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Genetic analysis of haemophilia A in Bulgaria

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Abstract

Background: Haemophilias are the most common hereditary severe disorders of blood clotting. In families afflicted with haemophilia, genetic analysis provides opportunities to prevent recurrence of the disease. This study establishes a diagnostical strategy for carriership determination and prenatal diagnostics of haemophilia A in Bulgarian haemophilic population.

Methods: A diagnostical strategy consisting of screening for most common mutations in the factor VIII gene and analysis of a panel of eight linked to the factor VIII gene locus polymorphisms was established.

Results: Polymorphic analysis for carrier status determination of haemophilia A was successful in 30 families out of 32 (94%). Carrier status was determined in 25 of a total of 28 women at risk (89%). Fourteen prenatal diagnoses in women at high risk of having a haemophilia A – affected child were performed, resulting in 6 healthy boys and 5 girls.

Conclusion: The compound approach proves to be a highly informative and cost-effective strategy for prevention of recurrence of haemophilia A in Bulgaria. DNA analysis facilitates carriership determination and subsequent prenatal diagnosis in the majority of Bulgarian families affected by haemophilia A.

Background

Haemophilia A is a common inherited disorder of blood clotting, inherited in a recessive X-linked pattern. The incidence of the disease is estimated at app. 1:8000 males.

Affected individuals develop a variable phenotype of hemorrhage into joints and muscles, easy bruising, and prolonged bleeding from wounds. The severity and frequency of bleeding in haemophilia A is inversely related to the amount of residual factor.

Haemophilia A is caused by deficiency of factor VIII, a cofactor in the activation of factor X at the middle stages

of the coagulation cascade. The gene coding for factor VIII is located in the subtelomeric Xq28 region, comprises 26 exons and spans 186 kb genomic DNA [1,2].

By the present moment over 400 mutations leading to haemophilia A have been identified [3,4]. De novo mutations in the factor VIII gene constitute a significant proportion of haemophilia A cases (app. 30% of all cases) [5]. Half of such mutations do not derive from a single germ cell but are attributed to a germline or somatic mosaic originating from a mutation during early embryogenesis [6,7].

In approximately 40–50% of the severe cases (25% of all cases) the underlying molecular defect causing haemophilia A is a gross rearrangement of the gene owing to intrachromosomal homologous recombination between inverted repeats located in intron 22. and outside the gene. Recombination leads to separation of exons 1. – 22. from exons 23. – 26. and positioning these in reverse orientation to the remaining part of the gene [8,9]. Several types of inversion have been recognized. Most often encountered is inversion type I, which involves the distal copy of the repeated unit, but types II (involves the proximal copy) and III (when more than two extragenic copies of the repeated unit are present) are also common in haemophilia A patients [10,11].

Transition mutations C→T (respectively, G→A) in CpG dinucleotides of factor VIII gene occur frequently [12]. Thus, screening for mutations abolishing or creating restriction sites in patients with severe haemophilia A can provide direct carrier detection. Screening for unknown mutations is also accomplished by various scanning methods (such as SSCA) and subsequent sequencing of fragments which show abnormal electrophoretic pattern [13,14].

Large inversions can be detected by either Southern hybridisation or nested RT-PCR spanning the breakpoint in intron 22. analysis and thus carrier and/or antenatal diagnosis is accomplished [10,15]. Because of the enormous variety of mutations producing the disease in the remaining 75% of cases (except for the large inversions), carrier identification and prenatal diagnosis can be accomplished by indirect detection using linked to the disease locus DNA polymorphisms. A number of RFLPs and tandem repeats have been identified within the genes of factor VIII and in its extragenic regions [16-23].

Rapid carriership determination and prenatal diagnosis are usually facilitated by a compound diagnostic strategy consisting of screening for the large inversions combined with DNA analysis of polymorphisms within and outside the factor VIII gene. DNA analysis using polymorphic markers linked to disease locus proves to be highly effective, as the proportion of cases in which linkage analysis fails is less than 20% of the affected families [24-26].

In Bulgaria, the incidence of haemophilia A is estimated to be about 1:18 000 live births (1:9000 males) [27]. The mainstay of treatment for bleeding episodes in Bulgarian haemophiliacs is "treatment on demand", e. g. application of the deficient factor at the first symptom of bleeding. Considering the short half-life of factor VIII in plasma, most of the time the haemophiliacs in Bulgaria live and function far below therapeutic levels of deficient factor. This poses a constant risk of sudden hemorrhage

that might be difficult to handle. Thus, despite recent marked improvement in treatment of bleeding disorders, for Bulgarian patients, haemophilia remains a severe, crippling, sometimes life-threatening disease.

Carriership of haemophilia invariably has influence on women's reproductive plans. It is documented elsewhere that approximately 50% of female relatives of haemophilia patients may decide consciously not to have children and relate their decision to fear of passing the disease to their children [28,29]. DNA analysis greatly improves the effectiveness of early determination of carrier status.

The main goal of this study was to investigate the genetic heterogeneity of haemophilia A in Bulgaria in order to construct effective diagnostic strategy for prevention of recurrent disease in families already affected.

Methods

DNA samples

Thirty-two haemophilia A families consisting of 162 members with 36 haemophilia A-affected males were included in the present study. Twenty-two of the families (69%) did not have previous history of haemophilia A. The distribution of clinical severity of haemophilia A was as follows: 23 patients (64%), severe (FVIII:C <1%); 8 patients (22%), moderate (FVIII:C 1 – 5%) and 5 patients (14%), mild (FVIII:C >5%).

Written informed consent was obtained from all haemophilia patients and their relatives that participated in the study. In the case of minors, informed consent for participation in genetic studies was obtained from their parents or legal guardians.

DNA was extracted from 5 ml peripheral blood or fetal material. For PCR purposes, DNA was prepared by proteinase K digestion/phenol extraction/ethanol precipitation as described in [30]. For genomic hybridisation, high molecular weight DNA was obtained by embedding leucocytes in low-melting agarose blocks and subsequent digestion by proteinase [30].

Fetal DNA was extracted from 3–10 mg chorionic villi obtained by chorionic villus sampling (CVS) at 10.–12. week of gestation (g. w.) or from 10–15 ml amniotic liquid obtained by amniocentesis at 16.–21. g. w., after informed consent.

Southern hybridisation for detection of factor VIII inversions

High molecular weight genomic DNA (3–6 µg) was digested with Bcl I, separated by agarose electrophoresis and blotted onto nylon membrane. DNA probe was obtained either by digestion of p542.16 with Eco RI/Sac I

[9]; or by amplification of the desired region of the intron 22. using genomic DNA from healthy male volunteers [31]. PCR fragment was purified by low-melting point agarose elution. Filling-end reaction was performed with Klenow fragment, though the probe can be used without the ends – blunting step. The hybridisation probes were labeled to high specific activity by random priming. Blots were hybridized at 64° C in modified Church's buffer: (5 X SSC, 50 mM Na₂HPO₄; pH = 7,2; 10% dextran sulphate; 6% SDS and washed twice with 2 X SSC, 0,1% SDS at room temperature, and twice with 0,1 X SSC, 0,1% SDS at the hybridisation temperature [9]. Autoradiography was carried out at -70° C.

Analysis of DNA polymorphisms linked to Factor VIII gene locus (gene tracking)

PCR analysis of tandem repeats

Tandem repeats in extragenic region of factor VIII gene (St14) and inside the gene (intron 13. (IVS13(CA)nSTR, IVS13STR), intron 22. (IVS22(CA)nSTR, IVS22STR), intron 25. (IVS25(CA)nSTR, IVS25STR) (Universitat fur Humangenetik-Muenster, Germany-personal communication) and intron 6. (IVS6(CA), IVS6STR) were used in the present study [16,21-23]. PCR products were separated in 6–10% nondenaturing (or sequencing) polyacrylamide gels and visualised by silver staining [32] or by autoradiography.

PCR-RFLP polymorphisms

Polymorphic sites in intron 18. (Bcl I RFLP); intron 19. (Hind III RFLP), intron 22. (Xba I RFLP) of the factor VIII gene were used in the present study [16,17,20,21]. PCR products were subjected to restriction with the relevant restriction endonuclease, separated in nondenaturing polyacrylamide gels and visualised by silver staining.

G/A polymorphism in intron 7. of the factor VIII gene

The region containing the polymorphic site was amplified according to [18]. Samples were denatured and electrophoresed on a 14% polyacrylamide gel (37,5:1 acrylamide:bisacrylamide) at 5° C for 20 h and visualised by silver staining.

Screening for mutations in the essential regions of the factor VIII gene

SSCA analysis

Exons 11., 14. and 24. of the gene of the Factor VIII gene were screened for mutations as described in [14]. PCR yielded 343 bp fragment for exon 24. and 445 bp fragment for exon 11., respectively. The large exon 14. was amplified as four overlapping fragments, app. 1000 bp long. The resulting amplification fragments were hydrolyzed with the Alu I. Digested samples were denatured and electrophoresed on a 14% polyacrylamide gel (37,5:1 acrylamide:bisacrylamide) at 8° C for 20 h and visualized by

silver staining. Sequencing of the fragments was performed by the dideoxy chain-termination method, on AbiGene automatic sequencer (Applied Biosystems).

Screening for restriction site abolishing mutations in exons 1. and 18

Screening for Taq I site abolishing mutations in exon 1. and Taq I/Rsa I site abolishing mutations in exon 18. of the Factor VIII gene was performed according to [12]. Briefly, the PCR amplification yielded 186 bp fragment for exon 1. and 291 bp fragment for exon 18., respectively.

Normally, Taq I digestion of the PCR fragment of exon 1. results in fragments 165 and 21 bp. C→T transition in exon 1. abolishes the existing Taq I restriction site.

In exon 18. normally exists 1 restriction site for Taq I (digestion pattern of 163 and 128 bp) and 2 sites for Rsa I (digestion pattern of 207, 57 and 32 bp). Transition C→T results in abolishment of the Taq I site and one of the Rsa I sites I (digestion pattern of 234 and 57 bp).

Results and discussion

Screening for most common mutations causing severe haemophilia A

Inversion mutation was found in 13 out of 29 severe and severe-to-moderate haemophilia A patients (45%, 0.95% CI: 28,4–62,5%). Ten of these carried the common type of inversion (type I), involving the distal extragenic copy of the repeated unit (77%), two patients (15%) – inversion, involving the proximal copy of the 9,5 kb repeat (type II) and 1 patient carried the rare type IIIB inversion.

Analysis of DNA polymorphisms linked to factor VIII gene locus

By genealogical data, 28 women (not including mothers of haemophilia A boys) were identified to be at risk to be carriers of haemophilia A.

Polymorphic analysis for carrier status determination of haemophilia A was successful in 30 families out of 32 (94%).

These families were informative by at least one marker locus. In 5 families (16%) only the extragenic DNA marker St14 was informative. Only 2 families (6%) were uninformative by all the markers used. Rates of heterozygosity for polymorphic markers used are shown in table 1.

Among markers internal to factor VIII, intron 22 dinucleotide repeat showed highest heterozygosity rate (H) of 0,63, followed by intron 13 repeat (0,46), that renders these two very useful and reliable for diagnostic purposes in Bulgarian population. Intron 22 STR was informative in 19 of 32 families (60%, 0.95% CI: 42,2–74,5%). Intron

Table 1: The panel of factor VIII gene linked polymorphisms, used as markers for carriership determination and prenatal diagnostics of haemophilia A in Bulgaria and their local heterozygosity rates.

Marker	ST14 VNTR	IVS22 STR	IVS13 STR	BclI RFLP	HindIII RFLP	XbaI RFLP	IVS25 STR	Int7 G/A	IVS6 STR
H*	0.78	0.63	0.46	0.35	0.35	n.a.**	0.25	0.19	0.02

*H: heterozygosity rate. ($H = 1 - 931p_i^2$); p_i – frequency of allele i. **The sequence containing the Xba I polymorphic site is located in the 9,5 kb repeated region of intron 22. Thus, flanking primers amplify together with the Xba I polymorphic site a fragment in the extragenic copies that usually lack the Xba I restriction site and, practically, genotypes +/- cannot be distinguished of genotype +/--. Thus, genotype of a female must be inferred of the genotype of her children [19].

13 STR was informative in 12 of 32 families (38%, 0.95% CI: 22,9–54,8%).

Biallelic polymorphisms Hind III and Bcl I RFLP follow as markers of choice in the Bulgarian population as their heterozygosity is 0,35. This marker system was informative in 14 of 32 families (45%, 0.95 CI: 28,1–60,7%).

Intron 25 dinucleotide repeat was informative in 7 of 32 families (21%, 0.95 CI: 11,0–38,7%). This marker exhibited a rare allele, (150 bp long, a CA₂₂ repeat) in the cohort of Bulgarian patients. This allele was observed only in one of the 65 independent X-chromosomes studied and is presumed to be quite rare (1,5%, 0.95 CI 0,27 – 8,22%). IVS25STR has limited applicability in most cases, because of its low heterozygosity rate (0,25). Nevertheless, as the gene for factor VIII is quite large and recombination within the limits of the gene itself is possible, when informative, IVS25STR may serve as an useful genetic marker in the distal part of the gene.

Intron 7 G/A SSCA polymorphism was informative in 6 of 32 families (19%, 0.95% CI: 8,9–35,3%). This marker exhibited in Bulgarian patients' heterozygosity rate even lower than the intron 25 dinucleotide repeat system (0,19). After typing with a panel of IVS22STR – HindIII/BclI system – Xba I RFLP – IVS25STR, intron 7 SSCA haplotype data does not add to the proportion of cases in which at least one marker is informative (Fig. 1).

IVS6STR dinucleotide repeat exhibits very low polymorphism in the Bulgarian haemophilic population. (heterozygosity rate of 0,02). We observed three alleles with 12–14 repetitions (CA)_(12–14). Single allele ((CA)₁₃) accounts for 99% of all alleles. This renders the marker less useful for the diagnosis of haemophilia A carriers.

St14 VNTR, with its large number of alleles (10 observed in this study out of 15 described in [21]) proves to be the most informative marker in the Bulgarian population. It was informative in 29 of 32 families (91%, 0.95% CI: 75,8–96,8%) and has the highest heterozygosity rate

(0,78) of all the polymorphic markers included in this study.

St14 may solve the diagnostic problem in cases when none of the intragenic markers is informative. As it is located approximately 2 map units apart from the factor VIII locus, recombination rate is estimated at 2% per meiosis. Rarely, recombination between marker locus and disease gene locus may occur. We have observed one such case (data not shown).

Screening for mutations in the essential regions of the factor VIII gene

SSCA analysis

SSCA can detect over 95% of causative mutations in moderate and mild haemophilia A and about 50% of the defects that cause severe haemophilia A (as large rearrangements account for app. 50% of the severe cases) [13,14,33].

Exons 11., 14. and 24. of the gene of the Factor VIII gene in 34 males with haemophilia A were screened by SSCA and subsequent sequencing. No pattern different from the normal was found.

Restriction analysis

Screening for Taq I abolishing mutations in exon 1 and Taq I/Rsa I site abolishing mutations in exon 18. of the factor VIII gene was performed. Two related patients were found to carry a Taq I site abolishing mutation in exon I, resulting in CGA-to-TGA (Arg to Stop) transition in the vicinity of transcription startpoint of the factor VIII gene. This molecular defect resulted in these patients in severe phenotype with practically undetectable plasma levels of factor VIII.

Conclusions

Severe form of haemophilia A accounts for over 60% of Bulgarian patients in this study. As no founder effect pattern could be established, this biased pattern may be due to underreferral of moderate and mild haemophiliacs. This may reflect the fact that some milder cases may escape clinical attention. In fact, some researchers estimate the

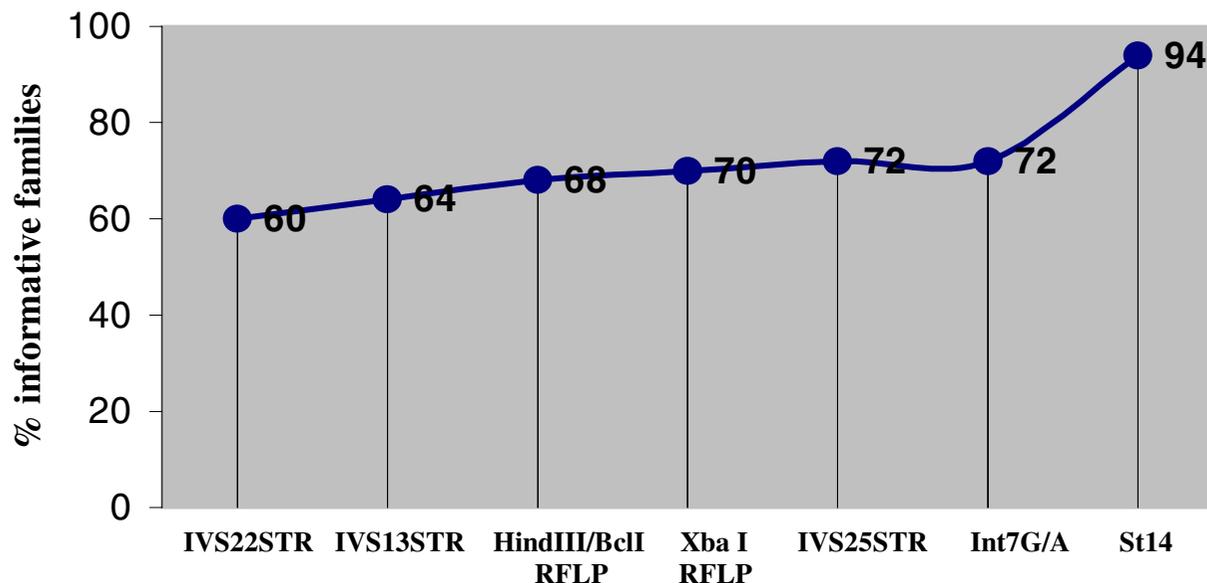


Figure 1

A plot of cumulative informativeness of panel of markers linked to factor VIII locus used for polymorphic DNA analysis of haemophilia A in Bulgaria. By polymorphic DNA analysis of the factor VIII gene locus carrier status of females in 30 Bulgarian families out of 32 (94%) could be determined. Intron 22. and intron 13. STR dinucleotide repeats used together are informative in 64% of haemophilia A families. Adding biallelic Hind III/Bcl I RFLP polymorphic system and Xba I PFLP results in further increase in percentage of informative families up to 70%. IVS25STR marker is informative in a limited proportion of cases (increases informativeness up to 72% of the families). Intron 7. SSCA haplotype data does not add to the proportion of cases in which at least one marker is informative. St14 tandem repeat exhibits the highest heterozygosity rate of all factor VIII gene linked markers and thus proves to be the most informative marker in the Bulgarian population. St14 is a marker of choice in cases when none of the intragenic markers is informative, nevertheless, its location apart of factor VIII gene poses risk of misdiagnosis due to marker-to-gene recombination.

real incidence of haemophilia A [1] to be 1:5000 males, that is, almost twice as high as the current value.

Gross inversions of the factor VIII were discovered in 45% of Bulgarian cases of severe and severe-to moderate haemophilia A (36% of all). In the remaining severe cases as well as in the moderate and mild haemophilia A, the analysis of polymorphisms linked to the factor VIII gene proved as an invaluable tool in carrier status determination and prenatal diagnosis. In Bulgarian patients, combined use of analysis of DNA polymorphisms linked to factor VIII locus and screening for inversion mutation failed only in 2 cases (6%).

Fig. 1. presents a plot of cumulative informativeness of the markers included in this study vs. increasing number of markers used for haplotyping. Additional number of intragenic markers does not increase the percentage of informative families beyond 72%. Including the external

marker St14 will raise the overall informativeness up to 94%, nevertheless, the risk of recombination between extragenic marker and disease locus may become significant depending on the number of meioses in question.

Carrier status was determined in 25 of a total of 28 women at risk (89%). One woman dropped out of study when she became pregnant. In 2 women (7%) DNA analysis gave no information about the type of mutation and tracking of the defective gene failed because of lack of heterozygosity of the polymorphic markers. In thirteen women carrier status of haemophilia A was ruled out. In the remaining 12 women, carrier status for haemophilia A was confirmed.

Fourteen prenatal diagnoses in women at high risk of having a haemophilia A - affected child were performed. These pregnancies produced 6 boys and 5 girls (two carriers, two noncarriers, and one where no further DNA anal-

ysis after fetal sex determination was carried out). Five of the boys were healthy. In one case with pregnancy with male fetus, a key recombination between extragenic and intragenic for Factor VIII gene polymorphic markers was discovered. As the haemophilic male was the first and only case in this family and no DNA was available from him, mutation screening could not be accomplished and no definite conclusion could be drawn from the polymorphic DNA analysis. The parents decided not to terminate the pregnancy, as the mother was in 22. g. w. by the end of analysis.

In two cases, the fetus was found to be at high risk to be affected by haemophilia A. In both cases, parents invariably chose termination of the pregnancy.

In one case CVS produced insufficient amount of material for DNA analysis and biopsy was repeated, which led to obstetrical complications, and, finally, to loss of fetus.

Screening for gross inversions disrupting factor VIII gene combined with analysis of a panel of DNA markers linked to the disease locus proves to be highly informative and cost-effective strategy for prevention of recurrence of haemophilia A in Bulgaria. This compound approach facilitates carriership determination and subsequent prenatal diagnosis in the majority of Bulgarian haemophilia A families.

List of abbreviations used in the text

H Heterozygosity rate

PCR Polymerase chain reaction

RFLP Restriction fragment length polymorphism

SSCA Analysis of conformation single-stranded DNA fragments

STR Short tandem repeat

VNTR Variable number of tandem repeats

Competing interests

None declared.

Authors' contributions

RP and SC carried out the DNA analysis studies. IK provided samples for analysis. All authors read and approved the final manuscript.

References

1. Scriver CR, Beaudet A, William SS, Walle D, eds: *The metabolic and molecular bases of inherited disease* VIIth edition. McGraw Hill Companies, Inc .

2. Gitschier J, Wood WI, Goralka TM, Wion KL, Chen EY, Eaton DH, Vehar G, Capon DJ, Lawn RM: **Characterization of the human factor VIII gene.** *Nature* 1984, **312**:326-330.
3. Kamball-Cook G, Tuddenham EG, Wacey AI: **The factor VIII Structure and Mutation Resource Site: HAMSTeRS version 4.** *Nucleic Acids Res* 1998, **26**(1):216-9.
4. Tuddenham EGD, Cooper DN, Gitschier J, Higuchi M, Hoyer LW, Yoshioka A: **Haemophilia A: a database of point mutations, deletions, insertions and rearrangements of the factor VIII gene.** *Nucl Acids Res* 1991, **19**:4821-4833.
5. Francis RB, Casper CK: **Reproduction in haemophilia.** *J Am Med Assoc* 1983, **250**:3192-5.
6. Brocker-Vriends AH, Briet E, Dreesen JC, Bakker B, Reitsma P, Pannekoek H, van de Kamp JJ, Pearson PL: **Somatic origin of inherited haemophilia A.** *Hum Genet* 1990, **85**(3):288-292.
7. Leuer M, Oldenburg J, Lavergne JM, Ludwig M, Fregin A, Eigel A, Ljung R, Goodeve A, Peake I, Olek K: **Somatic mosaicism in haemophilia: a fairly common event.** *Am J Hum Genet* 2001, **69**(1):75-87.
8. Naylor JA, Green PM, Rizza CR, Giannelli F: **Factor VII gene explains all cases of haemophilia A.** *Lancet* 1992, **340**:1066-7.
9. Lakich D, Kazazian HH Jr, Antonarakis SE, Gitschier J: **Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A.** *Nat Genet* 1993, **5**:236-241.
10. Goodeve A, Preston FE, Peake I: **Factor VIII rearrangements in patients with severe haemophilia A.** *Lancet* 1994, **343**:329-330.
11. Antonarakis S, Rossiter J, Young M, Horst J, de Moerloose P, Sommer SS, Ketterling RP, Kazazian HH Jr, Negler C, Vinciguerra C et al.: **Factor VIII gene inversions in severe haemophilia A: results of an international consortium study.** *Blood* 1995, **86**:2206-2212.
12. Reiner AP, Thompson AR: **Screening for nonsense mutations in patients with severe haemophilia A can provide rapid, direct carrier detection.** *Hum Genet* 1992, **89**:88-94.
13. David A, Moreira J, Laloz MRA, Rosa HAV, Schwaab R, Morais S, Diniz MJ, de Deus G, Campos M, Lavinha J, Johnson D, Tuddenham EGD: **Analysis of the essential sequences of the factor VIII gene in twelve haemophilia A patients by single-stranded conformational polymorphism.** *Blood Coagul Fibrinolysis* 1994, **5**:257-264.
14. Schwaab R, Oldenburg J, Laloz MRA, Schwaab U, Pemberton S, Hanfland P, Brackmann HH, Tuddenham EGD, Michaelides K: **Factor VIII gene mutations found by a comparative study of SSCP, DGGE and CMC and their analysis on a molecular model of factor VIII protein.** *Hum Genet* 1997, **101**:323-332.
15. Naylor JA, Green PM, Rizza CR, Giannelli F: **Analysis of factor VIII mRNA defects in everyone of 28 haemophilia A patients.** *Hum Molec Genet* 1993, **2**:11-17.
16. Peake I: **Registry of DNA polymorphisms within or close to the human factor VIII and factor IX genes. For the Factor VIII subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.** *Thromb Haemost* 1992, **67**:277-280.
17. Gitschier J, Drayna D, Tuddenham EGD, White RL, Lawn RM: **Genetic mapping and diagnosis of haemophilia A through a BclI polymorphism in the factor VIII gene.** *Nature* 1985, **315**:427-430.
18. Kogan S, Gitschier J: **Mutations and a polymorphism in the factor VIII gene discovered by denaturing gradient gel electrophoresis.** *Proc Natl Acad Sci U S A* 1990, **87**(6):2092-6.
19. Kogan S, Doherty M, Gitschier J: **An improved method for prenatal diagnosis of genetic disease by analysis of amplified DNA sequences.** *N Engl J Med* 1987, **317**:985-990.
20. Ahrens P, Cruze TA, Schwartz M, Rassmussen PB, Din N: **A new Hind III restriction fragment length polymorphism in the Haemophilia A locus.** *Hum Genet* 1987, **76**(2):127-28.
21. Richards B, Heilig R, Oberle I, Storjohann L, Horn GT: **Rapid PCR analysis of the St14 (DXS52) VNTR.** *Nucleic Acids Res* 1991, **19**(8): 1944
22. Windsor S, Taylor SA, Lillcrap D: **Multiplex analysis of two intra-genic microsatellite repeat polymorphisms in the genetic diagnosis of haemophilia A.** *Br J Haematol* 1994, **86**:810-815.
23. Lin SR, Lin SW, Hsu YC, Shen MC: **Identification of a new CA dinucleotide repeat in the human factor VIII gene.** *Br J Haematol* 2000, **111**(4):1256-9.

24. Dardik R, Peretz H, Usher S, Seligsohn U, Martinowitz U: **Current strategy for genetic analysis of haemophilia A families.** *Haemophilia* 1996, **2**:11-17.
25. Goodeve AC: **Advances in carrier detection on haemophilia.** *Haemophilia* 1998:358-364.
26. Peake I: **The molecular basis of haemophilia A.** *Haemophilia* 1998:346-349.
27. Anastassov A, Tankovski I, Nigno C, Guenova V, Lissitchkov T: **Epidemiology and characteristics of hemophilia in Bulgaria.** *Folia Haematol Int Mag Klin Morphol Blutforsch* 1971, **96**(1):113-8.
28. Tedgard U, Ljung R, McNeil TF: **Reproductive choices of haemophilia carriers.** *Br J Haematol* 1999, **106**(2):421-6.
29. Ross J: **Perspectives of haemophilia carriers.** *Haemophilia* 2000, **Suppl 1**:41-5.
30. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual Cold Spring Harbor, Cold Spring Harbor Press; 1989.*
31. Petkova R, Chakarov S, Kremensky M: **A Novel PCR – generated DNA Probe Used for Identification of the Most Common Defects Causing Severe Haemophilia A.** *Biotechnology and biotechnological equipment* in press.
32. Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC: **Analysis of the VNTR locus DIS80 by the PCR followed by high-resolution PAGE.** *Am J Hum Genet* 1991, **48**(1):137-44.
33. Higuchi M, Antonarakis SE, Kasch L, Oldenburg J, Economou-Petersen E, Olek K, Arai M, Inaba H, Kazazian HH Jr: **Molecular characterization of mild-to-moderate haemophilia A: detection of mutation in 25 of 29 patients by denaturing gradient gel electrophoresis.** *Proc Natl Acad Sci USA* 1991, **88**:8307-8311.

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