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Indirect Molecular Diagnosis of Congenital Factor XIII Deficiency by Candidate Microsatellites and Single Nucleotide Polymorphisms

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Abstract

Background: Congenital factor XIII (FXIII) deficiency is one of the rarest bleeding disorders with a prevalence of one per 2 million in the general population. The disorder is accompanied by a high rate of life-threatening bleeding. Due to normal results of routine coagulation tests, diagnosis of the disorder is challenging, but molecular methods can be used for precise diagnosis. Direct mutation detection is the standard method for confirmation of the disorder, but indirect molecular diagnosis can be used as a fast and cost-benefit choice. In the present study, we described indirect molecular methods for molecular diagnosis of congenital FXIII deficiency.

Materials and Methods: For this study, a comprehensive literature review was performed on PubMed, Embase, Web of Science, and Scopus databases using the following keywords: "indirect molecular diagnosis" with "rare bleeding disorder", "coagulation factor XIII/13 deficiency", "prenatal diagnosis" "rare bleeding disorder", "molecular diagnosis", "factor XIII/13 deficiency", "indirect molecular diagnosis" "carrier detection", and "factor XIII/13". These words were used separately and in combination with each other.

Results: A total of 293 papers were founded, among them 67 papers were selected for the study. Indirect molecular diagnostic approach can be done using candidate microsatellites and single nucleotide polymorphisms (SNP). This method can be used for prenatal diagnosis and carrier detection, especially in the areas with low economic resources. Polymorphic genetic markers associated with F13 gene like HumFXIII01, HumFXIIIA02, HumFXIIIB, rs7740009, and rs3024405 SNPs can be used for indirect molecular diagnosis of congenital FXIII deficiency. Finally, by comparing patient's polymorphic markers with healthy individuals, diagnosis can be made.

Conclusion: It seems that indirect molecular diagnosis is a relatively reliable and cost-effective method for diagnosis of congenital FXIII deficiency in the areas with low economic resources.

Key words: Factor XIII deficiency, Molecular Diagnostic Techniques, Microsatellite Repeats, Polymorphism

Introduction

Coagulation factor XIII (FXIII) or fibrin stabilizing factor is a protein with an essential role in the coagulation system, wound healing, angiogenesis, and pregnancy maintenance (1, 2). FXIII is a transglutaminase that circulates in the blood stream as a heterotetramer (FXIII-A2B2) and consists of two subunits; namely an enzymatic subunit (FXIII-A2) and a carrier (FXIII-B2) (3, 4). FXIII-A is produced by megakaryocytes, mast cells, platelets, and macrophages, while FXIII-B is only produced by hepatocytes (5). F13A and F13B genes are located in the chromosomal locations of 6p24-25 and 1q31-32.1, respectively (6, 7). FXIII-A subunit has two important regions: activation peptide and catalytic core.

Hence FXIII-A can be considered as the main FXIII subunit (8, 9).

FXIII deficiency is an autosomal recessive rare bleeding disorder (RBD) with a prevalence of one per 2 million in the general population (10, 11). FXIII deficiency is accompanied by a wide range of clinical manifestations, including intracranial hemorrhage (ICH), delayed wound healing, recurrent miscarriages, and umbilical cord bleeding (UCB) (12, 13). UCB with a prevalence of >80% is the most common presentation, while ICH is the main cause of morbidity and mortality (13, 14). About 80% of FXIII deficiency related deaths are attributed to ICH (15). The disorder is more common in the areas with a high rate of consanguineous marriages, such as Sistan and Baluchestan Province in southeast Iran (14, 16, 17). Routine coagulation tests are normal in FXIII deficiency (18). The most common diagnostic test for the disorder is clot solubility test, but it is not further recommended by the experts (19, 20). FXIII activity is recommended as first-line screening test for FXIII deficiency (21). Confirmation of the disorder can be done by molecular analysis of F13A and F13B genes (22, 23). Although the method of choice is direct sequencing of the genes, indirect molecular diagnosis is a fast and cost-effective alternative method. The aim of the current study was to describe indirect molecular diagnosis of congenital FXIII deficiency using selected polymorphic genetic markers.

Materials and Methods

A systematic search was performed on PubMed, Embase, Web of Science, and Scopus databases using the following keywords: “indirect molecular diagnosis” with “rare bleeding disorder”, “coagulation factor XIII/13 deficiency”, “prenatal diagnosis” “rare bleeding disorder”, “molecular diagnosis”, “factor XIII/13 deficiency”, “indirect molecular diagnosis” “carrier detection”, and “factor XIII/13”. In addition, the references to all retrieved studies were evaluated in order to find potentially relevant studies. These words were used separately and in combinations with each other. To avoid losing relevant information, no limitations were set in the search.

Results

In the present research, 293 studies had been found in initial search and 87 articles were excluded due to duplication. The abstracts of all remaining papers were carefully assessed, 122 further articles were excluded due to using indirect molecular diagnosis of unrelated disorders, being guidelines, or being non-English articles. By careful screening of all 84 remaining studies, 17 articles on molecular diagnosis of other inherited bleeding disorders and unrelated disorders were excluded. Finally, after filtering 67 studies were selected (Figure 1).

Structure, function, and activation of FXIII

FXIII protein with 731 amino acids is encoded by a 160 kb gene located on the petite arm of chromosome 6 (6p24-25). The protein has an activation peptide (residues 1-37) and 4 different domains, including β-sandwich (residues 38-183), central domain or catalytic core (residues 184-515), β-barrel 1 (residues 516-627), and β-barrel 2 (residues 628-731) (Figure 2) (1, 5).

The FXIII-B is known as the regulatory subunit of FXIII-A_2B_2 and acts as a transporter of FXIII-A subunit. Hepatocytes are exclusive producers of FXIII-B subunit. The *F13B* gene with 28 Kb length and 12 exons is located on the chromosomal region of 1q31-32.1. The *F13B* gene encoded a protein consists of 641 amino acids that has 10 similar repeated parts named Sushi domains or Glycoprotein I (GP-I). Different studies have shown that FXIII-B subunit enhances FXIII-A subunit stability and resistance against proteinases (1, 2) (Table I).

Coagulation Factor II (thrombin) is vital for early activation of FXIII. Therefore, at first step, thrombin interacts with FXIII-A subunit and cleaves arginine 37 and glycine 38 bond at the activation peptide N-terminal that separates activation peptide from FXIII-A subunit. Then, in the presence of calcium, separation of FXIII-B subunit from FXIII-A subunit is occurred (Figure 3). After FXIII activation, substrate is required for preceding the reaction. Fibrin is one of main substrates of FXIII and high concentrations of fibrin will reinforce the activation process of FXIII after recruitment of thrombin and calcium (3). FXIII is a member of transglutaminase family so it aggregates fibrins by lysyl epsilon iso-peptide bond (gamma_glutamyl) between a lysine amino acid of one fibrin monomer and a glutamine residue of another fibrin monomer. These cross-linkages will consolidate and insolubilize the fibrin clot (3) (Figure 3).

Diagnosis of factor XIII deficiency

In patients suspected to FXIII deficiency based on clinical manifestations, family history, and primary laboratory assessments, more specific tests are required for confirmation of the disorder (24). Although clot solubility test is the primary diagnostic test in most centers, FXIII functional assay is recommended as first-line screening test by the FXIII and fibrinogen scientific and standardization committee (SSC) of the international society of thrombosis and hemostasis (ISTH) (24). The committee is recommended an algorithm for diagnosis and classification of FXIII deficiency (1):

1. A quantitative FXIII activity assay as first-line screening test
2. Measurement of FXIII antigens ($FXIII\text{-}A_2B_2$, $FXIII\text{-}A_2$, $FXIII\text{-}B_2$) for subtyping of FXIII deficiency
3. Detection of autoantibody against FXIII (if acquired FXIII is suspected)
    a. Mixing study for detection of neutralizing antibodies
    b. Binding assay for detection of non-neutralizing antibodies
4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
5. Molecular studies for confirmation of FXIII deficiency

Although this algorithm is a standard diagnostic protocol, it is not applicable in all countries (21, 23).

Molecular diagnosis of factor XIII deficiency

Direct molecular diagnosis

In most developed countries, direct molecular diagnosis is commonly used (25). This method can be done by direct sequencing of *F13A* and *F13B* genes. Sanger sequencing is the most common DNA sequencing method for direct mutation detection, but this method cannot detect all disease- causing mutations (26, 27). Next-generation sequencing (NGS) or high-throughput sequencing is a more advance DNA sequencing technology and can increase the rate of mutation detection (28).

In patients with undetermined disease causing mutation, underlying mutation can be found by analyzing all exons, intron-exon boundaries, and promoter. If the disease-causing mutation is determined by a simple molecular technique, such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), tetra-primer amplification refractory mutation system-PCR (T-ARMS-PCR) or PCR-sequencing of an exon, the molecular diagnosis can be done (29, 30).

Indirect molecular diagnosis

In congenital FXIII deficiency, without a specific mutation and lack of direct sequencing facilities, indirect molecular methods can be used. Linkage analysis is used for indirect molecular diagnosis (29, 31, 32). During meiosis, genetic materials can be exchanged between homologous

chromosomes. This phenomenon is known as crossing-over, that results in recombinant chromosomes (33). The distance between two genes and sequences is an important factor in crossing-over, and near genes experience fewer crossing-over than far genes (34, 35).

For linkage analysis, suitable genetic markers, including microsatellites, single nucleotide polymorphism (SNP) ,and variable number tandem repeat (VNTR) should be selected (36, 37). These markers are the DNA sequences that inherited together. To select suitable genetic markers, some points should be considered (36, 37):

1. Selected markers should be near or adjacent to the affected gene
2. They should be informative and valid
3. Assessment of more genetic markers will increase reliability of results
4. Study of larger study population results is more reliable results
5. Generally, microsatellites are more reliable than SNPs.

Most important *F13A* and *F13B* gene polymorphic markers

A) Microsatellites

Microsatellites are highly repeated and extremely polymorphic sequences. The repeated sequences mostly consists of 2-7 bp that are repeated in a length of 50-150 bp (Short Tandem Repeats (STR)) (37, 38). STRs are dispersed all over the human genome and are highly prone to mutations and have some features like (37, 39, 40):

1. STRs are in different regions of the genome, especially near the genes or intergenic regions
2. These alleles have a unique pattern in different races and populations
3. Diversity and high rate of mutagenicity
4. High frequency of mutations rates across the whole genome.

These features make STRs more informative markers for indirect molecular diagnosis and linkage study than SNPs (41). STRs have better efficiency than VNTRs due to their small size. Therefore, if there is a small amount of DNA or if DNA is degraded, there is still a good chance to give the results. Polymorphic Information Content (PIC) is a parameter which is calculated by allele frequency in a population. This parameter is related to the mean of repeats lengths (40). If PIC be less than 0.7, it indicates that the selected STRs are informative (40). The most important STRs can be found at http://www.cstL.nist.gov/biotech/strbase.

A1) HumanFXIIIA01 (F13A01)

This STR polymorphism in *F13A* gene has AAAG repeated sequence at the beginning of 5′ untranslated region (UTR) (-2775/ATG) of the chromosomal location 6p24-25 (42, 43). FXIIIA01 is one of the most practical STRs in the genetic studies (44). For evaluation of STRs, the PCR product can be separated on 10% ethidium bromide stained poly acrylamide gel (43). More precisely light cycler or fluorescence base capillary electrophoresis can improve final results (Table II).

A2) HumFXIII A02 (F13A02)

This STR of *F13A* gene has AC (poly AC) repeat sequence in the intron number 8 of *F13A* gene (45). This STR was reported by Louhichi et al using tandem repeat finder (45).

A3) HumFXIII B (F13B)

This STR with TTTA repeat sequence located on of the *F13B* gene (38, 46). This STR is one of the most practical STR for indirect molecular diagnosis (44).

B*: Single Nucleotide Polymorphism (SNP)*

SNPs are single base-pair substitutions; the alteration of any one single nucleotide of A, C, G, and T to any other 3 nucleotides

(47, 48). If SNP is located in an important region of a gene like regulatory regions, it can influence the expression and function of the protein (49, 50). SNPs are different from pathogenic mutations. Unlike pathogenic mutations, SNP allele frequency is more than 1% in the general populations. Pathogenic mutations are occurred only in important gene regions (48, 51). SNPs can be used as genetic markers like STRs due to similar features they have in common, previously mentioned for STRs and even higher frequency of SNPs in the genomes can play an important role as a genetic marker in the genetic study (52-55).

B1) Candidate SNPs for *F13A* and *F13B* genes

With bioinformatics analysis, suitable SNPs for linkage analysis could be found. Several factors should be considered for selecting informative SNP, such as considered SNPs have to be located in the *F13A* (Chr6: 6144077-6320690) or *F13B* (Chr1: 197038740-197067266) genes. The SNPs also should be validated by Hapmap, Cluster, 1000 genomes and other similar project databases. Furthermore, to increase the power of the study, as a general rule, it's better to choose SNPs with global Minor Allele Frequency (MAF) more than 0.5. Higher MAF will increase the value of the study (47, 49, 56). If all items are considered, the opportunity for selecting informative and suitable SNPs for linkage analysis will increase (57-59). If all items are considered, the opportunity for selecting informative and suitable SNPs for linkage analysis will increase (57-59). After the selection of suitable SNPs in high probable mutagenic regions, a simple PCR-RFLP or PCR-sequencing can be used for SNP detection (48, 60). The candidate SNPs for the *F13A* and *F13B* genes are shown in table III and IV.

Utilization of polymorphic markers

Since, most of the FXIII deficiency causing mutations are located in *F13A* gene (1, 8), this subunit is more suitable for molecular analysis (61). STRs are more informative than SNPs for linkage analysis (62, 63). For linkage analysis, comparison of the polymorphic markers pattern of the suspected individual with normal population polymorphic markers pattern can determine molecular status of the suspected individual (64, 65). For better understanding, the chromosomal location of candidate STRs and SNPs of *F13A* and *F13B* genes are shown in Figure IV.

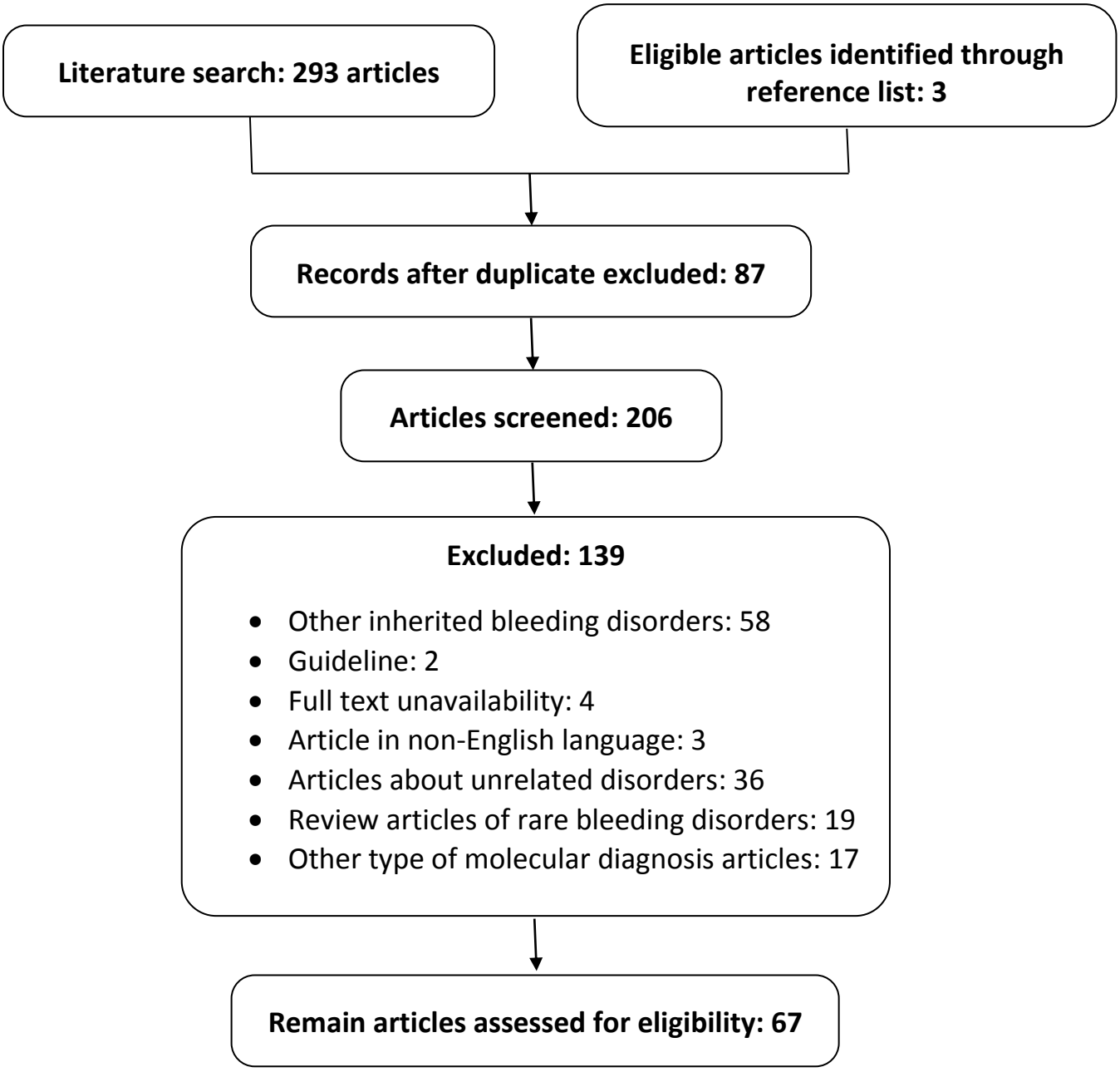

Figure 1. Schematic presentation of flow chart of systemic review.

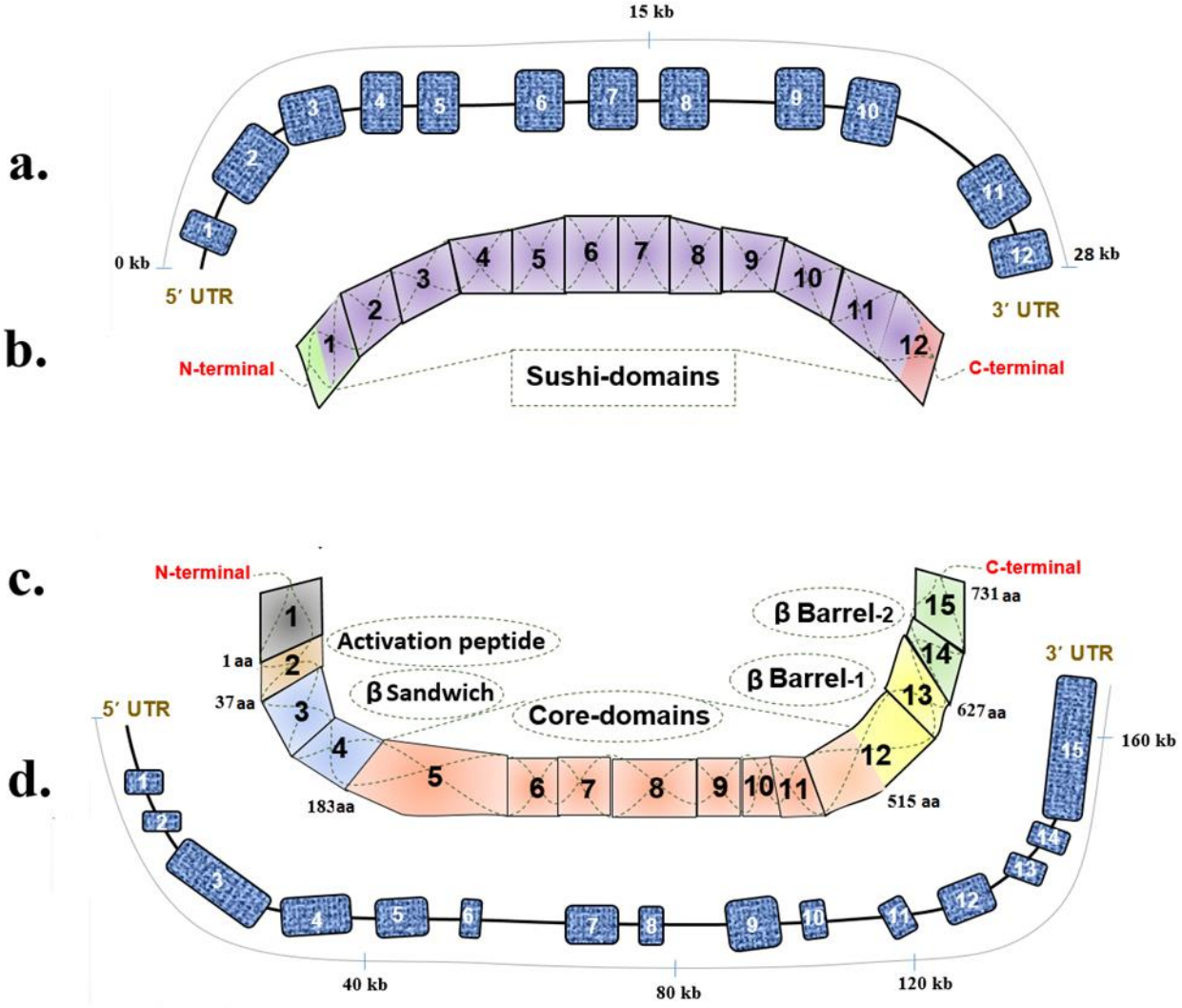

Figure 2. Schematic presentation of FXIII-A and FXIII-B subunit proteins and genes. a) *F13B* gene located on the chromosomal region of 1q31-32.1 b) FXIII-B subunit protein consists of 10 tandem repeats (sushi-domains) c) *F13A* gene located on the chromosomal region of 6p24-25 d) FXIII-A subunit protein consists of 5 domains, including activation peptide, β-sandwich, core domain or catalytic core, β-barrel 1 and β-barrel 2 . 5'-UTR: 5'-untranslated region, 3'-UTR: 3'-untranslated region, aa: amino acid- kb: kilo base pairs

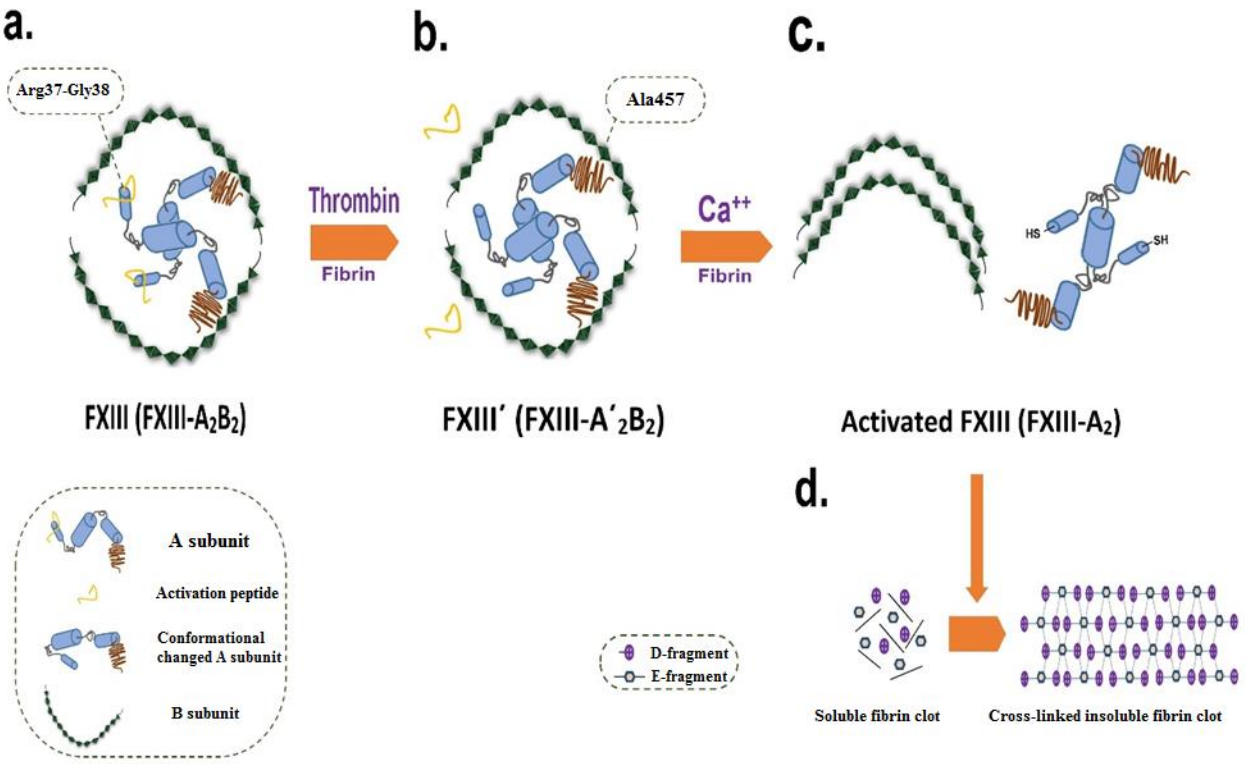

Figure 3. Schematic picture of coagulation FXIII activation proces. a) Coagulation FXIII consists of two catalytic A subunits and two transporter B subunits (FXIII- A2B2) b) Thrombin separates activation peptide from FXIII-A subunits by cleaving Arg37-Gly38 peptide bond (FXIII-A´2B2), c) Calcium releases FXIII-B subunits from FXIII-A subunits by attaching to Ala457 amino acid leading to FXIII activation (Activated FXIII), d) Activated FXIII changes soluble fibrins to insoluble fibrins and subsequently increases strength and consolidation of fibrin clot. FXIII: factor XIII, Arg37: Arginine 37, Gly38: Glycine 38, Ala457: Alanine 457, Ca: Calcium

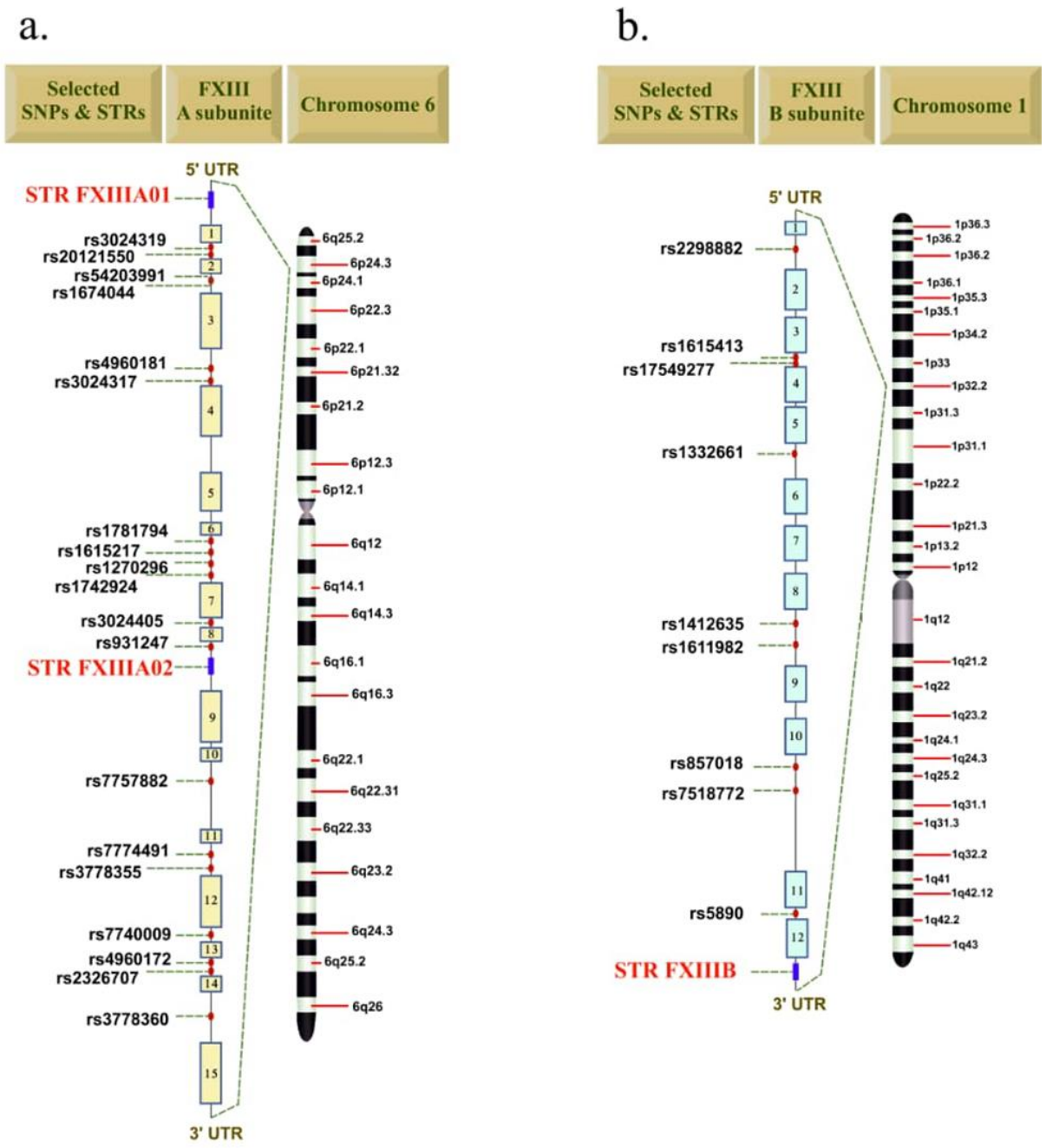

Figure 4. Schematic presentation of candidate STRs and SNPs of *F13A* and *F13B* genes. a) Selected STRs and SNPs of *F13A* gene b) Selected STRs and SNPs of *F13B* gene .STR: short tandem repeat - SNP: single nucleotide polymorphism- 5'-UTR: five prime untranslated region- 3'-UTR: three prime untranslated region. Designed by CorelDRAW

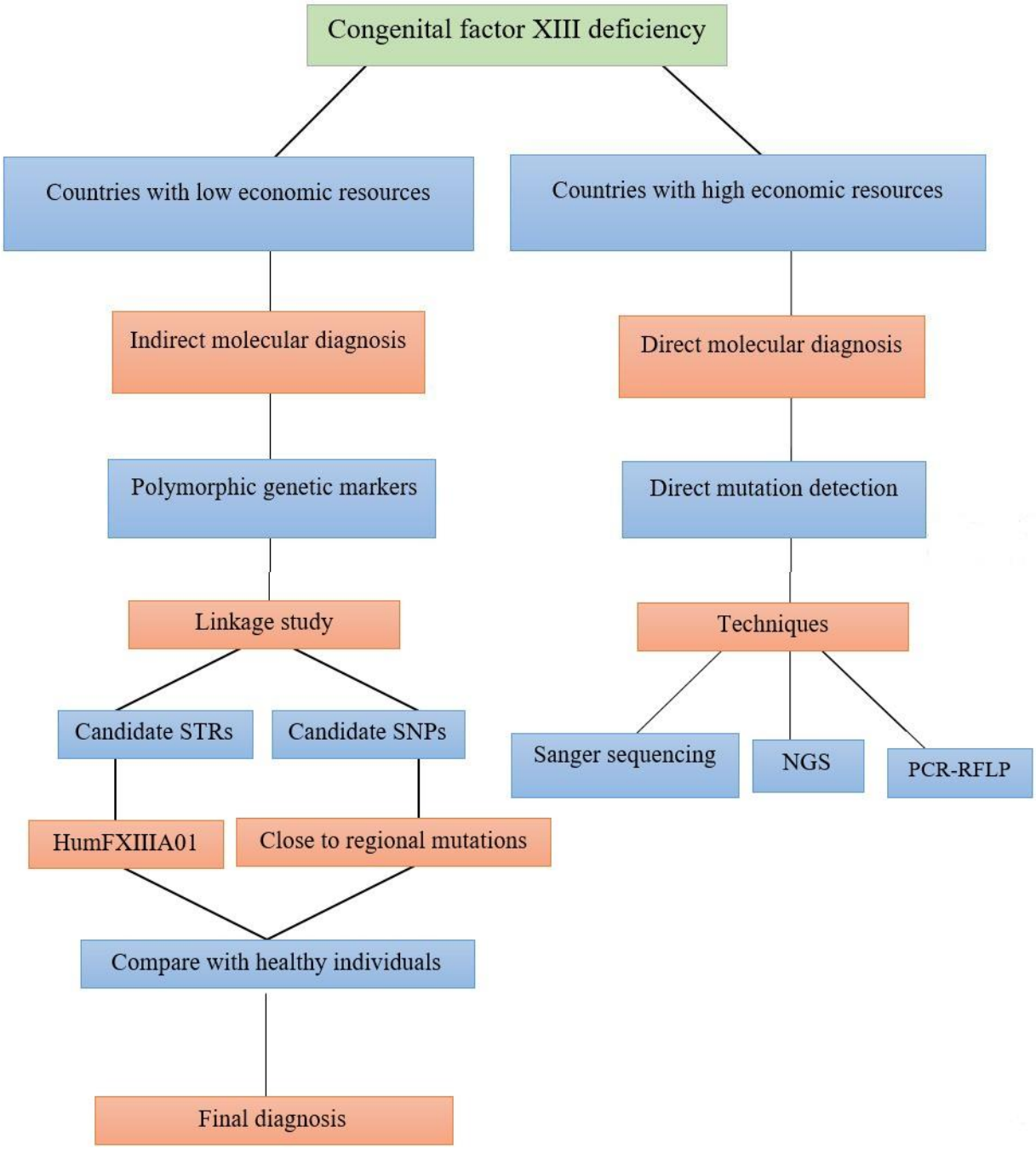

Figure 5. Indirect molecular diagnosis algorithm of congenital factor XIII deficiency by candidate microsatellites and single nucleotide polymorphisms. STR: short tandem repeat - SNP: single nucleotide polymorphism- NGS: Next Generation Sequencing-PCR-RFLP: Polymerase chain reaction-Restriction Fragment Length Polymorphism -HumFXIII01: Human Factor XIII01.

Table I: Comparison of the genetic features of A and B subunits

Features	A Subunit	B Subunit
Number of chain	Two A chains	Two B chains
Main role	Enzymatic- Catalytic	Transporter
Chromosomal location	6p 24-25	1q 31-32.1
Plasma concentration	Approximately 15 mg / mL	Approximately 21 mg / mL
Catalytic domain	Yes	No
Exons number	15	12
Molecular weight	80 KDalton	83 KDalton
Gene length	160 Kbp	28 Kbp
Amino acids number	731 amino acids	641 amino acids
Domains number	5 domains	10 domains
mRNA length	4 Kbp	2 Kbp
Producing cells	Hepatocytes, megakaryocytes, platelets, macrophages and mast cells	Hepatocytes
Transporter	NO	Yes
Plasma FXIII	A_2B_2 tetramer	B_2 dimer
Intercellular FXIII	A_2 dimer	No β chains
Pathogenic mutations	> 150	< 20

Table II: Characteristics of F13A and F13B genes short tandem repeats (STRs) (HumFXIIIA01-HumFXIIIA02-HumFXIIIB)

STRs	HumFXIIIA01 (F13A01)	HumFXIIIA02 (F13A02)	HumFXIIIB (F13 B)
Repeat sequence	AAAG	AC	TTTA
Chromosomal location	6p24-25	6p24-25	1q31-32.1
Gene bank accession number	M21986	*	M64554
Recommended primers	5′-TTTTCTCTGCCTTCCCATGT-3′ 3′-ATGCCATGCCAGATTAGAAA-5′	5′-GAGGTTGCACTCCAGCCTTT-3′ 3′-CCCCCAGTGCAGTGTTTTAT-5′	5′-TGAGGTGGTTGTACTACCATA-3′ 3′-GATCATGCCATTGCACTCTA-5′
Expected range allele	281-331	210-238	169-189
Primer concentration	0.1µM	0.1µM	1µM
Reference	(38, 42-44, 66)	(45)	(38, 46, 67)

STR: Short Tandrm Repeat, HumFXIIIA01: Human Factor XIIIA01, HumFXIIIA02: Human Factor XIIIA02, HumFXIIIB: Human Factor XIIIB, µM: Micromolar. * No accession number was reported for this STR and it was mentioned in article (45).

Table III: Candidate SNPs of F13A gene for linkage analysis

Name of SNP	Chr position	Validated					Global MAF	SNP to chromosome	SNP to Refseq gene
		Hapmap	1000G	Cluster	Frequency	2 hit allele			
rs7740009	6:6176347	+	+	+	+	+	0.4954	Forward	Reverse
rs3778360	6:6150132	+	+	+	+	+	0.4842	Reverse	Forward
rs1270696	6:6246406	-	+	+	+	+	0.4972	Forward	Reverse
rs3024405	6:6223749	+	+	+	+	+	0.4740	Reverse	Forward
rs4960172	6:6161429	+	+	+	+	+	0.4892	Forward	Reverse
rs1615217	6:6230327	-	+	+	+	+	0.4988	Forward	Reverse
rs931247	6:6200540	-	+	+	+	+	0.4579	Forward	Reverse
rs2326707	6:6157555	+	+	+	+	+	0.4732	Forward	Reverse
rs1742924	6:6242713	+	+	+	+	-	0.4914	Forward	Reverse
rs4960181	6:6269065	+	+	+	+	+	0.4854	Forward	Reverse
rs7774391	6:6176392	+	+	+	+	+	0.4940	Forward	Reverse
rs7757883	6:6189967	+	+	+	+	+	0.4553	Forward	Reverse
rs1781794	6:6241547	+	+	+	+	-	0.4888	Reverse	Forward
rs202215504	6:6318688	-	+	+	+	-	0.4982	Forward	Reverse
rs3778355	6:6176795	+	+	+	+	+	0.4615	Reverse	Forward
rs3024319	6:6319115	+	+	+	+	+	0.4639	Reverse	Forward
rs542039918	6:6311046	+	+	+	+	-	0.4980	Forward	Reverse
rs1674044	6:6310481	+	+	+	+	+	0.4535	Forward	Reverse
rs3024317	6:6319146	+	+	+	+	+	0.4688	Reverse	Forward

Table IV: Candidate SNPs of F13B gene for linkage analysis

Name of SNP	Chr position	Validated					Global MAF	SNP to chromosome	SNP to Refseq gene
		Hapmap	1000G	Cluster	Frequency	2 hit allele			
rs5990	1:197039461	+	+	+	+	+	0.4750	Reverse	Forward
rs1336661	1:197060215	+	+	+	+	+	0.4758	Reverse	Forward
rs17549277	1:197061108	-	+	+	+	-	0.4688	Reverse	Forward
rs1412635	1:197053918	+	+	+	+	+	0.4690	Reverse	Forward
rs1711983	1:197053638	+	+	+	+	+	0.4758	Reverse	Forward
rs7518773	1:197042506	+	+	+	+	+	0.4681	Forward	Reverse
rs229882	1:197065696	+	+	+	+	+	0.4589	Reverse	Forward
rs1615413	1:197061187	+	+	-	+	+	0.4756	Reverse	Forward
rs857018	1:197049268	+	+	+	+	+	0.4683	Reverse	Forward

Discussion

Congenital factor XIII (FXIII) deficiency is an extremely rare bleeding disorder (RBD) with an estimated prevalence of one per 2 million in the general population. This RBD is accompanied by clinical manifestations, such as intracranial hemorrhage (ICH), recurrent miscarriage, and umbilical cord bleeding (UCB) (5, 12). In the homozygote individuals, UCB and ICH are observed in 80% and 25-30% of the patients, respectively. UCB is the main clinical presentation and can be used for diagnosis of the disorder at time of birth. This clinical presentation is a life-threatening presentation, so timely diagnosis and appropriate management of this hemorrhage is crucial. Due to high rate of life-threatening bleeding in FXIII deficiency, regular prophylaxis is mandatory for all severely affected patients from time of diagnosis. Without regular prophylaxis, about one-third of patients with congenital FXIII deficiency will die before 40 years due to ICH. Primar prophylaxis with FXIII concentrate is a treatment choice in patients with severe congenital FXIII deficiency. Due to normal results of the routine coagulation tests, diagnosis of the disorder is challenging worldwide. Clot solubility test is the most commonly used diagnostic test worldwide. Due to low sensitivity and specificity of the test, it is not further recommended by experts for diagnosis of the disorder. FXIII functional assay should be used as a screening test for the disorder, but it is not available in all areas (1, 16, 17).

Confirmation of the disorder can be performed by molecular genetic analysis of *F13A* and *F13B* genes (24).

For FXIII deficiency diagnosis in developed countries, direct mutation detection can be performed. This process is possible by different molecular techniques like NGS and Sanger sequencing analysis. In the most of developing countries due to limited economic resources, direct molecular diagnosis are not executable easily, so the best strategy for FXIII deficiency prevention is prenatal diagnosis and carrier detection. To precede this purpose, using indirect molecular diagnosis like linkage study is suitable. We can use polymorphic genetic markers associated with *FXIII* gene like HumFXIII01, candidate STRs and SNPs in these methods.

Molecular testing has good progress in recent years and with high throughput sequencing technology, our understanding about the molecular basis of congenital disorders will increase, but most of these new technologies and equipment are not available in a large part of the world. For this part of the world and for those areas with limited resources, alternative molecular analysis, such as linkage analysis, can be used for molecular diagnosis of the congenital disorders. FXIII deficiency is a rare, but severe congenital bleeding disorders and early diagnosis of the disorder is mandatory to reduce the rate of morbidity and mortality in severely affected individuals. In a large part of the world, the only diagnostic test for this disorder is clot solubility tests. This test is quantitative and cannot detect all patients with severe FXIII deficiency and patients with mild and moderate factor deficiency as well as heterozygotes, but an indirect molecular analysis has this potential power to detect patients with any *FXIII* gene defects. This technique can be used for the the disorder confirmation, carrier detection, prenatal diagnosis (PND), and pre-marriage analysis (Figure 5). Although the indirect molecular study has low diagnostic power than advanced available molecular techniques, it can be used in a large part of the world as an alternative for timely diagnosis of congenital disorders, such as congenital FXIII deficiency.

Conlusion

It seems that indirect molecular diagnosis is a relatively reliable and cost-effective method for diagnosis of congenital FXIII deficiency in the areas with low economic resources.

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Conflicts of interest

There are no conflicts of interest.

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