Molecular Diagnosis of Haemophilia A in Clinical Practice

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SUMMARY
Understanding the pathogenesis of haemophilia A has allowed for detailed diagnosis of the condition at a molecular level. Evaluation of the interaction between factor VIII and von Willebrand factor has been utilised to distinguish haemophilia A from von Willebrand disease. The discovery of a wide spectrum of mutations in the factor VIII gene and their association with different severity of the disease has allowed for the development of a rational strategy for mutation detection in clinical settings. Characterisation of the genetic defects is required for carrier detection and antenatal testing, and this also helps to predict risk of factor VIII inhibitor development. Research is ongoing to establish less invasive prenatal testing and to move the testing to pregravid period. Key words: haemophilia A, von Willebrand factor, factor VIII, factor VIII inhibitor, mutation, inversion, polymorphism, prenatal testing, preimplantation genetic diagnosis.

PREDICTION OF CLINICAL SEVERITY
Plasma activity of factor VIII generally correlates well with clinical severity of the disease. Haemophilia A is classified as severe (FVIII<1%), moderate (1-5%) and mild (5-40%) to estimate future course of the disease.

In patients with severe haemophilia, frequent (several times a month) and spontaneous bleeding especially into weight-carrying joints and muscles should be expected. These patients represent about half of haemophiliacs and usually require long-term and expensive prophylactic administration of the factor VIII concentrates since early childhood. There is also an increased risk of the inhibitor development in these patients. Repeated bleeding with insufficient treatment leads to the development of haemophilic arthropathy and to severe derangement of the musculo-skeletal system. This may require an orthopaedic treatment, which is both demanding and expensive. Determination of carriers in these families has significant practical impact.

In the patients with moderate form of haemophilia, joint or soft tissue bleeding owing to minor injuries should be expected, usually several times a year.

Patients suffering from a mild form of haemophilia bleed mainly during surgical procedures performed without previous haemato logical preparation.

For clinical classification of haemophilia, determination of the factor VIII activity in plasma (FVIII:C) is sufficient, whether using one-stage (1, 2), two-stage (3) or chromogenic (4, 5) assay. New methods based on thrombin generation (6, 7) seem suitable also for laboratory follow-up of the efficacy of substitution treatment.

DIFFERENTIATION FROM VON WILLEBRAND DISEASE
Differentiation of haemophilia A from von Willebrand disease is a critical requirement for sound genetic counselling, owing to different mode of inheritance. It is also important for rational treatment of each of the diseases.

The stabilising influence of von Willebrand factor on the factor VIII in plasma is reflected by markedly decreased factor VIII (about 3% of normal) in the patients with complete deficiency of vWF.
of both gene and protein of the factor VIII are normal, while mutations in the vWF gene alter the ability of von Willebrand factor protein to bind and stabilise factor VIII (some of these mutations influence also the adhesive function of vWF).

A specific binding assay (vWF: FVIIIB) has been developed to detect diminished binding of normal factor VIII to tested von Willebrand factor (10). The principle and interpretation of the assay are explained in Technical Annex 1 and in the Fig. 1. When the functional assay is unavailable, the presence of specific type 2N mutations in vWF gene is sought (Technical Annex 2). However, negative finding does not completely exclude the type 2N vWD as a consequence of a novel mutation. Functional assay then needs to be performed.

**Prediction of Inhibitor Development**

In some patients the treatment with factor VIII concentrates induces specific alloantibodies that inhibit the function of factor VIII (inhibitor). The inhibitor significantly impacts on further treatment of the patients. The inhibitor arises mostly during the childhood in the course of the first 25 treatment days (11, 12). The occurrence of inhibitors has been reported in the range of 10–35% of severe haemophilic patients, depending on whether only clinically significant inhibitor with an impact on further therapy or also transient detection the antibodies is included. Risk factors associated with the inhibitor development have not yet been fully characterised. However, it is evident that they are both inborn and acquired in nature (13). It follows that in patients with increased genetic risk, the external risk factors should be minimised (desmopressin treatment (14), commencement of prophylactic therapy at higher age, limited switches between the factor VIII concentrates).

Estimation of the genetic risk is based on family history and knowledge of causal mutation. The presence of the inhibitor in a relative haemophilic is considered a risk factor. Extensive studies of a large numbers of patients with the inhibitor have permitted stratification of mutations in the factor VIII gene according to the risk of the inhibitor development, as is indicated in Tab. 1 (15). For instance, the intron 22 inversion – which occurs in almost half of the patients with severe form of haemophilia A – is associated with transient or permanent occurrence of the inhibitor in about one third of the cases. The highest risk is associated with gross deletions (inhibitor is present in about two-thirds of patients), which themselves are infrequent cause of severe haemophilia (16).

**Carrier Determination**

Haemophilia A is a gonosomal recessive disorder. Males usually suffer from bleeding while females are asymptomatic carriers. The investigation of carriership should be commenced in asymptomatic girls with family history of haemophilia at about 10 years of age. Determination is important for family planning, antenatal testing and for proper conducting the labour (care must be exercised to avoid risk of intracranial bleeding in a haemophiliac). Decreased factor VIII activity as observed in some carriers, is associated with increased risk of bleeding after teeth extraction or surgical procedures, and may lead to hypermenorrhea. A common cause of the decrease is non-random X-chromosome lyonization (17, 18), which leads to preferential utilisation of the damaged factor VIII gene. This can be verified by a laboratory test (Technical Annex 5).

In specific cases it is possible to identify carriers by simple pedigree analysis – “definite” carrier is the daughter of a haemophiliac, the mother of two haemophiliacs and the mother of a haemophiliac with family history of haemophilia traceable in the female line. Indirect methods of molecular genetics utilise polymorphic markers.
to trace the affected allele of the factor VIII gene throughout tested family (Technical Annex 4). This approach, however, may demand sampling of extensive number of family members. In addition, the indicated father is not always reliable. There is also a risk of error owing to recombination between the marker and causal mutation (<0.1% and 5% in case of use of intragenic or extragenic polymorphisms). The most reliable approach is direct determination of disease causing mutation. This is technically demanding and can be time-consuming. This approach allows for determination of carriers even among distant family members without sampling other relatives except of the haemophilic (Technical Annex 3). The formerly used ratio of factor VIII level and vWF (19) serves now for rough orientation only.

ANTENATAL TESTING

Invasive sampling such as chorionic villus sampling (CVS) or amniocentesis (AMC) carries about 1-2% risk of fatal and non-fatal complications to the foetus. It has to be performed by an experienced team. When indicating antenatal testing, this risk has to be weighed against the benefit - a possibility to make an informed decision regarding the abortion of the affected foetus, which is based on the results obtained from the sample (20, 21). As the severity of haemophilia remains stable within an individual family, the partners can base their decision on their own experience with the disease, while they are informed by a clinician about progress in haemophilia treatment. Prenatal testing is generally indicated in families with severe or moderate forms of haemophilia. In families with the mild disease such indication is rare. Preferably, the chorionic villus sampling is performed within the 10th–13th week of gestation. Indication for amniocentesis (between 15th–16th week) is warranted for pregnant women over 35 years of age or with another genetic disorder in family. The presence of Y chromosome in the tissue is detected rapidly by a PCR-based method (Technical Annex 6). If the foetus is male, the diagnosis of haemophilia is established either directly by proving the presence of causal mutation or indirectly by showing the presence of the affected allele (Technical Annex 3 and 4). The results are concluded within several days, as far as PCR-based methods can be used. The chromosomes are tested in parallel by cytogenetic methods to exclude presence of unexpected aberrations (results obtained after 2 or 3 weeks).

Occasionally, a carrier of severe haemophilia A is pregnant with a male foetus and at the same time she is homozygous (e. g. uninformative) in all polymorphisms while causal mutation has not yet been determined. In such cases chordocentesis is performed at 16th-18th week of gestation and antenatal diagnosis is established by factor VIII determination in the umbilical cord blood (22) (Technical Annex 7).

NOVEL APPROACHES

In female foetus the knowledge of antenatal diagnosis is not needed. It follows that, if the sex of the foetus was known, the invasive examination would not be indicated in about half of the cases. Therefore, less invasive procedures for sex determination are being developed, based on the venous blood sample taken from a pregnant woman at an early stage of gestation. This technique has been successfully used by our centre, so far on an experimental basis (Technical Annex 6) (23).

In principle, the assisted reproduction techniques shifted the decision regarding the fate of the affected embryo to the period before implantation into the mother’s uterus (Technical Annex 8) (24). At present in the Czech Republic, selection by the sex can be performed as an ethical makeshift solution. However, timed diagnosis could allow for the implantation of selected unaffected embryos of either sex. When established, this approach could be preferred by haemophilia families to current practice of antenatal testing associated with abortion of already developed though affected foetus.

TECHNICAL ANNEX

1. Determination of binding of factor VIII to von Willebrand factor

Specific functional assay based on the ELISA principle (see Fig. 1) allows detection of the decreased factor VIII binding to von Willebrand factor (25). The test is based on the patient’s plasma and requires 4 days. Von Willebrand factor from patient’s plasma is first immobilized on the solid phase and endogenous factor VIII is removed (Fig. 1A). Then the amount of added recombinant factor VIII that is bound to the binding sites on the examined molecule of von Willebrand factor, is determined (Fig. 1B). Control of the immobilisation efficacy of the tested vWF on the solid phase is performed (Fig. 1C). Patients with type 2N von Willebrand disease show decreased or undetectable amount of bound factor VIII (Fig. 1D).

2. Detection of selected mutations in the vWF gene

Mutations characteristic for type 2N von Willebrand disease are usually detected by direct sequencing of exons 18-21 and 24 gene for vWF. These exons code a binding site for factor VIII and contain several polymorphisms.

3. Detection of mutations in the factor VIII gene

Detection of causal mutation is technically demanding and time-consuming due to the considerable size of gene and high variability of genetic defects. First, the mutation is detected in a haemophilic who represents the given family (index patient). A definite carrier by pedigree analysis can supplement for the haemophilic, if he is unavailable *. Choice of a suitable method is guided by severity of the disease and pedigree analysis (Fig. 2). In the Czech Republic, the work has been facilitated by “National registry of patients with inherited coagulation disorders”, which is being created through a long-term collaboration of all nine haemophilia cen-

![Fig. 2. Strategy for mutation detection in the factor VIII gene](image-url)
tres in our country and is managed at ÚHKT (26). The registry comprises information regarding causal mutations detected in the index patients.

In almost half of the cases the cause of severe haemophilia A is an inversion of the factor VIII gene. The breakage site is commonly found in the intron 22 (about 45% of severe cases (27)), rarely in the intron 1 (2-5% of severe haemophilic cases), (28, 29)). Recently, it has become possible to detect both the inversions by PCR amplification of genomic DNA across the respective breakage sites (28, 30, 36). In case of the intron 22 inversion, very long and CpG rich segments need to be amplified by means of long-distance PCR (LD-PCR) (Fig. 3). In case of the intron 1 inversion, significantly shorter segments are amplified by multiplex PCR (M-PCR).

Except for the inversions, the haemophilia A causing mutations are usually unique and dispersed all over the entire length of the factor VIII gene. Their detection can be based either on the genomic DNA derived from peripheral leukocytes or on the mRNA ectopically transcribed in these cells (31). Amplification of the entire coding region (26 exons) together with the adjacent non-coding sequences can be performed. The other option is reverse transcription (RT-PCR).

4. Investigation of polymorphisms in the factor VIII gene
When utilising the indirect methods, the DNA of both parents of the suspected female carrier and possibly grandparents and other relatives is necessary in addition to that of the propositus and haemophiliac. The combined informativeness of the commonly used polymorphisms in the introns 19 (RFLP-Hind III) or 18 (RFLP-Bcl1) and VNTR-PCR in the introns 13 and 22 is about 80%. The polymorphisms in intron 7 and exons 14 and 26 may bring further information in some cases. Highly informative extragenous VNTR St14 (heterozygocity up to 90%) is burdened by high risk of recombination (3-5%) and is used in exceptional cases only.

5. Detection of non-random lyonization of chromosome X
The investigation is based on the fact that DNA of the inactivated X chromosome is modified by site specific methylation. This can be detected by restriction endonucleases sensitive to presence of the methylation. Besides the methylation site, the investigated genomic region should possess high frequency sequence variations, so that both chromosomes could be distinguished from each other in the majority of the tested carriers. The HUMARA gene (human androgen receptor) localised at Xq11 has been found to be a suitable locus (37). The investigation has to be carried on the genomic DNA.
6. Sex determination

The foetal sex can be determined by PCR based methods, utilising either Y chromosome specific sequence - SRY (38a) or a sequence in the amelogenin gene (38b)*, which is present on both sex chromosomes but differs in length. Advantage of the amelogenin locus is that in samples of either sex specific amplification is obtained. In contrary, lack of specific amplification may be either due to female sex or technical failure when SRY locus is employed. However, the SRY (but not amelogenin) gene can be utilised in highly sensitive real-time quantitative PCR (RQ-PCR) to detect trace amounts of Y chromosome in blood of a pregnant woman carrying male foetus (23).

7. Factor VIII determination in foetal blood

For the interpretation of factor VIII level (activity and antigen) in umbilical chord blood sample it is necessary to consider its developmental changes; factor VIII level is physiologically low (FVIII:C 25–80%, FVIII:Ag 11-46%) at the time of sampling (39, 40). Contamination of the sample with the mother’s blood or amniotic fluid has to be controlled. In the latter case, high amount of thromboplastin precludes correct determination of the factor VIII activity, while the factor VIII antigen remains unaffected. Estimation of the mother’s blood admixture in the foetal blood sample is based on differential acidoresistance of foetal and adult red cells (Kleihauer test) and the foetal and adult hemoglobin levels are determined.

8. Preimplantation genetic diagnosis

For the preimplantation genetic diagnosis (PGD), 1–2 blastomers are taken from the embryo at eight cells stage (24). Sex determination of the individual blastomer is performed by fluorescent in situ hybridisation (FISH). The development of reliable single-cell techniques suitable for preimplantation diagnosis of haemophilia is under way. The current approaches try to utilise the principles of diagnostic tests are based on the knowledge of molecular pathogenesis of haemophilia A (16). Diagnosis at the molecular level is necessary for genetic counselling and helpful for rational treatment. The methods and procedures are accessible in the Czech Republic to provide complex and reliable diagnosis of haemophilia A.

CONCLUSION

The principles of diagnostic tests are based on the knowledge of molecular pathogenesis of haemophilia A (16). Diagnosis at the molecular level is necessary for genetic counselling and helpful for rational treatment. The methods and procedures are accessible in the Czech Republic to provide complex and reliable diagnosis of haemophilia A.

Abbreviations

CCM - chemical cleavage mismatch
CNS - central nervous system
CSGE - conformation-sensitive gel electrophoresis
CVS - chorionic villus sampling
DGGE - denaturing gradient gel electrophoresis
DNA - deoxyribonucleic acid
FISH - fluorescent in situ hybridisation
HPLC - high performance liquid chromatography
HUMARA - human androgen receptor
LD-PCR - long-distance PCR
M-PCR - multiplex PCR
mRNA - messenger ribonucleic acid
PCR - polymerase chain reaction
PGD - preimplantation genetic diagnosis
PTT - protein truncation test
RFLP - restriction fragment length polymorphism
RQ-PCR - highly sensitive real-time quantitative PCR

RT-PCR - reverse transcription PCR
SRY - sex-determining region Y
SSCP - single stand conformation polymorphism
UKHT - Institute of Hematology and Blood Transfusion (Prague)
VNTR - variable number of tandem repeats
vWF - von Willebrand factor

REFERENCES


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