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Original Research Article

Molecular Study of the Correlation between Hemophilia and the Thrombophilic Risk Factors in Dohuk Province by using CVD Strip Assay.

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Abstract

Haemophilia A is an X-linked, recessively inherited bleeding disorder which results from deficiency of procoagulant factor VIII (FVIII). Forty hemophilic patients were enrolled in the current study. Blood sample were collected and DNA was extracted and subjected to multiplex PCR amplification using specific primers which resulted in bands containing all the suspected mutations associated with hemophilia; CVD Strip Assay was also used to differentiate the obtained mutations. The results showed that out of the (40) hemophilic individuals, the higher incidence (55%) was for the MTHFR (A1298C) heterozygotic type of mutation while the lower incidence (5%) for a mutation recorded for factor V (G1691A, leiden), factor V (H1299R, R2), prothrombin (G20210A), and facto XIII (V34L). For the Apo type mutation, B type hasn't been detected while E type observed in all cases; A1/A2 mutation created the higher incidence with 25% on the other hand, A3/A3 hasn't been detected and thus it represented 0% of all the Apo mutations.

Key Words: Hemophilia, MTHFR, Coagulation factor XIII, CVD Strip Assay.

الخلاصة

نزف الدم الوراثي نوع A هو من الاضطرابات الموروثة والمتعلقة بكروموسوم (٦) . ناتج من نقص في عامل التخثر شملت الدراسة الحالية (٤٠) مريض يعانون مرضا نزف الدم الوراثي وقد تم اخذ عينه دم من هؤلاء المرضى وبعد ذلك تم استخلاص الـ DNA لمضاعفة الجينات المطلوبة باستخدام تقنية multiplex PCR بوجود تنتج عن ذلك ظهور أشرطة تحتوي على أماكن الطفرات في المرضى المعنين بالبحث وكذلك تم استخدام تقنية CVD Strip Assay للتميز بين هذه الطفرات. أظهرت النتائج انه من بين هؤلاء (٤٠) مريض بوجود (٥٥%) منهم يعانون من طفرة A1298C MTHFR مع نسبة قليلة ٥% لطفرات مسجلة للعامل رقم ٥ (factor V G1691A, leiden), factor V (H1299R, R2) وعامل تخثر البروثروجية (G20210A) وعامل التخثر رقم ١٣ (V34L) كذلك لم يتم الكشف عن الطفرات Apo نوع B بينما تم الكشف عن نوع E من كل العينات من جهة اخرى سجلة طفرة A1/A2 نسبة عالية من الحدوث ٢٥% بينما لم تسجل أي نسبة لطفرة A3/A3 من كل العينات صفر %.

Introduction

Haemophilia A is an X-linked, recessively inherited bleeding disease which originates from deficient of coagulation factor XIII (FXIII). Victims always complain muscle and joint bleeding and easy bruising, the severity of signs is closely associated with the normal functioning of coagulation factor VIII in their blood. Factor XIII spans 186kb and consists of 26 exons, the size of which ranges from 69bp (exon 5) to 3.1kb (exon 14). Factor XIII mRNA is approximately 9kb in size and encodes a mature protein of 2332 amino acids [1]. The function of coagulation factor XIII (FXIII) is to stabilize clot by crosslinking fibrin chains during the final stage of the coagulation process [2].

The clinical outcome and the severity of hemophilia is greatly associated with the mutation type within the factor XIII gene. Furthermore, it has been stated that the clinical phenotype of hemophilia is markedly influenced by co-inheritance of the factor II G20210A variant or factor V G1691A mutation [3, 4].

Some scientists argue that the presence of multiple gene mutations in individuals affected by hemophilia has altered the longstanding paradigm that a diseases is the

attribute of single mutation in a single gene. A matter of interest is how the impacts of many mutations can be explained in terms of the participation of the intended single mutations at two or more sites. Inadequacies in the practices of mutation screening could primarily have contributed to frequently noticed discrepancies in the genotype-phenotype relationship [5].

Among the genetic factors responsible for hemophilic disorders are, single nucleotide polymorphisms (SNPs) in the genes for II (prothrombin), blood coagulation factors V (FV), plasminogen activator inhibitor-1 (PAI-1), and XIII (FXIII), ß-fibrinogen (FGB), 5,10-methylenetetrahydrofolate reductase (MTHFR), and apolipoproteins B (Apo B) and E (Apo E) resulting in cardiovascular diseases [6].

The aim of the present work is to study the incidence of the most common mutation in hemophilic patients by using CVD Strip Assay.

Materials and Methods:

Forty hemophilic patients were enrolled in the current study, they were previously diagnosed as local hemophilic patients and had recorded history in Kurdistan committee of hemophilia/Dohuk and thus they were continuously followed up and treated on schedules.

1) Sample collection and extraction of DNA

Blood samples were collected from these individuals and DNA was extracted by phenol -chloroform method as described by previous literature and kept at -20 for later use [7].

2) Cardiovascular Disease Strip Assay (CVD Strip Assay)

It was used to investigate the existence of any of the 12 mutations (FV G1691A (Leiden), FV H1299R (R2), PTH G20210A, Factor XIII V34L, ß-Fibrinogen −455 G-A, PAI-1 4G/5G, GPIIIa L33P (HPA-1), MTHFR C677T, MTHFR A1298C, ACE I/D, Apo B R3500Q and Apo E2/E3/E4) in hemophilic patients.

3) PCR amplification

Subjects were tested for Factor V, Prothrombin, MTHFR, and Apo gene mutations using the CVD Strip Assay (Vienna Lab, Austria) and the tests' protocols were followed as described by the manufacturer.

Multiplex-PCR was applied to amplify the genes of interest. The amplification conditions were of an initial step of denaturation at 94° C for 2 min, followed by 35 cycles for the CVD assay of denaturation at 94° C for 15s, annealing step at 58°C for 30s, extension at 72°C for 30s, and a final extension step at 72°C for 3 minutes [8].

4) Electrophoresis PCR products were run on 3% agarose gel to analyze the outcomes of amplifications [9]. Lastly, the products of amplification were selectively hybridized to a test strip which consisted of allele-specific (denoting wild or mutant type) oligonucleotide probes immobilized as an array of parallel lines. Streptavidin–alkaline phosphatase and color substrates were used to detect bound biotinylated sequences. Procedure strictly followed what was written by the manufacturer.

5) Interpretation of genotyping results

For each polymorphic site, one of three possible patterns may be obtained: normal, heterozygous, or homozygous mutant genotype. For the Apo E isoforms E2, E3 and E4, six possible homozygous and heterozygous Apo E genotypes (E2/2, E3/3, E4/4, E2/3, E2/4, and E3/4) could be obtained.

Results:

The results of the current study revealed that all the enrolled individuals had shown PCR profile relevant to hemophiliac patients, resulting bands are indicative of successful amplifications for the twelve genes of interest (Figure 1).

Applying the FV-PTH-MTHFR Strip Assay, out of the (40) hemophilic individuals 22 (55%) had shown MTHFR (A1298C) heterozygotic type of mutation; while the homozygotic type represented by 1 (2.5%) only. Moreover, MTHFR (C677T) mutation was represented by 10 (25%) and 4 (10%) samples for heterozygotic and homozygotic types, respectively.

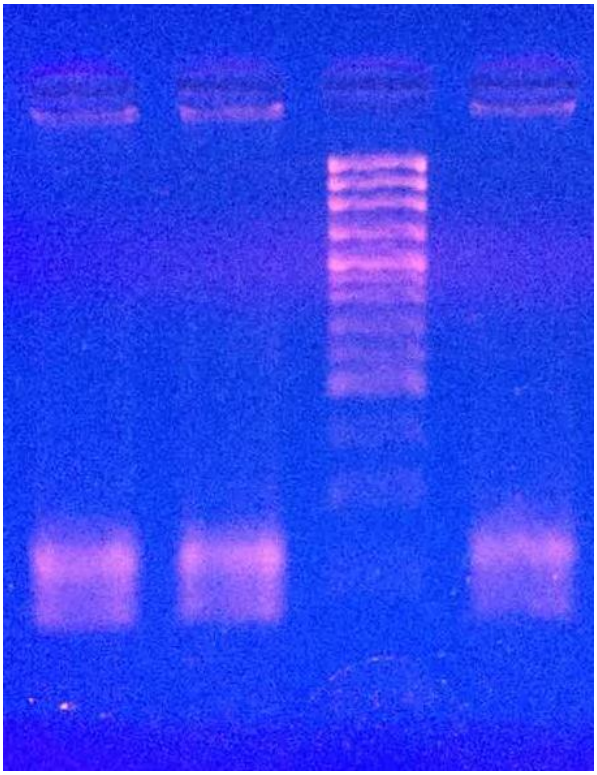

Figure 1: PCR products for the twelve mutant genes. Amplification bands were run on agarose 3%.

In addition, the results of the current study revealed high incidence of PAI-1 mutation and 20 (50%) of cases were diagnosed as affected by heterozygotic type of this mutation, on the other hand, homozygotes constituted 4 (10%) of patients. Interestingly, heterozygotic mutation in 5% of individuals was evidenced for three types of mutations namely, factor V (G1691A, leiden), factor V (H1299R, R2), prothrombin (G20210A), and facto XIII (V34L). Finally, the occurrence of Apo E mutations which are divided into four types represented by Apo E codon 112: TGC (A1), Apo E codon 112: CGC (A2), Apo E codon 158: TGC (A3) and Apo E codon 158: CGC (A4) is presented in table 1.

Table (1): Positive samples for Apo E mutation variants and their relevant percentages

Mutation type	Positive samples	%
Apo E (A1/A1)	6	15%
Apo E (A2/A2)	8	20%
Apo E (A3/A3)	0	0%
Apo E (A1/A2)	10	25%
Apo E (A1/A3)	2	5%
Apo E (A2/A3)	2	5%

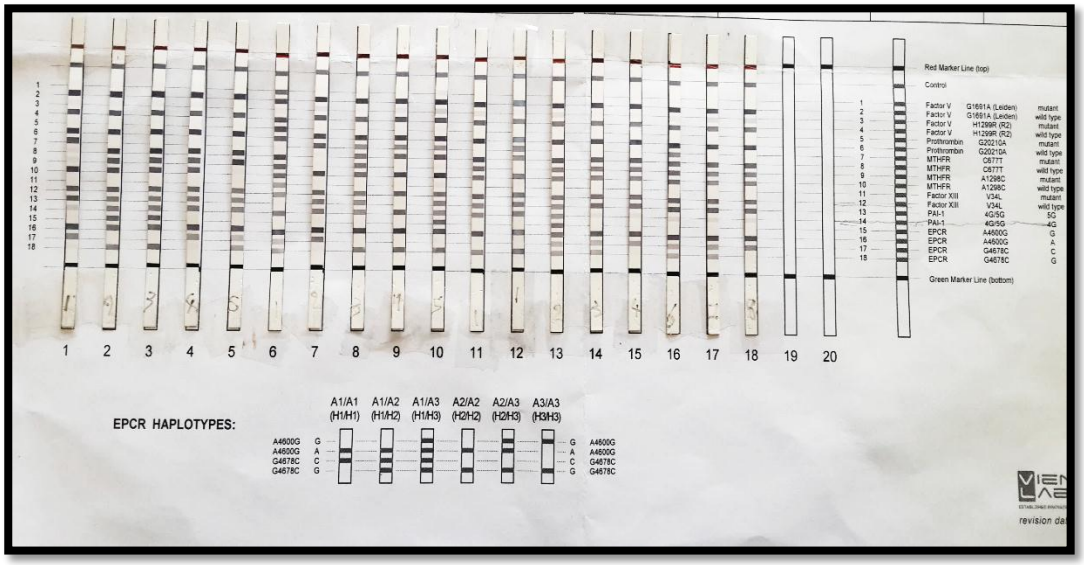

Figure (2): Design of CVD strip assay test used in the current study.

Discussion:

There is an increase diversity of the incidence of polymorphisms and prothrombotic risk alteration of genetic makeup worldwide. Data from different centers, countries, and ethnic population vary greatly [10]. The results of the current study were consistent with that of other researchers [11] who revealed mutations in hemophilia patients as follows: *prothrombin* G20210A (3%), *FVL* (14%), *MTHFR* C677T (42%), and A1298C (59%). It was also noticed that heterozygosity of *FVL* or *prothrombin* G20210A was almost linked to a mild or moderate, but not a severe, clinical manifestations [12].

Moreover, it was revealed that a homozygote point mutation (677: C>T) in the MTHFR gene (5-20% prevalence) lead to a temperature sensitive enzyme variant with decreased activity especially in Caucasians [13]. This was much lower than the figures reported by the present work and the discrepancy can be justified as a result of population mixture or ancient migratory activity or, it is a technical effect depending on search itself, design of the study and exploratory methods [14, 15, 16].

On the contrary, a correlation between prothrombotic genetic variants (FVL, FVR2, FII G20210A, FXIII Val34Leu, b-fibrinogen G455A, HPA 1b/1b, PAI-1 4G/5G, MTHFR A1298C and MTHFR C677T) and clinical presentation of hemophilic patients haven't been found while in the current work a strong association has been established between the above mentioned mutations with hemophilia.

Another study reported higher prevalence of 57% for the MTHFR C677T mutation [17] than both heterozogotic (25%) and homozygotic (10%) types of the same mutation found in the current study. Consistently, the same author stated that 70% of the cases of the MTHFR mutations occur simultaneously with other types of mutation such as Factor V Leiden and this finding came in line with the results of the present study. In previous work [18], percentages of 2.02% and 4.8% were reported for FII (G20210A) and FV (G1691A), respectively; these findings were close to those of the present work in which a 5% of affected individuals had these types of mutation.

The existence of what is called modulating genes or prothrombotic risk factors (Factor II 20210A, Factor V G1691A, and FV Leiden) results in attenuation of hemorrhagic signs such as onset of bleeding attacks and frequency of hemarthroses as well as therapeutic requirements [19, 20]. Previous literature reveal that the contribution of prothrombotic genetic risk factors to the wide range variability of the clinical presentation of hemophilias has yet to be determined. It can be difficult to compare the results of studies

in this field because low number of cases sometimes, the reproducibility of clinical data is poor, the prothrombotic markers investigated in different studies are variable, in some research, HA and HB patients are poorly differentiated, and HA patients with varying FVIII levels are compared. It can be stated that, multicenter studies need to be accomplished to obtain conclusions on the relationship between phenotypic presentation of hemophilia and prothrombotic gene variants [21].

The existence of multiple mutations has a direct effect on genetic diagnostic approaches as well as genetic counseling strategies. Based on the wide world knowledge, genetic screening should not terminate after an individual mutation has been detected. As hemophilia genetic testing translocate from the research phase to the clinical field, it will become more difficult to ascertain that affected populations receive the intact notifications about the limitations of this test. Moreover, a screening for mutation is offered by the commercial genetic testing only, rather than the whole gene sequencing, which may appear more cost-effective to a victim. Despite not established, the presence of multiple gene mutations may be considered as a marker of disease intensity and therefore denote patients who would most make use from more directed treatment and prevention strategies. With more and more illnesses being linked to multiple mutations, the idea of 'mono factorial disease' or 'monogenic trait' becomes vague and the concept of Mendelian inheritance in the near future may become a non-absolute attitude even in a monogenic diseases like hemophilia [22].

In conclusion, a correlation has been found between hemophilia and some mutations causing cardiovascular risks.

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