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RESEARCH ARTICLE

TWO NOVEL MUTATIONS IN SIX EXONS OF FACTOR VIII GENE IN SUDANESE PATIENTS WITH HEMOPHILIA A.

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Abstract

Hemophilia A (factor VIII deficiency) is the most common hereditary disorder of blood coagulation. It is due to the absence or decreased function of coagulation factor VIII, resulting from mutations in the factor VIII gene. The aim of the study was to screen the factor VIII gene mutation among Sudanese patients with hemophilia A.

This analytical cross sectional study conducted in Khartoum teaching hospital in patients with hemophilia A who attended to hemophilia center, 10 patients with hemophilia A were selected, 5 ml of blood samples were taken in K_2 EDTA for DNA extraction for the molecular studies and 5ml tri sodium citrate for APTT, PT, factor VIII assay and factor VIII inhibitor.

For the molecular studies a master mix and conventional PCR were used. Twelve primers were used for screening of six exons sequence by sequencer. For exon screening for mutations 120 PCR products were sequenced and analyzed by BLAST and FASTA in the NCBI web site, in which query and subjects procedure was used. PCR products were tested by agrose gel electrophoreses and gel documentation system.

The result showed All the 10 patients were males. For exons sequencing there were deletions mutations in three out of ten samples (30%), in exons 11, 23 and exon 24.

Introduction:-

The hemostatic system, consisting of the blood vessels and their content, blood, plays a crucial role in human survival. The importance of the plasma coagulation system in protecting life by preventing further blood loss following transsection of a blood vessel is well recognized. Blood is usually maintained in a fluid state, without evidence of bleeding or clotting. The presence of an X-linked pattern of inheritance of a bleeding diathesis in families, referred to as hemophilia, has been recognized for hundreds of years (1,2).

Inherited disorders of blood coagulation are due to the lack of synthesis or to the synthesis of a dysfunctional molecule of one and, in rare instances, more than one coagulation factors. Although uncommon, these disorders have provided a great deal of information about the normal physiology of blood coagulation. Factor VIII and factor IX deficiencies are inherited as a sex-linked trait. Von Willebrand disease (vWD) is inherited in an autosomal

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dominant manner, whereas all the other abnormalities of coagulation factors show an autosomal recessive pattern of inheritance. Many of the coagulation proteins have been cloned and their amino acid sequences are known. (3).

Hemophilia A:-

Hemophilia A (factor VIII deficiency) is the most common hereditary disorder of blood coagulation. It is due to the absence or decreased function of coagulation factor VIII, resulting from mutations in the factor VIII gene (3). The prevalence is 30-100 per million populations. The inheritance is sex-linked but up to 33% of the patients have no family history and result from spontaneous mutation. The factor VIII gene is situated near the tip of the long arm of the X chromosome (Xp2.6) and extremely large consisting of 26 exons. The factor VIII protein include triplicate regions A1, A2, and A3 with 30% homology with each other, a duplicated homology region C1and C2 and a heavy glycosylated B domain which is removed when factor VIII is activated by thrombin (4). Approximately half of the patients have missense, frame shift mutations or deletions in the factor VIII gene. In others a characteristic 'flip-tip, inversion is seen in which the factor VIII gene is broken by an inversion at the end of the X chromosome. This lead to severe form of hemophilia A (4,5).

Factor VIII:-

Factor VIII Gene:-

The human factor VIII gene was cloned between 1982 and 1984 by Gitschier and colleagues at Genentech Incorporation. (6): at the time the gene was the largest described (186kb). Mapping positions the factor VIII gene in the most distal band (Xq28) of the long arm of the X chromosome (7). Analysis of the gene reveals 26 exons, 24 of which vary in length from 69 to 262 base pairs (bp): the remaining much larger exons, 14 and 26, contain 3106 and 1958 bp respectively (the large majority of exon 26 is 3' un-translated sequence) *(*8).

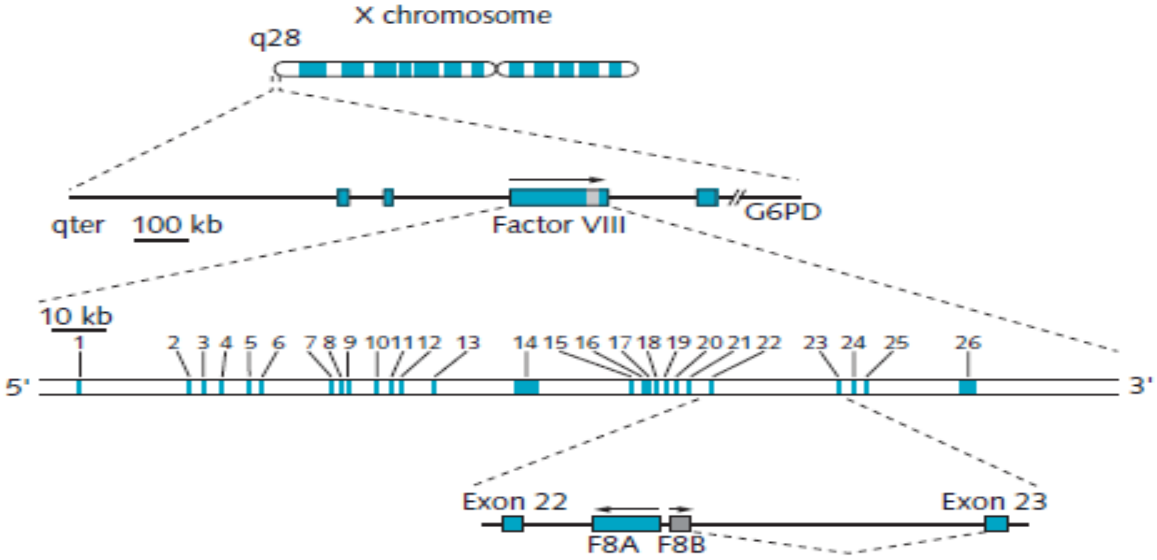

Figure (1):- Schematic representation of the chromosomal localization and structure of the factor VIII gene. (8)

The spliced FVIII mRNA was approximately 9kb in length and predicts a precursor protein of 2351 amino acids of the introns, 6 are larger than 14 kilo bases (kb). Unusually, the intron separating exons 22 and 23 (IVS22) contains a CpG island associated with two additional transcripts, termed factor VIII A (9) and factor VIII B (10). Factor VIII B was a transcript of 2.5kb and was transcribed in the same direction as the FVIII gene, using a private exon plus FVIII exons 23-26. Factor VIII A however contains no introns and was transcribed in the opposite direction to the FVIII gene: furthermore, two additional copies of factor VIII A have been found approximately 400kb telomeric to the FVIII gene (9) figure (1.1): these factor VIII A copies are implicated in almost half of severe hemophilia A via a partial inversion mechanism. The functions if any of the factor VIII A and factor VIII B transcripts and their potential translated products are unknown, although factor VIII A transcripts have been found in a wide variety of tissues.

Methodology:-

Sampling: -

Blood samples were taken under aseptic condition and tourniquet by vacutainers needle from the forearm vein. EDTA blood samples were used for DNA extraction for genetic testing. The samples were taken in anticoagulant disodium or potassium. Then were mixed gently. Tested immediately or storage in special ice containers in – 80 degree centigrade till tested. (11)

DNA extraction:-

DNA was extracted by using the EDTA blood samples and blood genomic DNA isolation kit (Kaminneni's life science company India). The DNA isolation kit provides a simple nontoxic method for quickly and efficiently isolation high molecular genomic DNA from whole blood. The advantage of this method was the neat of DNA of A260/A280 value of (1:80). Yield of DNA more than 50 micrograms from 0.3 ml of whole blood. The entire isolation takes only one hour and did not required phenol or chloroform.

The DNA isolation kit based on separating contaminating protein from DNA by salt precipitation, and the procedure involves digestion of cellular proteins using concentrated sodium chloride. (12)

Protocol of PCR for exons:-

Twelve primers (forward and reverse) for factor VIII gene sequence will be used for certain exons and sequencing to detect non sense mutation. For factor VIII gene mutations, we initially focused on six coding regions (exons 4, 8, 11, 18, 23 and 24). The reason for studying these exons was their smaller sizes and high mutational rates. (13)

Primers were shipped from applied biosystems USA at 10 nmol concentration, dried with distilled water and use ampli Taq polymerase and taq buffer (New England Biolabs), (Applied biosystems)

- 5 µl of 5X Amp *Taq* Reaction Buffer were used.
- 0.75 µl of 10 mM dNTPs were added.
- 3 µl of 10 µM Primer mix.
- 1 µl of Template DNA was added.
- 1 µl of Amp *Taq* DNA Polymerase.
- Nuclease-free water up to 25 µl was added.

An ice was used in a very quick process to avoid cross reactivity of the PCR and preheated lid thermal cycler Bio Rad (model T100) initial denaturation 94 ^{o}C for 2 minutes then followed by 30 cycles of 94 degrees C for 30 seconds, (49 – 53 ^{o}C) for 45 seconds and 72 degrees C for one minute 30 seconds. Final extension of 72 degrees C for 5 minutes. (14,15)

Table (1):- primer set forward and reverse for exons 4, 8, 11, 18, 23, and 24 showing product size, and primer annealing temperature. (14,15)

Primer set	Primer sequences	Product size (bp)	Annealig temp
F8EX4F F8EX4R	CATGTTTCTTTGAGTGTACAGTGG TTCAGGTGAAGGAACACAAATG	372 406	49
F8EX8F F8EX8R	CACCATGCTTCCCATATAGC ATGGCTTCAGGATTTGTTGG	484 518	49
F8EX11F F8EX11R	CCCTTGCAACAACAACATGA TTTCTTCAGGTTATAAGGGGACA	362 396	50
F8EX18F F8EX18R	TGGTGGAGTGGAGAGAAAGAA AGCATGGAGCTTGTCTGCTT	362 396	54
F8EX23F F8EX23R	TTGACAGAAATTGCTTTTTACTCTG TCCCCCAGTCTCAGGATAACT	294 328	50
F8EX24F F8EX24R	ACTGAGGCTGAAGCATGTCC CCCAACCACTGCTCTGAGTC	250 284	53

Then agrose gel electrophoreses 1% gel and 70 volts for 45 minutes to see if PCR was optimized to see the bands of exons by gel documentation system BIO RAD (Gel Doc X100).

Agrose gel electrophoreses:-

Agarose gel electrophoresis was the easiest and most popular way of separated and analyzed DNA. Here DNA molecules were separated on the basis of charge by applying an electric field to the electrophoresis apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process was called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide. (16)

Principle of sequencing:-

Sequencing samples using ABI 3130 sequencer (4 capillary) , 3730Xl (96 capillary) electrophoresis instruments. Along with samples, the "internal sequence control"(pGEM) was processed this was used to ensure the quality of the complete DNA Sequence process including the cycle sequencing, purification and loading on to the Sequencer . The internal sequence control worked well and the pGEM data was enclosed.

Results:-

Table (2) :- Frequency of exons mutation in the study.

	Mutation	Percent
Mutation	3	30%
Non mutant	7	70%
Tota of patients	10	100%

Table (3) :- Frequency of exons mutation in 120 PCR products.

	Mutation	Percent
Mutation	3	2.56%
Non mutant	117	97.46%
Total of PCR products	120	100%

Table (4) :- Exons mutation types and amino acid changes.

Mutant exon	Type of mutation	Nucleotide change position	Codon change
Exon 11	Deletion	T at 57	TTT to ATT
Exon 23	Deletion	T at 2151	TTC to TCA
Exon 24	Deletion	A at 2198	GAA to GAT

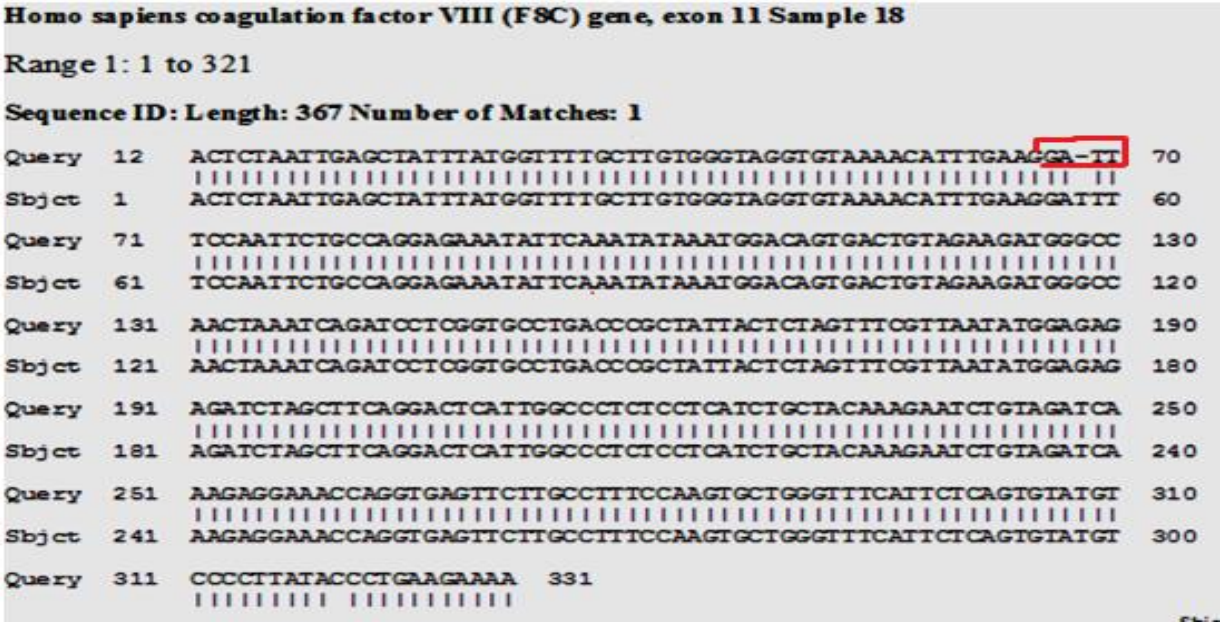

Figure (2): subject query alignment BLAST NCBI website Forward Primer exon 11 shows deletion mutation, T from the position 57 TTT Phenylalanine to codon ATT Leucine)

Figure (3):- subject query alignment BLAST NCBI website Forward Primer exon 23 shows deletion T from the position 563, codon change was TTC phenylalanine to TCA Serene amino acid change at 2151.

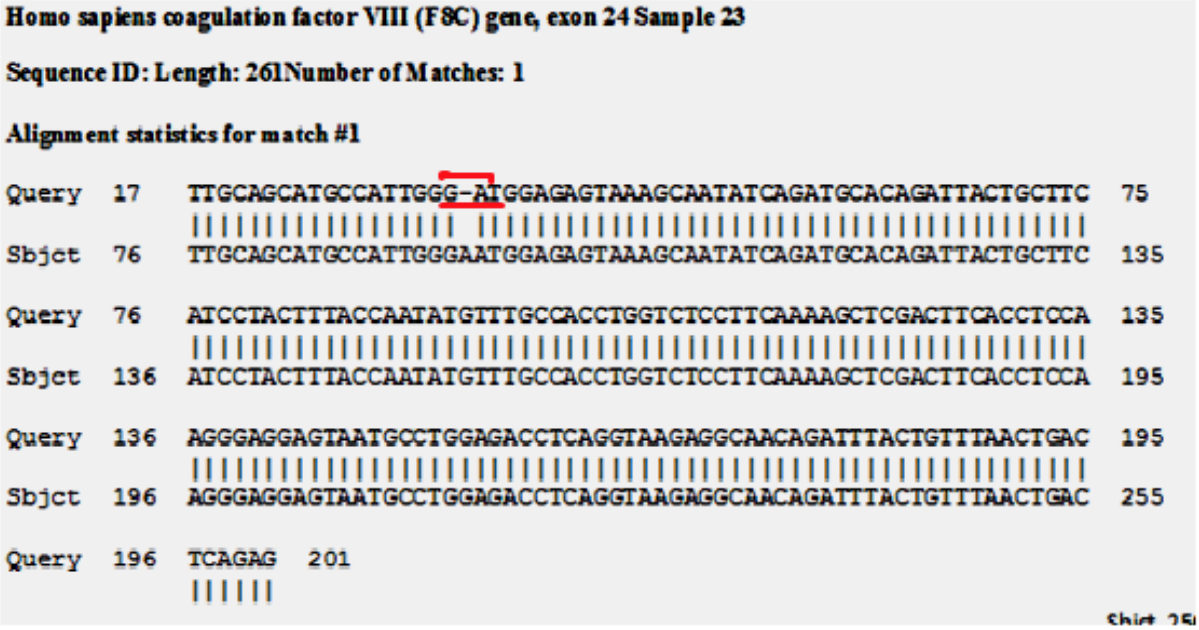

Figure (4)**:-** subject query alignment BLAST NCBI website Forward Primer exon 24 shows deletion, A from the position 95 codon GAA Glycine to GAT Aspartic acid at position 2198.

Discussion:-

Determining the sequence of bases in DNA become a major challenge of contemporary biology, and the desire to sequence the complete human genome has spawned the Human Genome Project (17). In the past, slab gel electrophoresis dominated DNA sequencing; now capillary electrophoresis was becoming the dominant technique. In the future, hybridization and mass spectrometry may replace electrophoresis for comparative or diagnostic sequencing, but electrophoresis will probably remain the major tool for de novo sequencing. The earlier history of the DNA sequencing, including the use of cross linked gels for capillary electrophoresis, has been reviewed. (17)

12 primers (forward and reverse) from applied biosystems company USA. For factor VIII gene mutations, a six coding regions exons (4, 8, 11, 18, 23 and 24) were choose, because of their smaller sizes and high mutational rates (13). Two types of PCRs amplification were used one master mix reagent from applied biosystems company USA cat no (4398876). The study group were 10 patients samples run with PCR, protocol of master mix was used. Second type was conventional PCR was used. The optimization of the PCR protocol begin from the annealing temperature which calculated by prime 3 software program, till the PCR give a clear bands and a 120 PCR product with their primers (forward and reverse) were labeled and shipped to Vimta laboratories India in 4 ^{o}C.

The mutation detection by alignment of origin sequence (14) (subjects sequence) and query sequence that done by the sequencer. Those two sequence alignment by the NCBI program nucleotide BLAST and FASTA after analysis we find most of results were typical sequence between query and subject see figure (3.1) and three new mutations out of the 10 samples were discovered in three exons 11, 23 and 24 as followed: all mutations were deletions, exon 11, exon 23, and exon 24. figure (3.2) and figure (3.3), and figure (3.4) respectively. Those mutation s were sever cases negative for inhibitor.

In exon 11 the deletion mutation at the position 57 of the subject sequence in factor VIII gene in Hemophilia A data base research site UK , the mutation lies in the heavy chain in the A 2 domain of the factor VIII gene. Codon change from TTT to ATT this deletion changed the amino acid phenylalanine (Phe) to Leucine (Leu) at the position 520 of amino acid gene sequence.

Deletion in exon 23 at the position 563 of the subject sequence in factor VIII gene in Hemophilia A data base research site UK, the mutation lies in the light chain in the C2 domain of the factor VIII gene. Codon change TTC to TCA this deletion changed the amino acid phenylalanine (Phe) to Serene (Ser)) at the position 2151 of amino acid gene sequence. These two mutations were not reported in The Hemophilia A Mutation, Structure, Test and Resource Site HAMSTeRS UK (18) and Factor VIII variant data base Royal free London NHS foundation trust (www.factorVIII db.org/).

Deletion in exon 24 at the position 95 of the subject sequence in factor VIII gene in (Hemophilia A data base research site UK) the mutation lies in the light chain in the C2 domain of the factor VIII gene. Codon change GAA to GAT this deletion changed the amino acid Glycine (Gly) to Aspartic acid (Asp) at the position 2198 of amino acid gene sequence this mutation was reported in hemophilia A database website.(18,19,20)

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