The Association between Prothrombin Gene G20210A and Factor V Leiden Mutation in Women with Complications of Pregnancy in Baghdad Province

المصاحبة بين طفرة جين ألبروثرومبين G20210A والعامل الخامس لايدن في النساء أللواتي يعانين من مضاعفات الحمل في مدينة بغداد

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Abstract

Factor V Leiden (FVL) (G1691A) and prothrombin gene (G20210A) mutations are the most common inherited forms of thrombophilia. The main objective of this study is to analyze the association between inherited thrombophilia FVL mutation and prothrombin G20210A mutation and recurrent pregnancy loss (RPL) among women suffering from complications of pregnancy. The study included 40 buccal swab samples collected from women at the reproductive age complications; abruption placenta 12.5%, dead fetal 37.5%, and recurrent spontaneous abortions (RSA) 50% in comparison with 30 women who had one or more normal pregnancies from Elwiyah Obstetric teaching hospital through the period from mid of September 2014 to the mid of March 2015. The median age of patients was 32 years (range: 19–42 years) while for the control group, it was 28 years (range: 17–41 years). Out of 40 women, 65% had one pregnancy loss and 35% for more than two previous pregnancy losses. According to the time of pregnancy losses, 22(55%) women had early pregnancy loss (EPL), and 18 (45%) women had Late Pregnancy Loss (LPL). For FVL mutation detection the restriction fragment length polymorphism (RFLP) was used. DNA fragment of interest was amplified by PCR and then subjected to digestion by *MnlI* specific restriction enzyme. For prothrombin G20210A mutation detection different PCR products were generated using a set of primers in A/A, G/G, A/G alleles. Out of the 70 samples tested for FVL mutation no homozygous FVL mutation was detected and Prothrombin gene mutation G20210A was totally absent among patient and control.

Key words: gene polymorphism, factor V Leiden, Prothrombin gene, obstetrical complications, abortion

الملخص

تعد الطفرات الوراثية الحاصلة في جين العامل الخامس لايدن (FVL) (G1691A) وجين البروثرومبين (G20210A) هي من أكثر الأشكال شيوعا لمرضى الثرومبوفيليا. الهدف الرئيسي من هذه الدراسة هو تحليل العلاقة بين الإصابة بمرض الثرومبوفيليا وعلاقته بالطفرات الحاصلة في كل من جين FVL وجين البروثرومبين G20210A في النساء اللواتي يعانين من مضاعفات الحمل، إذ شملت الدراسة جمع 40 عينة بشكل مسحة فموية من النساء اللواتي يعانين من إحدى مضاعفات الحمل والتي تضمنت انفكاك المشيمة 12.5٪ والجنين الميت 37.5٪ والإجهاض التلقائي المتكرر 50٪ لهذه الدراسة، ومقارنة النتائج مع 30 امرأة ممن لهن واحد أو أكثر من حالات الحمل العادية، من مستشفى العلوية للولادة التعليمي خلال الفترة من منتصف شهر أيلول 2014 الى منتصف شهر اذار2015، وكان متوسط عمر المرضى 32 عاما (المدى: من19- 42 سنة). في حين كان متوسط عمر الأصحاء 28 عاما (المدى: من17-41 سنة). من 40 امرأة، كان 65٪ منهن فقدن الحمل للمرة الأولى و 35٪ منهن فقدن الحمل لأكثر من مرة. 22 امرأة تعرضن لفقدان مبكر للحمل، و 18 امرأة في وقت متاخر من الحمل. للتحري عن الطفرة في جين العامل الخامس لايدن استخدمت تقنية التباينات في الطول باستخدام تقنية (RFLP) أذ يتم تضخيم جزء من ألجين ثم تعريضه لعملية الهضم من خلال أنزيم القطع*MnlI*. للتحري عن الطفرة في جين البروثرومبين ضخمت قطع مختلفة باستعمال مجموعة من ألبرايمرات المتخصصة لتحديد أليلات A/A, G/G, A/G. من 70 عينة تم اختبارها ل FVL جين لم يتم الكشف عن وجود أي طفرة فيه وكذلك بالنسبة للطفرةG20210A للبروثرومبين أذ يمكن اعتبار النمط الوراثي لهما غائبا من نساء المجتمع العراقي.

الكلمة الدالة : تعدد الأشكال الجيني، جين العامل الخامس لايدن، جين ألبروثرومبين، مضاعفات الحمل، الإجهاض

Introduction

Recurrent pregnancy loss (RPL) represents a major health problem with two to three or more losses in up to 5% of women of reproductive age and is actually one of the most common causes of female sterility [1]. Thrombophilia, both acquired and hereditary, has been implicated in the increased susceptibility to adverse pregnancy outcomes such as fetal loss recurrent spontaneous abortions (RSA), abruption placenta, intrauterine growth restriction (IUGR) and pre-eclampsia [2]. Hereditary thrombophilia is a genetic disorder of blood coagulation resulting in an unusual hypercoagulation state, which in turn can result in abnormal implantation and may manifest as spontaneous loss [3].Genetic thrombosis risk factors include a sequence variant in the prothrombin (20210G>A) and factor V Leiden (1691G>A) genes. The functional consequence of the mutation that occurs is an impaired in activation of Factor V (also known as "activated protein C resistance"), Factor V Leiden is a single point mutation involving a guanine to adenine transition at position 1691 in exon 10 of the factor V gene, which leads to the synthesis of a variant factor V molecule. A FVL mutation resulting in a substitution of Glu to Arg at position 506 in the protein prevents cleavage of factor V at this site by APC and thus results in a delay of inactivation. As a result, clotting becomes less inhibited [4] resulting in increased thrombin generation. The second most frequent thrombophilia is a single nucleotide substitution (G20210A) in the promoter region of the gene for the key coagulation factor, prothrombin. This prothrombin gene mutation (PGM) results in an increase in the concentration of prothrombin [5]. It is determined that a variant of coagulation factor V, designated as factor V Leiden, is basically a genetic polymorphism. This is a SNP polymorphism in the 506th cod on, which triplet CGA for arginine replaces the CAA triplet for glutamine. Under normal conditions, APC proteinbinds to factor V and cuts it into two inactive fragments. It is determined that the Leiden variant is resistant to APC protein, which prolongs the action of factor V. The result is a continuation of prothrombin activation and continuously maintain coagulation cascade [6-7]. Its frequency in European populations is 1-5%, while it is very rare in people of African or Asiatic origin, this polymorphism is almost absent. It is estimated that this polymorphism originated 21- 24000 years ago [6-7].

Factor V Leiden and prothrombin G20210a mutations have been identified as high risk factors for venous thromboembolism (VTE) among Caucasians. In fact, the relative risk of venous thrombosis is increased approximately 4- to 8-fold in individuals who are heterozygous for the FVL mutation and up to 80-fold in individuals who are homozygous [7-8] .The prothrombin G20210A mutation increases the relative risk of venous thrombosis by approximately three fold [9].

The prothrombin G20210A mutation involves guanine to adenine substitution at nucleotide 20210 of the prothrombin gene [10]. The mutation is associated with an increased plasma concentration of prothrombin, which leads to an increased potential for thrombin generation [11]. The main objective of this study is to analyze the association between inherited thrombophilia FVL mutation and prothrombin G20210A mutation with RPL frequency.

Materials and Methods

Sample collection (Buccal swab)

Samples were collected from 40 women who had one of the above-mentioned obstetrical complications (abruption placenta, fetal growth retardation, and stillbirth) and 30 women who had one or more normal pregnancies from Elwiyah Obstetric teaching hospital through the period from mid of September 2014 to the mid of March 2015, the volunteers recruited were asked to rinse their mouth with tap water, 30 sec before sampling of Buccal swabs, to avoid the contamination as a result of food particles. For each individual, both sides of buccal mucosa were wiped with a cotton swab for 15 sec.

Detection of FVL and prothrombin G20210A mutations

DNA was extracted using organic phenol-chlorophorm method with modification [12]. The concentration of DNA sample was determined using the Nano Drop ND-1000 spectrophotometer (Nano Drop Technology. Detection of FVL and prothrombin G20210A mutations was based on examination the size of the polymerase chain reaction (PCR) products following DNA amplification of the target sequence of the factor V gene and factor II gene respectively. Factor V Leiden mutation detection was done by performing PCR-restriction fragment length polymorphism (RFLP) with *MnlI* specific restriction enzyme while allele-specific PCR was done to detect prothrombin G20210A mutation. The following primers were used according to [13]. Factor V Leiden Mutation detection by FVA 5' GGA ACA ACA CCA TGA TCA GAG CA -3', and FVB 5' TAG CCA

GGA GAC CTA ACA TGT TC -3'. The prothrombin G20210A mutation detection by PRC 5' CTC CAA ACT GAT CAA TGA CCT TC -3' PFC 5' TCT AGA AAC AGT TGC CTG GCA -3' for common gene (c) produce 220 bp, Mismatched in one nucleotide in antisense primers were used to amplify both mutant (M) 340 bp, and normal (N) products 340 bp, PRN 5' CAC TGG GAG CAT TGA GGC AC -3', PRM 5' CAC TGG GAG CAT TGA GGC AT -3 primers sets. Two reaction sets was performed for each sample (Normal reaction contain: 0.5 µl PFC primer, 0.5 µl PRC primer, 0.5 µl PRN12.5µl of 2X Master mix, 6µl nuclease free water µl, 5µl template DNA), and (Mutant reaction contain: 0.5 µl PFC primer, 0.5 µl PRC primer, 0.5 µl PRM primer, 12.5µl of 2X Master mix, 6µl nuclease free water µl, 5µl template DNA). The amplification cycle for both FVL and prothrombin G20210A mutation detection was optimized for several trial till reach to the optimal condition as the following: 1- pre-denaturation at 95°C 5 minutes,2- secondary denaturation at 94°C 15 seconds,3- annealing at 64°C for FVL, and at 62°C for prothrombin gene for 15 sec, 4-extention at72°C 30 sec (steps 2 -4was repeated for 40 cycles), 5-final extension at 72°C for 1 minute and holding at 4°C. After the PCR-RFLP and allele-specific PCR were performed, 7.0 µl of the sample products were electrophoresed in a 3% agarose gel. The DNA ladder and samples were run at 70 V for 120 minutes. Finally, the bands were visualized using an image analyzer. Detection of FVL mutation involves the amplification of a 287 bp-long fragment, which yields in the production of various numbers of fragments after restriction with 0.5µl *MnlI*, 0.5µl Bovine serum albumin, 2µl of 10X buffer, and 10µl of PCR products of FVL gene. Normal alleles with G/G at nucleotide 1691 yielded the following 3 fragments: 37 bp, 93 bp, and 157 bp. As FVL mutation abolishes the *MnlI* restriction site, a homozygous A/A yields only 130 bp and 157 bp fragments. In heterozygous alleles with genotype G/A, all 37 bp, 93 bp, 130 bp, and 157 bp fragments are produced. For prothrombin G20210A mutation detection, electrophoresis yields a 220 bp fragment and a 340 bp fragment in Normal (N) and 220bp in mutant (M) reactions respectively when a normal G/G allele is present at nucleotide G20210A. In homozygous mutant A/A alleles, both 220 bp and 340 bp fragments are produced in M reaction while none is produced in N reaction. On the other hand, heterozygous G/A alleles yield 219 bp and 340 bp fragments in M reaction and a 340 bp fragment in N reaction.

Results and discussion

During the study, taking into account the age of the women, the obstetrical complications in pregnancy and frequency of pregnancy loss of embryos, and studying the impact of these factors on the rate of emergence of both FVL and prothrombin G20210A mutation. The obstetrical complications showed in Figure (1), abruption placenta represented 12.5%, while dead fetal was represented 37.5%, and the recurrent spontaneous abortions (RSA) was represented 50% of the subject study.The median age of the patients women was 32 years (range: 19–42 years) while median age of the control group was 28 years (range: 17–41 years). Out of 40 women, 65% had one pregnancy losses and 35% for more than two previous pregnancy losses. According to the time of pregnancy losses, 22 (55%) women had Early Pregnancy Loss (EPL) < 7 weeks, and 18 (45%) women had Late Pregnancy Loss (LPL) >7 weeks as shown in Figure 2. The association of inherited markers of thrombophilia in a 40 pregnant Iraqi women with a history of multiple pregnancy loss was conducted. Out off 70 tested cases all had normal allele while no homozygote or heterozygous FVL mutation were detected the results was showed in Figure (3 and 4).

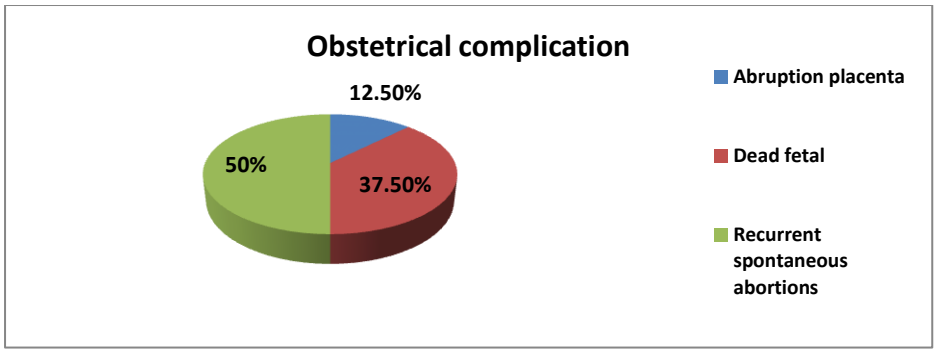

Fig. (1): The percentage of the obstetrical complication in 40 women

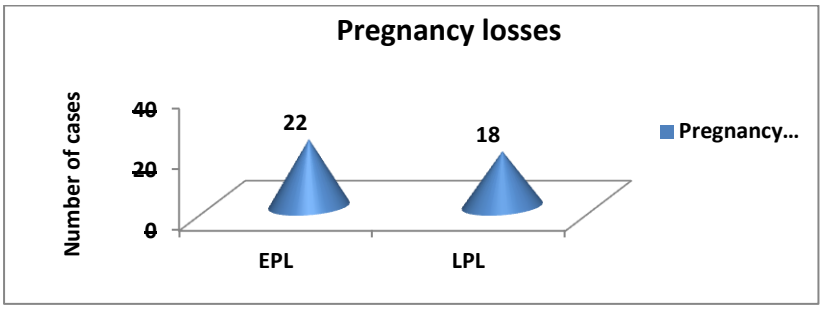

Fig. (2): The number of women who had the early pregnancy loss, and the late pregnancy loss according to time of pregnancy losses.

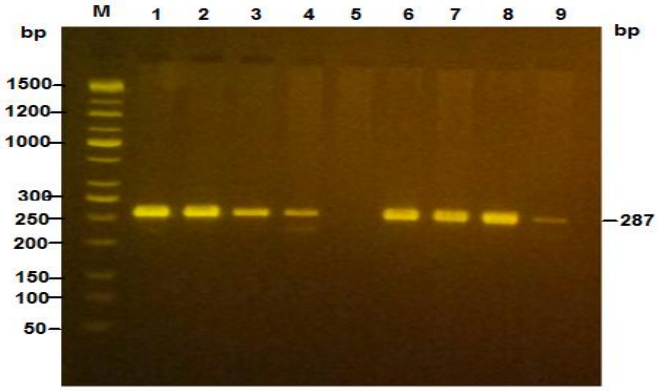

Fig. (3): Electrophoresis analysis of PCR product FVL gene fragment 287 bp ,using 2% Agarose stained with red safe DNA dye and electrophoresed by 3vol/cm in TBA buffer ,Lane M-50 bp DNA ladder.Lane (1,2,3,4,) show samples of healthy group and (6,7,8,9,) sample of patients .

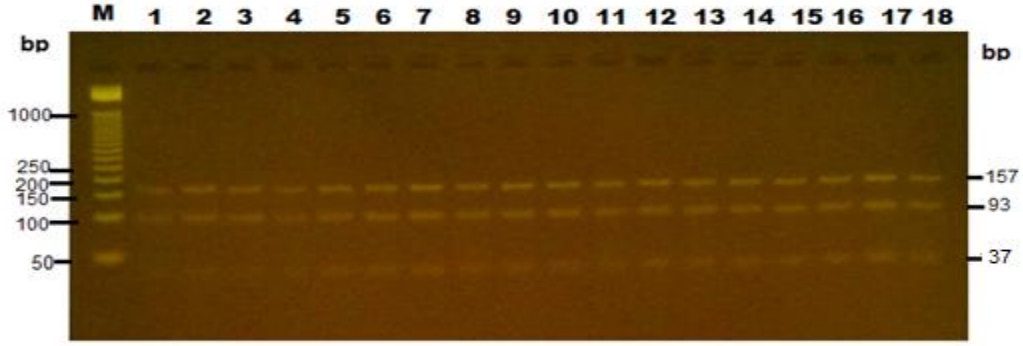

Fig. (4): The gel-electrophoresis result of RFLP using 3% Agarose stained with red safe DNA dye and electrophoresed by 3vol/cm in TBA buffer. Lane M-50 bp DNA ladder, Lane (1,3,5,7,10,11,14,16,17) patient sample and lane (2,4,6,8,9,12,13) healthy people .PCR product restricted with *MnII* restriction enzyme showed normal allele G/G.

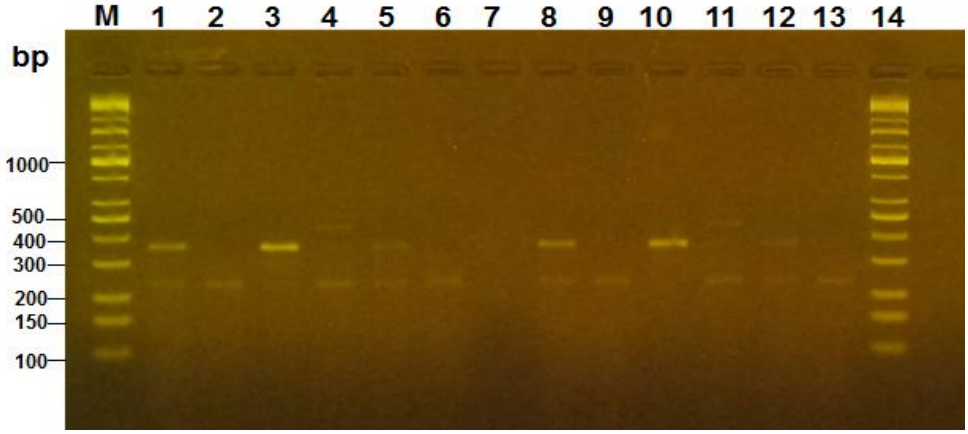

Fig. (5): The gel-electrophoresis result of prothrombin G20210A mutation in 3% agarose stained with red safe DNA dye and electrophoresed by 3vol/cm in TBA buffer. Lane M-100 bp DNA ladderelectrophoresis, lane 1,3,5,8,10,12 showed Normal reaction result (220 bp, and340bp for sample 11,3,15,8,11,33,) respectively lane 2,4,6,9,11,13 showed Mutant reaction result (220 bp for sample 11,3,15,8,11,33,) respectively.

Also no prothrombin G20210A mutation was detected in any patients or control groups, which indicates that FVL and G20210 prothrombin gene mutations are infrequent in Iraqi women as mention as in figure (5). Our findings were very closed to Abdullah's *et al* .,[12] study results that was conducted on healthy Indians in Malaysia , they was reported (5.6%) heterozygous for FVL mutation, and agree with Vora *et al*.,[13] study on Indian people as they found that (3.4%) homozygous FVL mutation were positive for factor V Leiden mutation compared to 1% of controls. These findings are in complete agreement with previous reports that found prothrombin G20210A mutation to be totally absent among non-Caucasians patients, Ahmed *et al*.,[14].Greer [15] study on Caucasian population was found that the inherited thrombophilia is common in the Caucasian population with a prevalence of up to 15% Our result was indicated that there is no association between the prothrombin G20210A mutation and pregnancy loss, and this result is agrees with Robert *et al.,* [16], and with Sharma *et al* [17] that the factor V Leiden and G20210 prothrombin gene mutations are infrequent in Indian patients with portal vein thrombosis PVT. Thus, these mutations are unlikely to be responsible for PVT in the Indian population .We concluded that there was no association between the prothrombin G20210A mutation and any individual obstetric complication, including pregnancy loss, preeclampsia, abruption, and SGA neonates in Iraqi patients. This low-risk results among studies may explained by the different ethnic groups that found in Iraqi population. The variability of result in different study appeared due to the small size of samples that have been used to conduct these studies and the diversity of ethnic groups. Each of these factors introduces potential bias. It is striking that the only large case–control study may be confident to find the association between the prothrombin G20210A mutation and SGA neonates.

Conclusion

This study indicated that FVL and G20210 prothrombin gene mutations are infrequent in Iraqi women.

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