Evaluation the role of PAI_1 polymorphism in thrombous formation of type II diabetes mellitus patients

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Abstract

This study was designed to investigate the role of polymorphisms of plasminogen activator inhibitor-1 PAI_1 gene in development of vascular thrombosis(VTE,MI,PE)in patients with T2DM using Amplification-Refractory Mutation System ARMS_PCR to detect change at the promoter region of PAI-1 gene at site (_675). Results of statistical analysis for ARMS-PCR method showed that frequency of genotypes in two study groups were 38 samples 54% heterozygous,5 samples 7%homo4Gand27 samples 39%homo5Gin Group1,while Group2 contain 39 samples 56%heterozygous,7 samples 10%homo4Gand 24 samples 34% homo5G. The current study found that differences in genotypes distribution between groups was not significant (P<0.07) and that’s mean two variables were independent. Estimation the effect of pathogen (T2DM,thrombous) and PAI_1 genotypes and their interaction on PAI_1 concentration in serum, in Group1 significant effect recorded at the level(P<0.05) for factor 1,2 and their interaction on PAI_1 concentration ,higher levels of protein in serum shwoed in patients with 4G/4G genotypes.

Introduction

Type 2 diabetes mellitus T2DM diagnosed by measuring blood glucose during fasting period blood glucose FBG, whose level in healthy people may be (3.5-5.5) mmol/L (Shaw & Tanamas, 2012). As a concept, its conditions affect the body’s ability to regulate the level of sugar in the blood, In 2017, the number of people with type I and II was estimated at 425 million people around the world (Saeedi et al., 2019). Approximately 80% of deaths in patients with T2DM are due to thrombosis and cardiovascular disease CVD (Stegenga et al., 2008). Recently, genetic studies revealed the association of more than 80 susceptible T2DM loci, these loci were identified as large groups of specific ethnic groups (Andersen et al., 2016). T1DM and T2DM are characterized by a defect in insulin signaling that stimulates glucose transport to muscles, liver and adipose tissue under physiological conditions, where the peptide hormone stimulates glucose uptake in T2DM With or without obesity (Al Alawi et al., 2018).

Thrombosis risk is influenced by the balance between plasminogen activator inhibitor-1 PAI_1 and tissue plasminogen activator t_PA, an increase in the PAI_1
levels in plasma can induce a hypercoagulable state and facilitate thrombous formation (Verkleij et al., 2011). Fibrinolytic system activated by conversion of plasminogen to plasmin by t_PA or u_PA and inhibited by PAI_1, furthermore increased PAI_1 levels associated with impaired activity of fibrinolytic system in coronary artery disease (Jung et al., 2018). PAI-1 is released by vascular endothelial cells VECs, adipocytes, hepatocytes, fibroblasts, cardiomyocytes, and platelets, in healthy humans levels of it in plasma top to t_PA by a ratio of 4:1in which most of PAI_1 filtered by liver, in pathologic cases its secretion can be arranger by proinflammatory factors such as insulin and TNFα (Cesari et al., 2010).

PAI-1 is an independent risk factor for cardiovascular disease as it was recently shown that increased expression of the PAI-1 gene in the walls of blood vessels leads to the formation of a rupturable plaque and thus Acute coronary syndrome ACS (Zakrzewski et al., 2016). Genetic polymorphism is defined as the presence of at least two variants within the gene sequences or chromosome structure observed with a frequency of 1% or higher (Daly, 2010). SNPs formed as a result of a change, and they are one of the prominent sources of variation in the human genome and are described as an excellent genetic indicator, its may fall within the gene sequences or within its sequences, as they may arise in non-coding regions of the genome that do not often have a known effect on the phenotype of the individual, as their effect depends on its location and can have different results on phenotypes (Ismail & Essawi, 2012). Studies have indicated a relationship between PAI-1 4G/5G polymorphisms with many diseases such as CAD, cardiovascular disease, asthma, high blood pressure, hypertension, stroke, obesity, rheumatoid arthritis and osteoarthritis (Ozgen et al., 2012). The current study aims to evaluation of the Role of Plasminogen Activator Inhibitor_1 gene polymorphisms in thrombous formation in Patients with T2DM by using ARMS_PCR Technique.

Materials and methods

The study included (140) individuals, their ages between (38-85) years, distributed into two groups. Group 1 (70 individuals) a group of patients with T2DM and blood clots (MI, PE, DVT) entrants in the intensive care units (CCU) in Baghdad and Tikrit hospitals, Group2 (70 individuals) it included healthy individuals, venous blood (5ml) collected in gel tubes and isolated serum to measurement PAI_1 concentration by used analysis kit of Jordanian Al-shkairate establishment for medical supply for ELISA technology,(3ml) were collected and placed in tubes containing anticoagulant (K2EDTA) and kept at a temperature of (_20) until it was used in the molecular aspect. Genomic DNA was isolated from blood samples in Group1 and Group2 using ready-made ReliaPrep™ Blood gDNA Miniprep system analysis kit of Promega company. After DNA extraction completed samples migrated on gel electrophoresis (1%) to ensure presence or absence of genomic DNA by using red safe stain. After migration was completed, the gel was
transferred to the imaging device to see the resulting bands that indicate the presence of DNA.

The primers used in the study were prepared from Macrogen/korea company and were dissolved using Nuclease free water to reach the final concentration (100pmol/µl). Next step included preparing working solution by adding (10µl) of previous solution to (90µl)Nuclease-Free Water to reach a primer concentration (10pmol/µl) for use in PCR reaction. ARMS_PCR were used in this study to detected PAI_1 gene polymorphism, four primers were used, 4G (5_GTC TGG ACA CGT GGGGA_3) and 5G allele (5_GTC TGG ACA CGTG GG_3)as well as upstream (5_AAGCTT TTA CCA TGG TAA CCC CTGGT_3) and downstream primer (5_TGC AGC CAG CCA CGT GATTGT CTA G_3), PCR program contain 30cycles first step was Initial Denaturation 95°C for 5min, the second step Denaturation at 95°C for 30 sec third step Annealing at 55°C for 30 sec and forth step was Extension at 72°C for 30 sec,fifth step Final extension at 72°C for 7min and the last step was Hold 10°C for 10min, step 2,3,4 contain 30cycles while steps1,5,6 contain 1cycle (Shaghaghi et al., 2014). At the end of PCR reaction Agarose gel electrophoresis(2%) used to detect bands and genotypes for each sample.

Results and Discussion
when PCR reaction end, the products migrated onto gel electrophoresis (agarose2%) and then detected by gel documentation system, as aresult when the special primers bind and amplified the bands size 138bp,139bp determined the presence of 4G,5G allele, respectively in samples of patients and healthy individuals,figure(1,2) this results are in agreement with the findings of Shaghaghi et al (2014).

Figure(1).Result of migration heterozygous 4G/5G genotypes of PAI-1 gene on agarose (2%) using ethidium bromide stain for patient samples. The M symbol refer to molecular
weight index (1000bp) lines (1,4,5,8) represents 4G/5G genotypes (138,139bp) and lines (2,3,6,7,9) represent 5G/5G (139bp) homozygous genotypes. Bands with size (257 bp) refer to replication of the positive control primers.

Figure(2). Result of migration heterozygous 4G/5G genotypes of PAI-1 gene on agarose (2%) using ethidium bromide stain for healthy samples. The M symbol refers to molecular weight index (1000bp). Lines (63, 65, 66, 67, 69) represents 4G/5G genotypes (138,139bp) and lines (64, 68, 70) represent 5G/5G (139bp) homozygous genotypes. Bands with size (257 bp) refer to replication of the positive control primers.

The significant differences between genotypes in two study groups were tested by used chi-square test at the probability level (p<0.05), Group1 showed presence (38) samples (54%) 4G/5G genotypes and (5) samples (7%) 4G/4G genotype and (27) samples (39%) 5G/5G genotype, while Group2 results showed presence (39) samples (56%) 4G/5G genotype, (7) samples (10%) 4G/4G genotype and (24) samples (34%) 5G/5G genotype, as a result the difference in genotype distribution between groups was not significant (P<0.07) that’s mean that two variables were independent, Table(1) this results agreed with Shaghaghi et al (2014).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Genotypes</th>
<th>Observed genotypes</th>
<th>Genotype frequencies</th>
<th>Allele frequencies</th>
<th>Expected genotype frequencies</th>
<th>Expected genotypes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G/5G</td>
<td>77 individuals</td>
<td>0.55 (55%)</td>
<td>-</td>
<td>0.47</td>
<td>65 individuals</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Homo 4G</td>
<td>12 individuals</td>
<td>0.09 (9%)</td>
<td>0.4 (36%)</td>
<td>0.13</td>
<td>18 individuals</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (1) Results of statistical analysis of genotypes distribution in PAI_1 gene using ARMS PCR
<table>
<thead>
<tr>
<th>Homogeneous</th>
<th>Individuals</th>
<th>Genotype</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Genotype Frequency</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td></td>
<td>4G/5G</td>
<td>0.54</td>
<td>0.36</td>
<td>0.46 (54%)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homo 4G</td>
<td>0.07</td>
<td>0.34</td>
<td>0.11 (7%)</td>
<td>8 Indiv.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homo 5G</td>
<td>0.39</td>
<td>0.66</td>
<td>0.43 (39%)</td>
<td>30 Indiv.</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>4G/5G</td>
<td>0.56</td>
<td>0.47</td>
<td>0.33 (56%)</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homo 4G</td>
<td>0.1</td>
<td>0.38</td>
<td>0.14 (10%)</td>
<td>10 Indiv.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homo 5G</td>
<td>0.34</td>
<td>0.62</td>
<td>0.39 (34%)</td>
<td>27 Indiv.</td>
</tr>
</tbody>
</table>

Genotype frequencies for two groups were (55%) for heterozygous, (9%) for 4G/4G and (36%) for 5G/5G genotype, while allele frequencies in two groups were (36%) for 4G allele and (64%) 5G allele. Hardy weinberg equation used to calculated expected genotype frequencies which was (0.47) for heterozygous genotypes, (0.13) for 4G/4G genotype and (0.40) for 5G/5G genotypes. This study results agreed with results of Isordia-Salas et al (2009).
Results of this study showed no significant differences of genotype variable between Group1 and Group2. This may explained by the fact that polymorphism of PAI_1 gene may not be the only reason for the occurrence of Venous thrombosis embolism VTE in patients with T2DM, frequency of 5G allele in current study was higher compared to 4G although 4G allele and 4G/4G genotype have important and binding role for increased gene expression of PA_1 and protein secretion in plasma of patients with MI, DVT, and PE this is associated with disturbance of fibrinolysis system and coagulation formation,4G/5G polymorphism binding with modification its level in plasma and as a result 4G/4G genotype linked with hypofibrinolysis and caused intravascular complication that’s lead to thrombous formation and inflammatory diseases (Shaghaghi et al., 2014). results reached by Liang et al (2015) not compatible of our results and found that 4G/4G genotype frequency was higher in individuals with positive history for coronary heart disease CHD compare with those without this history, individulas with 4G/4G genotype have higher PAI_1 activity three times compare with those with 5G/5G genotype so that 4G allele linked with MI disease.

current study results of showed increased in frequency of 5G allele (64%) comparsion to 4G allele (36%) among two study groups, however 4G allele have astrong effect on protein secretion that may be have arisk factor for development of many diseases such as Familial Mediterranean Fever, Acute Myocardial Infarction, Coronary artery disease and T2DM, our finding agreement with the results founded by Bonyadi et al (2013) and Lima et al (2011).the results of current study was not compatible with results of Mohammad et al (2020) who reached that frequency of 4G allele increased comparsion to 5G in MI patients and there is a link between 4G allele and high levels of protein in serum thus increased the risk of MI.

The results of the study showed a significant increase at the probability level (p<0.05) in the concentration of PAI-1(ng/ml) in the group of patients (6.68±0.60) compared to the healthy controls (3.72±0.85), where the results explain the existence of significant differences between the two groups of patients. And healthy ones for the measured physiological variables, Fig(4).The results of current study agreement with results founded by Schneider & Sobel (2012) showed an increase in concentrations of PAI-1 in blood and arterial walls of people with obesity, metabolic syndrome and T2DM, where it was found that the increase the expression of PAI-1 is stimulated by insulin resistance IR or its rise, Which is the contributing factor to risk of CAD in patients with diabetes T2DM.
Several studies agree with the results of the current study, including what Sakurai et al (2020) indicated about presence of a significant increase in concentration of PAI-1 in T2DM patients compared to healthy as a result of many factors, including high glucose level, visceral obesity, insulin resistance, lipid disorder, Enhancement of the fibrinolytic system by reducing PAI-1 using empagliflozin as a therapeutic method to prevent CVD in T2DM patients. Results of current study was compatible with what reached by Kearney et al (2017) indicated that PAI_1 concentration linked with risk of CAD in which increased in survivors MI pateints or those with recurrent MI, PAI_1 secretion from endothelial and adipocytes working as primary inhibitor of fibrinolysis in T2DM.

Current study agreement with the result of Grant (2007) found that increased levels of PAI_1 may be independent risk factor to improved T2DM in healthy individuals and suggested that PAI_1 can considered an early danger indicator to improved metabolic syndrome and T2DM. Depending on Liang et al (2015) results PAI_1 polymorphisms may be not effected on PAI_1 concentration but on its response to some factors, so that elevated level of this protein may be result from VLDL_C,IL_1 in case of 4G allele, high levels of protein may caused from severe shock in pateints carried 4G allele compare with 5G allele, so that can describe polymorphism as polyresponse and the difference in protein concentration between two alleles be more clear in presence of natural or pathogenic factors that stimulated its secretion.

Figure(4) Figure (1): The results of the physiological variable PAI–1 test in patients group Group1 and healthy group2, different letters = significant differences
Reference


