variants present in only one patient. Eight variants were present in [?] two patients; all were found in patients who were related family members with hemophilia (e.g. brother, cousin; table 12). As expected, the majority of the new variants found were located in exon 14 for hemophilia A and in exon 8 for hemophilia B.

F8 – Hemophilia A

With respect to hemophilia A (n=73), 39 new variants were SNVs, 27 were deletions, two were complex mutations, four were duplications and one was an insertion (see Table 1). In mild and moderate hemophilia A, all new alterations were missense variants. Table 1 gives more detailed information on variant type and effect in all variants found in the cohort.

Variants that could be classified directly as causing severe hemophilia were: deletions (n=27), duplications (n=4), insertions (n=1) and complex mutation type (n=2) causing frameshift, small and large structural changes (reported in Table 2) and variants causing nonsense effect (n=11) (reported in Table 3).

SNVs leading amino acid alterations (missense) and splice site mutations underwent *in silico* analysis with prediction programs, as described above. Twenty-three of 26 variants causing missense mutations (Table 4) and seven of nine splice site variants (Table 5) could be confirmed as causing hemophilia. The five variants were classified as non-disease causing or likely benign (Tables 6 and 7). In total, 73 of 78 new variants found in this cohort were found to be causative of hemophilia A. Inhibitors were diagnosed in 18 patients carrying one of these 73 new variants, all found in patients with the severe form of the disease, with the exception of one variant p.Glu409Lys found in moderate hemophilia.

Hemophilia B

With regard to hemophilia B, in total 19 variants were found (Table 8): 13 in severe hemophilia B, three in moderate hemophilia B and one in mild hemophilia B patients. Similarly to hemophilia A, duplications causing frameshift (n=3), insertions (n=1), SNVs with nonsense effect (n=3), duplications causing small insertions/duplications (<50bp, in-frame; n=1) and frameshift and deletions causing frameshift (n=4), all caused severe and one moderate phenotype of hemophilia B and were regarded to cause hemophilia B without further investigations (Table 9). Five out of the eight variants with missense effect could be classified as disease-causing by *in silico*analysis (Tables 10 and 11), resulting in a total of 17 new variants found to cause hemophilia B. In one new variant an inhibitor was reported.

Discussion

Mutation analysis in hemophilia has become a standard procedure over the years, giving confirmation of the suspected disease, making carrier diagnosis possible and enabling identification of variants with inhibitor risk. In this study encompassing data from 1681 children included in the PedNet Registry with hemophilia A and B, we can report 90 causative new variants in *F8* and *F9*. These variants were not previously reported in the HGMD or CHAMPS, CHMBS and EAHAD hemophilia variant databases. The new variants were frequently found in exon 14 in hemophilia A and exon 8 in hemophilia B as expected, since both are the

largest exons in *F8* and *F9,* respectively. No 'hotspot' was identified, and the new variants occurred in all variant types following the general spectrum of variants types in hemophilia A and B. This is in line with a report from Johnsen *et al* ., in which 3000 hemophilia patients were investigated with NGS and 285 new variants were found in all variant types and *F8* or *F9* loci (Johnsen*, et al* 2017).

In hemophilia A, 73 of 78 and in hemophilia B, 17 of 19 new variants were found to be causative. However, seven variants could not be proven to be causative by *in silico* analysis: five variants in mild hemophilia patients (three hemophilia A, two hemophilia B), one in a severe hemophilia A patient having another causative mutation and one in a severe hemophilia A patient without other explanations. In general, probable disease-causing variants are identified in approximately 95% of hemophilia A cases and in almost all patients with hemophilia B (Swystun and James 2017). It is a known phenomenon that *in silico*analysis – even if combined – can be non-conclusive and should be seen as only one of the steps in categorizing variants as described by the ACMG. Another explanation could be misdiagnosis due to overlapping phenotypes, e.g. von Willebrand disease causing low FVIII levels, as well as deep intronic variants altering mRNA splice sites.

Since the data were retrieved from the PedNet hemophilia foundation with 31 centers over the last two decades, reporting of genotype may vary from centers over time. External quality assessment can be used to ensure good quality genetic reports, which have become more complex during the last decades: the availability and range of methodologies for genetic diagnosis for hemophilia increases, e.g. by implementing NGS, the evaluation of genotyping accuracy, standardization of nucleotide and protein variant descriptions using Human Genome Variation Society (HGVS) nomenclature has to be used correctly, and variant pathogenicity assessment strategies such as *in silico* analysis have become essential(Gomez*, et al* 2019). To ensure high-quality reporting in the Pednet Registry, all reports were re-evaluated and updated with HGVS nomenclature and classification in 2018/2019 (den Dunnen 2017) by a genetic laboratory technician and two MDs (Lund University, Malmö/Lund, Sweden). A regular update of genetic reports is planned for the Pednet Registry and all new reports to be included are re-evaluated continuously.

While hemophilia genetic variant databases are very useful, it should be noted that they have certain limitations too. Reporting in hemophilia variant databases, such as EAHAD, CHAMPS and CHBMPS, does not require a specific standard on genetic reports and usually no follow-up reports or re-evaluations are carried out. Also, the update of these registries is not performed continuously and by now, according to their website information and personal communication, the CHAMPS/CHMBPS database had the latest update in December 2014 and the EAHAD variant database in 2017. To be sure that a new variant is being presented, a literature search has to be performed additionally. The HGMD bases the entries on published variants; however, not every new variant is published in a medical journal and HGMD contain large number of misclassified variants similar to scientific literature. The definition of variant type and effect differs between databases and publications and adaptations are needed for comparisons. There is no requirement to classify variants by the guidelines or to prove specific variants – such as missense or splice site – as likely causative by prediction programs, even if most new reports will follow these standards today. Most probably, some variants in the databases reported are polymorphisms not causing hemophilia, which was also suggested by another group 'My Life, Our Future initiative', finding 11 earlier reported variants unlikely to cause hemophilia (Johnsen*, et al* 2017).

In 19 variants, inhibitor development was reported: 18 variants in hemophilia A patients (18/73; 24.7%), and one in a patient with severe hemophilia B (1/17; 5.9%). Although the new variants only represent a subgroup of our population-based registry, this follows the expected rate of inhibitor formation for hemophilia A and B. The highest rate of inhibitors was found in hemophilia A as expected: most inhibitors occurred in deletions causing large structural change (100%), followed by complex variants (50%), variants with nonsense effect (45.4%), in variants with duplication (33%), variants with splice site effect (27.2%) and variants with deletions causing frameshift (18.2%). Inhibitors were rarely reported in variants with missense effect. This is in line with earlier publications (Gouw*, et al* 2012).

Information on inhibitor formation in specific variants is important for clinical practical issues. The use and start of prophylaxis can be influenced if the variant is known to cause inhibitors, especially in the light of

new emerging non-factor therapies in hemophilia that could avoid early exposure to FVIII (Mahlangu, *et al* 2018). In mild and moderate hemophilia A, it is known today that these patients have a relevant risk for the development of inhibitors during their lifetime (Eckhardt, *et al* 2013). A known risk of inhibitor development could lead to the recommendation of the use of DDAVP as a first-line therapy, which enhances the internal release of FVIII in mild hemophilia A patients and avoids the formation of inhibitors.

In conclusion, we report 90 new causative variants in hemophilia A and B, with detailed information on severity, factor level and inhibitor formation. This will lead to further guidance for genetic laboratories and the treating physician and these findings can be implemented in variant databases. The strength of our study is the uniform collection of variants in a large cohort with regular re-evaluation of genetic reports following international guidelines. The study highlights the need to re-evaluate and update earlier genetic reports both locally, but also in variant databases in the light of changed nomenclature, the use of population database data e.g. gnomAD, *in silico* prediction and new sequencing techniques, such as NGS or MLPA.

Acknowledgements

This study is supported by the PedNet Haemophilia Research foundation and by grants from The Swedish Research Council (2015-02957). Unrestricted sponsorship for the foundation is currently received from: Bayer AG, Novo Nordisk Healthcare AG, Pfizer SRL, CSL Behring GmbH, Swedish Orphan Biovitrium AB, Takeda, Hoffmann La-Roche.

We greatly appreciate the support of the PedNet Foundation staff members, especially Marloes de Kovel and Ella van Hardeveld.

Disclosures and competing interests

All authors report no conflicts of interest for this study.

Data Availability Statement

The data that support the findings of this study are available from the registry of the PedNet Haemophilia Research Foundation. Restrictions apply to the availability of these data, which were used under license for this study. Data are available from the authors with the permission of PedNet Registry Foundation (www.pednet.eu).

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