Genetic study of ITGA2 polymorphisms and impact on diabetic retinopathy risk in Al-Anbar population

Estudio genético de polimorfismos ITGA2 e impacto en el riesgo de retinopatía diabética en la población de Al-Anbar

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Abstract

This study aimed to explore an accurate estimate of the association between ITGA2 gene polymorphisms and diabetic retinopathy risk in the population of Al-Anbar province.

Methods: The study was designed as a case-control study which included 75 persons, who were divided into two groups 25 were healthy control, while 50 were patients with Type II diabetes mellitus, and the later patients were subdivided into two groups, 25 with diabetic retinopathy and 25 without diabetic retinopathy. Blood samples were collected in Al-Nahrain Eye Specialty Center in Al-Ramadi city, west of Iraq, for the period from the seventh of September to the twenty-fifth of December 2019.

Results: The wild genotype CC/GG and mutant homozygous TT/AA genotype for C807T/G873A SNPs appeared in equal number in controls with the presence of TT/AA genotype in half of the control and most of the patients. While the heterozygous CT/GA genotype was present in approximately in the same proportion in controls and patients. There was a high relationship between C807T and G873A SNPs with the progression of diabetic retinopathy disease with X^2(15.83 and 13.722) and a significant P value (<0.003 and <0.008) for both SNPs, respectively. Both SNPs were in a high linked disequilibrium with D' (0.99), D value (0.15) at significant P<0.0001.

Conclusion: Mutant homozygous pattern for both 807TT/873AA SNPs, appears as a risk factor for diabetic retinopathy development in Al-Anbar population with co-dominant models as a genetic model for both SNPs behavior to transmit the disease.

Resumen

El objetivo principal de este estudio fue explorar una estimación precisa de la asociación entre los polimorfismos del gen ITGA2 y el riesgo de retinopatía diabética en la población de la provincia de Al-Anbar.

Métodos: El estudio se diseñó como un estudio de casos y controles que incluyó a 75 personas, que se dividieron en dos grupos, 25 eran de control de salud, mientras que 50 eran pacientes con diabetes mellitus tipo II, y esos pacientes se subdividieron en dos grupos (25 con retinopatía diabética). y 25 sin retinopatía diabética). Se recolectaron muestras de sangre en el Centro de Especialidades Oculares de Al-Nahrain en la ciudad de Al-Ramadi al oeste de Irak durante el período del 7 de septiembre al 25 de diciembre de 2019.

Resultados: El genotipo salvaje CC / GG y el genotipo TT / AA homocigoto mutante para los SNP C807T / G873A aparecieron en igual número en los controles con la presencia del genotipo TT / AA en la mitad del control y en la mayoría de los pacientes. Mientras que el genotipo heterocigoto CT / GA presenta aproximadamente en la misma proporción en controles y pacientes. Hubo una alta relación entre los SNP de C807T y G873A con la progresión de la enfermedad de la retinopatía diabética con X2 (15,83 y 13,722) y un valor de P significativo (<0,003 y <0,008) para ambos SNP, respectivamente. Ambos SNP se encontraban en un alto desequilibrio ligado con D '(0,99), valor D (0,15) en P significativo (0,000.).

Conclusión: patrón homocigoto mutante para ambos SNP 807TT / 873AA, aparece como un factor de riesgo para el desarrollo de retinopatía diabética en la población Al-Anbar con modelos codominantes como modelo genético para el comportamiento de ambos SNP para transmitir la enfermedad.

Introduction

Diabetic retinopathy (DR) is a vasculopathy that affects the fine vessels in the eye and is a leading cause of preventable blindness globally. Forty to 45% of diabetic patients are likely to have DR at some point in their life; however, fewer than half of DR patients are aware of their condition[1]. Integrin 2 1 is a glycoprotein which acts as a receptor for collagen/laminin (similarly identified as very late activation antigen-2VLA-2 or the platelet glycoprotein (GP or Ia/IIa), is expressed on a wide-ranging variety of cell types, megakaryocytes, epithelial cells, endothelial cells, fibroblast and platelets. Therefore, platelet glycoprotein (GP) plays a vital role in platelet adhesion and aggregation, for the development of thrombosis and hemostasis. Variation in platelet GP density becomes a risk factor for hemostatic abnormalities and this platelet 2 1 density is genetically determined. GPIa/IIa intermediates platelet adhesion to collagen, thus, 2 1 receptor contributes significantly to platelet function in vivo[3]. ITGA2 gene, the gene encoding integrin 2 1 has several polymorphisms. Some of these gene polymorphisms, such as Bgl II polymorphism of subunit of 2 1 integrin has been associated with the extent of platelet adhesion to collagen which might have a significant influence on platelet function[4]. Glycoprotein expression level, which are located on the platelet surface relates to allelic polymorphisms of the ITGA2 gene located on a fifth chromosome 5q11.2. Numerous single nucleotide polymorphisms (SNPs) for the ITGA2 gene have been fundamental, the two associated dimorphism inside the coding area of the ITGA2 gene at the location of C807T and G873A have been identified. These two SNPs have G to A polymorphism at 873 position and C-T polymorphism at 807 position. The expression level of the ITGA2 on platelet surface has an important association with these SNPs polymorphisms[5]. The two silent bi-allelic polymorphisms on the subunit alpha2 for the 2 1 integrin have been recognized; CC807TT localized on the seventh exon, and GG873AA is localized on the eighth exon, these two silent mutations are in whole linkage disequilibrium (LD), has identified to be related with density expression of the 2 1 integrin on platelet surface[6]. The DNA sequence alternatives recognized contain two variations in an amino acid encoding area of alpha2 at the nucleotide C807T (TTT/TTC at codon Phe[224]) and nucleotide G873A (ACA/ACG at codon Thr[246]) for cDNA sequence, however, the specific variations of amino acids do not alter the amino acid sequence of the 2 protein[7]. Several studies show the relations between the ITGA2 polymorphism and the progress of diabetic retinopathy (DR), A higher frequency of BglII (+/+) genotype of gene polymorphism of 2 1 integrin gene was found in Japanese and Caucasians T2DM with DR compared with patients without DR[8,9]. The precise mechanisms involved in aetiopathogenesis of DR are not known, therefore studies are required for the analysis of these genes to have better understanding of pathophysiology of diabetes. Thus, this study was designed to assess the association between ITGA2 gene polymorphisms and diabetic retinopathy, by using a technique of Allele-Specific Polymerase Chain Reaction (AS-PCR) to detect the genotypes and alleles frequencies in these two SNPs (C807 T and G873A) in the population of Al-Anbar province.

Materials and methods

The present study was designed as case-control which includes 75 persons divided into two groups, 25 subjects were healthy control, while 50 were patients with Type II diabetes mellitus. Patients were subdivided into two groups (25 with diabetic retinopathy and 25 without diabetic retinopathy). The mean age of participants was 51.68 years. Blood samples were collected in Al-Nahrain Eye Specialty Center in Al-Ramadi city west of Iraq for the period from the seventh of September to the twenty-fifth of December 2019. Subjects received informed written consent before collection of blood samples. A questionnaire survey was conducted according to the requirements of the research outlines and ethics. All patients underwent a complete ophthalmic examination and were examined by a certified ophthalmologist. A biochemical analysis includes blood glucose level, HbA1c, and serum cholesterol level to determine their impact on diabetic retinopathy. DNA was extracted from peripheral leukocytes using, by using standard procedure using Genomic DNA Mini Kit (Add Prep/ Korea) following the manufacturer's guidelines. Polymerase amplification of specific alleles (PASA) was used for genotyping of GPIa, the technique which depends on a precisely designed primer AS-PCR uses the two forward primers for each SNP (one primer for mutant genotype and the other primer for wild genotype) and one reverse primer for both of two forward primers (Table 1).

Thus, two reactions in two tubes achieved for two SNPs, the 807C and 807T, were multiplexed in the first tube, while 873G and 873A were amplified in the second tube. 25 µL PCR reactions was prepared as following: first reaction (Taq Green Master mix 12.5 µl, 1 µl from first forward primer, 1µL from second forward primer, 1µL from reverse primer (Intron GR), 5µL from genomic DNA, 4.5 from Free nuclease water). The second reaction (Taq Green Master mix 12.5 µL, 1µL first forward primer, 1µL from second forward primer, 1µL from reverse primer (EXON8R), 5µL DNA, 4.5 Free nuclease water). Reactions were multiplexed in Polymerase chain reaction MultiGene Optimax thermal cycle using 35 cycles of the following: initial denaturation at 94°C for 2 minute and (denaturation step at 94°C for 30sec, primer annealing at 61°C for 1 minute, extension step at 68°C for 1 minute and Final extension at 68°C for 5 minutes). 10µL from AS- PCR products were loaded into 2% agarose which was stained with red safe for electrophoresis. Analysis of samples demonstrated three probable genotypes is revealed in figure 1[5].

Table 1. Primers sequence

Primer	5 Sequence 3	SNPs	Product size (bp)
Forward 1 G allele	GGT GGG CGA CGA AGT GCT AGG	C807T	232
Forward 2 T allele	ATGGTGGGGACCTCACAAACACATAT		
Intron GR	GAT TTA ACT TTC CCA GCT GCC TTC		
Forward 1 C allele	GTG GGG ACC TCA CAA ACA CAT GC	G873A	113
Forward 2 A allele	GGT GGG CGA CGA AGT GCT AGA		
Exon8R	CTCAGTATATTGTCATGGTTGCATTG		

Statistical analysis

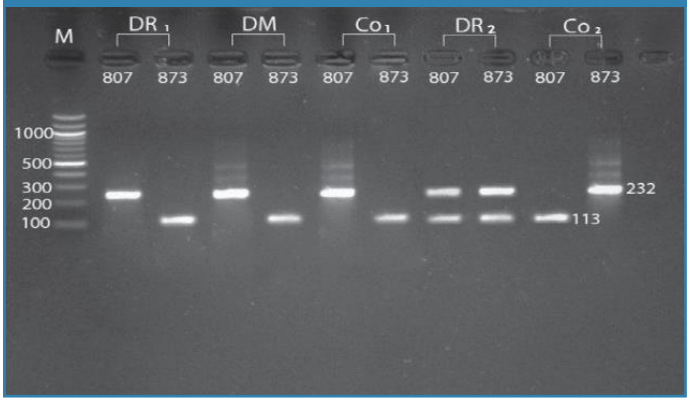
The statistical analysis was achieved using the SPSS software (Version 21.0; SPSS Inc., Chicago, IL, USA). The chi-square is used to estimates the relationship between DR and 807T/873A alleles. AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) were used to select the best inheritance model that explains the genetic behavior of the SNPs. The adjustment was made for risk factors: sex, hypertension (yes/no) and smoking (yes/no), total serum cholesterol, hyperglycemia. "www.bioinfo.iconcologia.net/SNP stats (2018).

Results and Discussion

ITGA2 polymorphism and it's susceptibility to diabetic retinopathy

The clinical significance of the ITGA2 polymorphisms was assess so the study was designed as a case-control study. The results by agarose gel electrophoresis for C807T and G873A appeared in figure 1. The genotype pattern of (C/C) for C807T homozygous (Wild type) appeared in band 113 bp the same as the genotype pattern of G873A (A/A) homozygous (Mutant type). The heterozygous genotype pattern (CT/GA) for C807T and G873A SNPs appeared in two sites (113 bp and 232 bp) on gel electrophoresis (113 and 232).

Figure 1. Products of the ITGA2 gene analyzed by used AS-PCR, the products appeared on 2% agarose gel electrophoresis which stained with red safe dye. With a 100bp ladder as a standard size. DR and DM patients and control$_1$ are homozygous mutant pattern 807TT/873AA, control 2 homozygous wild-type pattern 807CC/873GG, and DR2 are heterozygous genotype pattern 807CT/873GA.



Genotype and allele frequencies for C807T and G873A SNPs of ITGA2 gene

In the present study genotype and allele frequencies were calculated according to Hardy-Weinberg law to determine the ratio of each allele and the distribution of those alleles and genotypes in the population. In addition to exploring the relationship of different alleles with diabetic retinopathy occurrence. The study revealed a frequency of the mutant type allele T in the patients was about 0.87 and a frequency of a similar allele in healthy controls was 0.50. While the wild type allele C in patients was 0.13, in comparison to healthy controls, which was more frequent 0.50 for C807T SNPs. The wild-type genotype CC frequency was 0.05 in patients, while was more frequent in controls 0.44. The genotype CT frequency in patients was 0.17, whereas in the control was 0.11. The frequency of mutant genotype pattern TT was about 0.78 in patients in contrast with healthy control, 0.44 for C807.

Allele frequencies in G873A SNPs were 0.39 for wild type G allele in controls and 0.12 in patients, while the mutant allele A was 0.61 in the control and 0.88 in patient's groups. The G873A SNPs genotype frequencies were as the following: the homozygous mutant genotype AA was 0.56 in the control and 0.82 in the patient's groups, while the heterozygous AG genotype was 0.11 in control and 0.13 in patients. Homozygous wild-type GG was 0.33 in control and 0.05 in patients.

A study on the Egyptian population to determine the relationship between DR and integrin alpha2 beta1 showed that there is a relationship between DR development and the alpha2 beta1 integrin polymorphisms, which is in agreement with the present investigation[11]. The results of the study on the Swedish population supported the working hypothesis which suggests that there is a significant correlation between DR development and alpha2 beta1 integrin polymorphism[12]. A study in England population done to explore the linkage between the alpha2 beta1 (C807T/G873A) integrin polymorphisms and its relationship with retinal vein occlusion showed that there was a relationship between CT/GA genotype and retinal vein occlusion occurrence[13]. Both C807T and G873A SNPs for ITGA2 frequencies have deviated from Hardy-Weinberg equilibrium with ($P<0.05$) in the Al-Anbar population and this might be due to common consanguinity marriage in our population.

It is noted in the present study that the homozygous mutant patterns TT/AA for 807 and 873 SNPs were dominant in most of the patients and half of the control group. There are many explanations for these findings, one of this might be due to consanguinity marriage. Because the samples were collected from Al-Anbar province. The society in Al-Anbar is a clan society. Therefore, the inbreeding marriage abounds in this province and the marriage inside one population also is dominant. The consanguinity marriage helps focus the genes inside the population. Therefore, there is no genetic mixing up between the population, with no renewal genetic material, the same genes will be transmitted from one generation to another, the frequency of the gene is high in the population and the likelihood of alleles appearing is great. For these reasons, the genetic structure for the individual will stay constants across generations.

First-cousin marriage rates between) 29%–35.6%) and overall consanguinity rates, (47%–60%) in Iraq population[14].

The relationship between C807T and G873A SNPs and diabetic retinopathy development

Chi-square analysis showed a highly significant association between C807T, G873A SNPs and diabetic retinopathy progression ($X^2 = 15.83$, d.f = 4, $P< 0.003$) and ($X^2= 13.722$, d.f= 4, $P< 0.008$), respectively. Several studies showed that the Phe (/TTC/TTT) due to the T/C transition at nucleotide C807T and Thr (ACG/ACA) due to the G873A transition. Therefore, these two nucleotides are in linkage disequilibrium, the wild type 807C allele is linked with the wild type 873G, and existing in the indi-

vidual lead to decreases of the integrin alpha2 beta1 copies on platelet surface while the mutant type 807T is linked with the mutant allele 873G, which lead to an increased number of integrin alpha2 beta1 on the platelet surface. So, platelet resulting from 807 T/873A donors adhere significantly quicker than platelets from 807C/873G. These increases lead to the accumulation of collagen on the platelet surface and finally to thrombus[15-17]. A higher frequency of TT genotype of gene polymorphism of 2 1 integrin gene was found in Caucasians population with T2DM who have diabetic retinopathy compared with diabetic patients without have diabetic retinopathy and was considered a risk factor for diabetic retinopathy disease[18]. A similar polymorphism has been studied on C807T and G873A SNPs in Japanese[8], and Suzhou Han population[19], who showed that the polymorphism in intron-7 of the alpha subunit of collagen receptor encoded by alpha2 beta1 gene makes retina vulnerable during hyperglycemia. The present study is in agreement with all studies mentioned above and supports the working hypothesis which states that there is an association between C807T/G873A SNPs and the development of diabetic retinopathy. Our result disagreed with a study on the Northeastern Mexico population, which showed that there was no relationship between 807 and 873 SNPs for the ITGA2 gene and the progression of diabetic retinopathy. These disagreements may be due to that the heterozygous genotypes were prevalent in most of DR and non-DR patients' group for this population[20]. While the present study has shown that the mutant homozygous genotypes TT was dominant in the patient's group.

Also, this study was in disagreement with a study in French diabetic patients which does not support the impact of ITGA2 C807T polymorphism in the development of retinopathy[10]. This contrast might be due to the ethnic difference between the French population and the Iraqi population or the cause of disagreement due to the frequency of mutant genotype TT was appeared in a low ratio in patients and control groups while the wild CC genotype was dominant in DR patients and controls group. Also, the heterozygous genotype appeared in the equal ratio in both patients and control group and the SNP (C to T) did not occur in most of the patients.

Haplotype Frequency Analysis

A haplotype is a combination of a group of alleles for cluster-related genes that occupy connecting spots on the chromosomes which are commonly inherited together as one part. The individual pattern may be at several or a single chromosomal site, which depends on the recombination that happens between them[21].

The present study treats two polymorphisms of the ITGA2 gene, which is located on the fifth chromosome. The first SNPs included C and T alleles and the second SNP included G and A alleles. The frequency of TA haplotype was 0.87 and 0.5 in patients and controls, respectively; while the CG haplotype frequency was 0.1 in patients and 0.39 in controls. The CA was 0.02 in patients and 0.1 in control, whereas the TG haplotype has never shown any ratio. The results of this study can be described by these new patterns (haplotypes) development, there is high recombination between mutant allele T807 and A873, and this may give them the capacity to transmit constantly together from one generation to another. Likewise, the recombination between wild-type alleles was high to allow them to transmit together. While the CA haplotype (C is a wild allele for C807A SNP and A mutant allele of G873T SNP) showed low recombination to enable them to transmit together through different generations in patients and controls, while TG haplotype was never showed any recombination.

Linkage disequilibrium plays an important role in genetic maps and the identification of numerous genetic disorders. The principle of LD is based chiefly on the distance that splits the alleles into different loci and the haplotype frequencies[23].

Table 2. Linkage disequilibrium estimation

SNPs	D'	D	R	P value
807	0.9997	0.1521	0.8804	0.000
873				

The investigation had tested two SNPs located at one chromosome five Linkage disequilibrium depended on the value of D' for determination of the degree of LD, which supposed D' close to 0.0 for no LD and D' close to 1.0 for high LD ratio. The results showed there is a high linkage disequilibrium between C807T and G873A with D value (0.1521) and D value (0.9997) at (P<0.000), this confirm that their position on the same chromosome, thus it can transmit together by one parent. Five genetic models were generated to determine the behavior of SNPs inheritance (Co-dominant, dominant, recessive, over-dominant, and log-additive) to determine which best model fit the SNPs inheritance. The AIC and BIC showed the best model was the co-dominant inheritance. In the Pakistan population, research accomplished to determine the association of alpha2 beta1 integrin polymorphism with diabetic retinopathy in DM patients, revealed the co-dominant model was a model which inherits the DR disease[25]. An investigation in the Tunisian population, which showed the dominant model, is a significant model for the inheritance of diabetic retinopathy disease[26].

Conclusion

The mutant homozygous genotypes frequencies TT/AA appeared in close to half of the controls and most of the patients with the high association for DR progression in the Al-Anbar population. From five genetic models to explained SNPs behavior a co-dominant model is the genetic model. There is a high linkage disequilibrium between C807T/G873A SNPs allows them to transmit together by one parent.

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