

The origin of mutation in sporadic hemophilia B

Research program

Research group

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Purpose

The general purpose of this research program is to improve the genetic counselling and diagnosis of carriers of sporadic haemophilia B.

Specific research questions:

1. What is the origin of mutation in sporadic cases of haemophilia B (*i.e.* haemophilia is not known in the pedigree). The frequency of mothers of a sporadic case who are carriers vs. non – carriers?
2. The mothers who are carriers – where did the mutation occur? A study of three generations to reveal grandparental male or female origin of mutated X-chromosome.
3. The mothers who are not carrier by conventional Sanger sequencing – what is the frequency of mosaicism, *i.e.* mutations are carried in part of the somatic or gonadal cells not revealed by conventional diagnostics?
4. Which is the preferred method to diagnose mosaicism in a clinical setting - SNP (single nucleotide polymorphisms), microsatellites (MS), droplet digital PCR or NGS (Next generation sequencing) by Ion Torrent sequencing?

Survey of the field / background

Haemophilia A and B are hereditary, X-chromosomal recessive bleeding disorders caused by absent, deficient or dysfunctional factor VIII (FVIII) and factor IX (FIX), respectively. Depending on the concentration of FVIII or FIX coagulant activity in blood, the disorders may be classified as severe (<1% of normal activity), moderate (1-4%) or mild (5-25%). In Sweden we have approximately 800 patients (belonging to 375 families) with haemophilia A and approximately 170 patients (belonging to 100 families) with haemophilia B. Patients with

clinically severe haemophilia receive prophylactic treatment with FVIII/FIX concentrate. Untreated the patients will develop spontaneous bleeds in particular in joints and muscles and are at the risk of life-threatening intracranial hemorrhages.

Approximately 60% of newly diagnosed cases of haemophilia A or B are sporadic cases, *i.e.* hemophilia is previously not known in the family. We have recently, by haplotyping using SNPs and microsatellites, studied Swedish families with haemophilia B carrying identical mutations and have investigated whether the mutations are recurrent mutations, *i.e.* are unrelated new mutations, or are identical by descent, *i.e.* share a common origin (1-3). We found that approximately 50% of families having the same mutation, had true unique mutations while the remaining were related, due to a “founder-effect, without knowing it. This background information will now allow a 100% correct identification of a sporadic case of haemophilia B and thus a study group without selection bias to study the origin of mutation in hemophilia B and the phenomenon of mosaicism.

The frequency of mosaicism in haemophilia A has been analysed previously using rather unsensitive allele-specific PCR. In total 13% of the 61 analysed families showed the presence of somatic mosaics. They were present exclusively in the 32 families with point mutations (25%) (4). Recently we published a study on the origin of mutation in sporadic haemophilia A (5). Microsatellite haplotyping determined if the X chromosome was of grandpaternal or -maternal origin. Droplet digital polymerase chain reaction (ddPCR) was used in a pilot study to reveal mosaicism mutations not found on conventional DNA sequencing. In 28/45 families the mother carried the mutation and in 5/28 families the grandmother was also a carrier. The X chromosome was of grandpaternal origin in 17/23 cases. In 17/45 families the mother was a non-carrier. Digital droplet PCR (ddPCR) was used and was found to be a powerful new analysis method for mosaicism in HA with detection limit of 1/10.000 cells compared to conventional Sanger sequencing with about 1/10 cells (5).

The frequency of mosaicism in hemophilia B has not been studied systematically in a here-generation cohort and only one report from the 90s, based on the UK haemophilia B Registry, suggested it to be rare (<6.25%) (6). The frequency of carriers as well as frequency of mosaicism are important to know in the genetic counselling of haemophilia B families and are crucial in diagnosis of carriers and carriers’ decision to perform prenatal diagnosis (PND). The aim of this study is to make a similar investigation of sporadic families of haemophilia B as we previously did on sporadic cases with haemophilia A and also use new powerful technologies.

Working plan

Study group.

Sporadic cases of severe and moderate haemophilia B (index case), mothers and grandparental mothers and fathers of the index case (family of three generations with at least one living grandparent). Families will be recruited from the Hemophilia Centres in Malmö, Gothenburg and Stockholm in Sweden and Copenhagen and Aarhus in Denmark. Applications will be send to respective Ethical Review Boards and informed written consent will be obtained.

Methods and equipment.

DNA. Blood samples/mouth swabs will collected from relatives in three generation pedigrees and DNA will be isolated using QIAmp DNA Blood Maxi Kit (Qiagen, Hilden, Germany).

All samples will be screened for mutations, the segregation of the mutation carrying X-chromosome will be determined by microsatellite markers and mosaicism will be studied in all non-carriers.

Mutation screening. The screening for F9 mutations will be done by conventional Sanger sequencing and deletions/duplications by multiplex ligation-dependent probe amplification (MLPA).

Microsatellite markers. The inheritance/segregation pattern of the mutation-carrying chromosomes will be determined by genotyping a set of microsatellite markers for the index patient, the carrier mother and the grandparents. The microsatellite markers are amplified using Veriti 384 polymerase chain reaction (PCR) machines (Applied Biosystems, Foster City, CA, USA), PCR products are separated by capillary electrophoresis on an ABI 3130 XL sequencer (Applied Biosystems) and the genotyping data analysed using GeneMarker Software (SoftGenetics, USA).

Droplet digital PCR (ddPCR) will be used to reveal potential mosaicism in the non-carrier mother. The ddPCR analysis will use a QX100 ddPCR system from Bio-Rad (Bio-Rad, Hercules, CA, USA) and TaqMan genotyping to quantify the mutant alleles. TaqMan systems will be designed for mutations to be investigated using RealTimeDesign Software, BioSearch Technology (<https://www.biosearchtech.com/display.aspx?pageid=54>), checked for cross-hybridization using NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and ordered from DNA Technology A/S (Risskov, Denmark). The ddPCR reaction mixture containing template DNA, 1xSupermix (Bio-Rad), 900 nM of each primer and 250 nM of each probe is mixed with droplet generation oil in a DG8 disposable droplet generator cartridge using a QX100™ Droplet Generator (Bio-Rad). The generated droplets are transferred to an Eppendorf 96-well twin.tec PCR plate and heat-sealed using pierceable foil seal. PCR amplification will be performed in a Veriti® 96 PCR machine (Applied Biosystems). Droplets are counted in a QX100™ droplet reader and the data analyzed using QuantaSoft software (Bio-Rad).

Next generation sequencing (NGS) will be performed on an Ion Torrent PGM sequencer. Target sequences will be amplified, sequence templates created by limiting dilution and sequenced on a chip. The applicability of this universal mutation detection system will be evaluated for its usefulness in detection of mosaics (as well as in addition the causative mutation) in comparison to ddPCR.

All equipment for performing the studies is available in our own laboratories in Malmö and Kristianstad and all processes have been validated.

Clinical significance

This research program has a “translational structure” since it bridges from basic scientific questions to clinical applications, combines Lund University with Kristianstad University College.

The origin of mutation and frequency of mosaicism in sporadic hemophilia B cases will be of great importance in our genetic understanding of the disease and, translated to the clinic, add valuable knowledge to the genetic counselling and genetic diagnosis in the families for the future. It will also be of particular interest in the developing world if our studies on origin of mutation will help to make a simple pedigree analysis of a sporadic case more accurate and thus enable a more selective approach to DNA-analysis.

Preliminary results

Our previous studies on haplotypes of haemophilia A and B has enabled us to select a group of “true sporadic cases” when “unknown familiar cases” have been excluded. We are not aware that any other group have done such haplotyping on a whole defined population which will provide us with a most valid study group.

A pilot study using ddPCR of 5 different *F8* mutations and artificially created mixtures of wild-type and mutant alleles has confirmed that the use of ddPCR for detection of mosaics is highly sensitive and precise. ddPCR uses allele-specific probes specific for the mutation in the family and will allow detection of very low proportions of the mutation. Our pilot study clearly showed that ddPCR allows detection levels in the range 0.01% and the use of ddPCR for detection of mosaics represent a paradigm shift in the analysis of mosaicism since it is highly sensitive and precise compared with previous methods.

AmpliSeq-based re-sequencing using Ion Torrent next generation sequencing has produced high quality data in a number of projects performed in the Kristianstad laboratory. The lab has more than 3 years of experience of managing and analysing next generation re-sequencing data.

References

1. Halldén C, Nilsson D, Säll T, Lind-Halldén C, Lidén AC, Ljung R. Swedish hemophilia A mutations. *J Thromb Haemost.* 2012; 10(12):2503-2511. (Impact Factor 6.081)
2. Halldén C, Mårtensson A, Nilsson D, Säll T, Lind-Halldén C, Lidén AC, **Ljung R.** Origin of Swedish hemophilia B mutations. *J Thromb Haemost.* 2013; 11(11):2001-8. (Impact Factor 6.081)
3. Mårtensson A, Letelier A, Halldén C, Ljung R. Mutation analysis of Swedish haemophilia B families - high frequency of unique mutations. *Haemophilia.* 2016 May;22(3):440-5. doi: 10.1111/hac.12854. Epub 2015 Nov 27. (Impact Factor 2.603)
4. Leuer M, Oldenburg J, Lavergne J-M, Ludwig M, Fregin A, Eigel A, **Ljung R**, Goodeve A, Peake I, Olek K. Somatic mosaicism in Hemophilia A: A fairly common event. *Am J Hum Genet.* 2001;69:75-87. (Impact Factor 10.542)
5. Mårtensson A, Ivarsson S, Letelier A, Manderstedt E, Halldén C, Ljung R. Origin of mutation in sporadic cases of severe haemophilia A in Sweden. *Clin Genet.* 2015 Dec 10. doi: 10.1111/cge.12709. [Epub ahead of print] (Impact Factor 3.931)
6. Green PM1, Saad S, Lewis CM, Giannelli F. Mutation rates in humans. I. Overall and sex-specific rates obtained from a population study of hemophilia B. *Am J Hum Genet.* 1999 Dec;65(6):1572-9.