The Association of Prothrombin Gene Mutations and Cytomegalovirus Infection with Abortion Among Iraqi Women

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Abstract:
Abortion is categorized as the termination of conception caused by the failure or removal of the embryo from the uterus before the conclusion of pregnancy. Microorganisms and genetic factors are two of the many factors associated with abortion. Cytomegalovirus is a widespread congenital virus infection pathogen that affects a wide variety of people. The prothrombin gene is one of the essential causes that trigger blood clotting and the function of abortion women, therefore the aim of the study is to detect and associate Cytomegalovirus and prothrombin gene mutation (Gene ID: 14061 in NCBI) with abortion through genetic and immunological methods. Five ml of whole blood was collected from an intravenous puncture and divided into two tubes, one with EDTA and one without (plain tube) from 74 women with an abortion history as a patient group and 74 women without an abortion record who had at least one successful fertility as a control group. Allele-specific PCRs are used to amplify gene regions with genetic primers containing prothrombin gene polymorphisms. Current results have shown the greatest risk of abortion was observed in women patients with IgG seropositivity in 65 women with frequency (87.8%) and the lowest rate of abortion was in IgM seropositivity in 3 women with frequency (4.1%) and 6 (8.1%) were positive for IgM and IgG indicating they have both an old and recent infections. Furthermore, allele-specific PCRs are used to amplify prothrombin G20210A polymorphism. The result of this study demonstrated there is no association between prothrombin genotype level frequency and abortion in CMV-infected women. While, there is a highly significant association between A and G Alleles combinations and abortion in CMV-infected women.

Keywords: Abortion, Cytomegalovirus, Polymorphisms, Pregnancy, Prothrombin gene.

Introduction:
Pregnancy is the fertility period in which a mother obtains a live infant by implanting a fertilized zygote during the copulation . Pregnant women face several problems, with morning sickness being the most complicated and common problem. Abortion is defined as the death of a fetus weighing less than 1000 grams before the fetus reaches the age (23-24) weeks .

Although abortion has many causes, more than 50 percent of cases remain idiopathic . In the first trimester of pregnancy, the bulk of miscarriage happens in almost 80% of unintended fetal death due to symptoms such as bleeding and discomfort during pregnancy that contributes to extreme maternal anxiety.

Cytomegalovirus is a common congenital viral infection pathogen that affects a wide range of people worldwide (85%) . Human Cytomegalovirus (HCMV) infection is usually asymptomatic and ends in a life-long latent infection . The high prevalence of congenital infections, which can lead to severe congenital abnormalities, is a major public health concern during puberty and adolescence .

Increased incidence of both spontaneous and birth defects is also associated with maternal genetic susceptibility . Examples of mutation are the prothrombin G20210A, Factor V Leiden (FVL), and Methylenetetrahydrofolate Reductase . Prothrombin G20210A is the second most frequent genetic abnormality in women with pregnancy-related thromboembolism following FVL mutation . The prothrombin mutation G20210A requires the substitution of guanine for
adenine at nucleotide site 20210 in the untranslated region at three ends of the prothrombin gene. So, the transition of amino acid arginine to Glutamine is at site 457 of the prothrombin gene. The prothrombin gene contains 21,000 base pairs (bp), including 14 exons and 13 introns, and is located in chromosome number 11.

The study aims to detect and correlate the genetic variation of the prothrombin gene mutation, as well as its association with abortion, using genetic and immunological methods.

Materials and Methods:

Samples collection
The study is a correlational analysis that was carried out by collecting data from maternity and children’s hospital consultations, Al Hashimiya General Hospital in Babylon Province, besides several medical labs in the provinces of Babylon, Wasit or Diwaniyah, between the period from September 2020 to December 2020. The control group included seventy-four healthy women between the ages of 18-50 years, while the patient group included seventy-four women who suffered from recurrent pregnancy loss with an age range between (15-45) years. However, the included criteria like fetal chromosomal defects and deformities of the uterus who take the drug and congenital malformations of the fetus and abortion in the second and third trimester must be met.

Blood collection
Five ml of whole blood was collected from an intravenous puncture and divided into two tubes, one with EDTA and one without (plain tube). Specimen of the blood of each participant is obtained after consent.

Serology test
Serum was separated for serological immunology analysis to detect CMV in serum by the mini-Vidas technique kit IgM and IgG CMV mini-Vidas (Biomerieux, France) according to the manufacturing instructions.

DNA extraction
Blood was collected to use the DNA extraction kit (Intron Biotechnology, Korea) according to manufacturing procedure and kept at -20°C for further analysis. The UV spectrophotometer analysis is used to measure the concentration and the purity of DNA samples.

Genetically test
The identification of the prothrombin gene mutation (G20210A) was verified by the amplification of the mutated allele (M reaction) and the normal allele (N reaction) by allele-specific PCR. The primers used in this study are based on what has previously been identified for identifying mutation (G20210A) (Tab. 1).

<table>
<thead>
<tr>
<th>primer</th>
<th>Sequence (5’……..3’)</th>
<th>Tm</th>
<th>GC%</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC (Reverse)</td>
<td>CTC CAA ACT GAT CAA TGA CCT TC</td>
<td>61.1</td>
<td>43</td>
<td>219</td>
</tr>
<tr>
<td>PFC (forward)</td>
<td>TCT AGA AAC AGT TGC CTG GCA</td>
<td>59.4</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>PRN (Reverse)</td>
<td>CAC TGG GAG CAT TGA GGC AC</td>
<td>62.5</td>
<td>60</td>
<td>340</td>
</tr>
<tr>
<td>PRM (Reverse)</td>
<td>CAC TGG GAG CAT TGA GGC AT</td>
<td>60.5</td>
<td>55</td>
<td>55</td>
</tr>
</tbody>
</table>

Allele-specific PCR protocol
Via preparing two tubes for each sample, labelling one of them as (M) and the other is (N) for each tube for each patient and control, 5μl of Master Mix, 8μl of isolated DNA solution, nuclease-free water, and 2μl of PFC were added to each tube, while 2μl of PRC, and 2μl of PRM in (M) tube, and just 2μl of PRN in (N) tube. After that, 6μl from DNA product were taken and electrophoresed in a 1.5% agarose concentration at 75 V for 55 min, and the UVP trans illuminator device observes the DNA bands and captures the gel images.

Program Allele-specific PCR system
The amplification cycle was detected by using the prothrombin G20210A mutation detection as in the following: 5 minutes at 95°C, 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C (steps 2 to 4, 40 times), 5 minutes at 72°C and 4°C.

Detection of (G20210A) mutation
The homozygous A/A allele yields only 219bp and 340bp fragments from electrophoresis in the (M) reaction, while the heterozygous G/A allele yields 219bp and 340bp fragments in the (M) reaction, and the 340bp fragment in the N reaction. however, mild allele G/G In both the (M) and (N) reactions, 219bp and 340bp fragments are formed.

Statistical analysis:
A statistical study was conducted using the Statistical Package for Social Sciences (SPSS version 26, Inc., Chicago, IL, USA) and the Microsoft Excel Worksheet. The results and
examples of the current study were analyzed. P-value significance was determined when the value less than 0.05 (P<0.05), Odds Ratio (OR), and 95% Confidence Interval. 

Results:
The two classes of seropositivity rates of IgG- and IgM unique to CMV were reviewed in a total of 148 studies. The results of the patient women involved showed that 65 (87.8%) were positive for IgG, indicating an old infection, 3 (4.1%) were positive for IgM, indicating a recent infection, and 6 (8.1%) were positive for both IgM- and IgG, indicating an old and recent infection. Whereas healthy women are negative for both IgM and IgG (Fig. 1).

Maternal age was divided into 5 major groups include (15-21) years recorded patients women (17.6%) and (21.6%) recorded control women, (22-28) years recorded patients women (47.3%), and (29.7%) recorded control women, (29-35) years recorded patients women (29.7%) and (32.4%) recorded control women, (36-42) years recorded patients women (4.1%) and (9.5%) recorded control women, (43-49) years recorded patients women (1.4%) and (6.8%) recorded control women (Fig. 2).

According to the results of the PCR analysis for amplification of the prothrombin G20210A mutation, 1(1.35%) of patient women afflicted with Cytomegalovirus have the AA allele as homozygous, 54(72.97%) of patients have the GG allele, and 19(25.68%) of patients have the AG allele as heterozygous, as seen in Fig. 3. In addition to the control group, which did not have the AA allele, 64(86.49%) had the GG allele, and 10(13.51%) had the AG allele as heterozygous (Fig. 4.)
While prothrombin G20210A mutation revealed that 21 (14.19%) patient women were infected with Cytomegalovirus, and 10 (6.76%) healthy women have A allele and 127 (85.81%) patient women infected with Cytomegalovirus, and 138 (93.24%) healthy women have G allele. The prothrombin (G20201A) genotype level frequency with abortion in CMV-infected women was (P=0.7749; OR = 1.61; CI95%= 0.060-43.249). While the prothrombin allele level frequency is linked with abortion in CMV-infected women (P=0.0408; OD= 2.28; CI95%= 1.0349-5.0313) (Tab. 2).

**Table 2. Allele and genotype frequencies of prothrombin gene mutation among patients with Cytomegalovirus and healthy women. (P ≤ 0.05; OR; 95% Confidence Interval (95% CI))**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients (74)</th>
<th>Control (74)</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>1.35</td>
<td>0</td>
<td>0</td>
<td>0.7749</td>
</tr>
<tr>
<td>AG</td>
<td>19</td>
<td>25.68</td>
<td>10</td>
<td>13.51</td>
<td>0.4407</td>
</tr>
<tr>
<td>GG</td>
<td>54</td>
<td>72.97</td>
<td>64</td>
<td>86.49</td>
<td></td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P value</td>
</tr>
<tr>
<td>A</td>
<td>21</td>
<td>14.19</td>
<td>10</td>
<td>6.76</td>
<td>0.0408</td>
</tr>
<tr>
<td>G</td>
<td>127</td>
<td>85.81</td>
<td>138</td>
<td>93.24</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion:**

The association between seroprevalence rates of anti-CMV IgM and IgG, maternal age, and prothrombin gene mutation with repeated abortion is a real and present danger on pregnancy complications. CMV is the most frequent source of congenital infectious diseases, hence more than
70% within the first three months of pregnancy can result in abortion 22.

In the current research, the number and proportion of both CMV-IgG and IgM seropositive women who had abortions are determined in table 1. The highest rate of IgG specific to CMV recording percentage seropositivity rates at 65 women with a frequency of 87.8%, was observed approach to the previous result given by Yasir et al., 2020 23. Using the mini-Vidas method, the results show the highest IgG specific to CMV was recorded, indicating that the infection is old or latent. While the findings of this research differ from the previous study advanced by Anwar and Al-Bayati, 2017 24, where the results are the highest IgM specific to CMV recorded using the ELISA method. Thus, the prevalence of IgM antibody to CMV indicates that latent CMV are infections or re-infection with a new strain of the virus.

While an elevated proportion of abortion was observed in the group of patients (22-28) aged 47.3% more than other ages, because of their more probable chance than older age groups of pregnancy. This result was similar to the previous result given by Kadhim et al., 2020 25. While the findings of this research differ from those of the previous study advanced by Saad et al., 2021 26. According to the findings, the majority of primary CMV infections occur in early adulthood, as elevated CMV seroprevalence is found in younger women who become infected mostly in childhood or early adulthood. In comparison to older women, younger women have a lower recurrence percentage of abortion 27.

The prothrombin G20210A mutation is a genetic mutation that contributes to thrombophilia, which is associated with a dangerous rise in frequent abortion, including embryo death 28. The results of the present research indicate no relationship between the frequency of the prothrombin (G20210A) genotype level with miscarriage in women infected with CMV. An approach has been observed to the previous result given by Rafiei et al., 2017 29, where the results disagree with the connection between this polymorphism and abortions. Although the conclusions of this research differ from others a previous study performed 2011 by Gihan Gawish et al. 30, where they pointed that the effects of the G20210A genetic variant in the patient sample were more prevalent than in the control group. The findings' variability can explain the disparity in the race, sample size, and other genetic and environmental influences that interfere with the RPL.

**Conclusion:**

It’s concluded from the results of the current study, there are no significant differences across genotypes AA, AG, and GG This implies that there is no association between the prothrombin (G20210A) genotype level frequency and abortion in CMV-infected women. While the combination of the A allele and the G allele of the prothrombin (G20210A) is found to be associated with abortion in CMV-infected women.

**Authors' declaration:**

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for republication attached with the manuscript.
- Authors sign on ethical consideration’s approval
- Ethical Clearance: The project was approved by the local ethical committee in University of Al-Qasim Green

**Authors' contributions:**

E.F.T., A.G.H. and A.K.A. contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript

**References:**


علاقة الطفرة الجينية للبروثرومبين وعدوى الفيروس المضخم للخلايا بالإجهاض بين النساء العراقيات

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الخلاصة:
يُصنف الإجهاض على أنه إنهاء الحمل الناجم عن فقدان الجنين أو إزالة الجنين من الرحم قبل إتمام الحمل. هناك عوامل مختلفة ترتبط بالإجهاض منها الكائنات الحية الدقيقة والعوامل الوراثية. أن الفيروس المضخم للخلايا هو أحد مسببات العدوى الفيروسية الخلقية التي تسبب مجموعة واسعة من الأشخاص. يعتبر جين البروثرومبين (معرف الجين: 14061 في NCBI) أحد الأسباب الأساسية التي تؤدي إلى تخثر الدم وظاهرة الإجهاض، وبالتالي فإن الهدف من الدراسة هو اكتشاف وربط الفيروس المضخم للخلايا وطفرة جين البروثرومبين بالإجهاض من خلال الأساليب الجينية والمناعية.

تم جمع 5 مل من حجم الدم الكامل من خلال وخز الوريد وتم تقسيمها إلى أنبوبتين، أحداهما تحتوي على EDTA والأخرى دون (أنوب عادي) من 74 امرأة لديهن تاريخ إجهاض كمجموعة مريضات و74 امرأة بدون سجل إجهاض مع واحد على الأقل من الخصوبة الناجحة كمجموعة تحكم.

تُستخدم PCRs الخاصة بالأليل لتضخيم مناطق الجينات باستخدام بادئات جينية تحتوي على تعدد أشكال G20201A البروثرومبين. أظهرت النتائج الحالية أن أكبر خطر للإجهاض لوحظ في النساء المصابات بتفاعلية مصلية IgG في 65 امرأة مع تردد (87.8%) وكان أقل معدل للإجهاض في إيجابية مصلية IgM في 3 امرأة بمعدل تكرار (4.1%) و(6.1%) كانت إيجابية لـ IgG و-IgM.

أظهرت نتيجة هذه الدراسة عدم وجود علاقة بين تكرار مستوى التفاعل الجيني للبروثرومبين والإجهاض في النساء المصابات بفيروس CMV في حين أن هناك علاقة ارتباط ذات دلالة إحصائية بين مجموعات الأليلات G و-IgG و-IgM في النساء المصابات بفيروس CMV.

الكلمات المفتاحية: إجهاض، فيروس مضخم للخلايا، تعدد الأشكال، الحمل، جين البروثرومبين.