Association of Alpha-2 Beta-1 Integrin Polymorphism with Retinopathy in Diabetic Patients

Nadeem Afzal1*, Nagi AH2, Muhammad Imran3, Abu Zafar4, Amtul Musawar5, Romeeza Tahir1, Sarwar Abbas6, Afia Abbas4, Shakeela Zaman6 and Shah Jahan1

1Department of Immunology, University of Health Sciences, Lahore, Pakistan
2Department of Pathology, University of Health Sciences, Lahore, Pakistan
3Centre for Research in Endocrinology and Reproductive Sciences, Department of Physiology and Cell Biology, University of Health Sciences, Pakistan
4Amin Hayat Memorial Diabetic Centre, Lahore, Pakistan
5Services Hospital, Lahore, Pakistan
6Children Hospital and Institute of Child Health, Lahore, Pakistan

Abstract

Aim: Diabetes mellitus (DM) is a health concern because it leads to complications such as retinopathy and nephropathy. Pakistan has 6.9 million DM affected people that will be doubled by 2025. A study was designed to determine the role of genetics in diabetic retinopathy in Pakistani type-2 diabetes mellitus (T2DM) patients.

Material and methods: It was a cross-sectional case-control study that included 236 subjects, divided into Group I (54 volunteers without diabetes), Group II (30 T2DM patients without retinopathy) and Group III (152 T2DM patients with retinopathy). A BglII polymorphism of α2β1 integrin gene was genotyped in all these individuals by using RFLP method.

Results: The frequency of BglII polymorphism (++ and +-) genotypes was found significantly different between group I and group III individuals.

Conclusion: BglII (+/+, +/-) genotypes, gender of subject, age at the onset of diabetes, and duration of diabetes is associated with development of diabetic retinopathy in Pakistani T2DM patients.

Keywords: Diabetes mellitus; DNA; Polymorphism; PCR; RFLP

Introduction

Diabetes mellitus has increased significant burden on human health because it leads to numerous and serious complications such as retinopathy, nephropathy, and cardiovascular diseases. There are many risk factors that are associated with diabetes and its complications such as diet, sedentary lifestyle, age, obesity and genetic profile of a person [1]. According to W.H.O. 1999 [2], diagnosis of diabetes should consist of both fasting and 2 hour post (75g) glucose load. Type 2 Diabetes mellitus (T2DM) is one of the leading causes of morbidity and mortality in the world. It is estimated that this disease affects 240 million people worldwide and this number would be increased to 380 million by 2025, with the 80% of burden in low and middle income countries [3]. In the United States of America, the prevalence of diabetes was estimated as 13.7% and 11.7% among men and women of ≥30 years of age, respectively, while it is the sixth leading cause of death in the US [4]. Even if, the prevalence of obesity remains stable, until 2030 the number of diabetes patients will be more than double as a consequence of population aging and urbanization [5]. Regarding diabetes, Pakistan belongs to high prevalence area, currently having 6.9 million affected people that will be doubled by 2025 and it may affect 11.5 million people [3].

T2DM is more common and it occurs mainly in adults but now it is being recognized as epidemic of young. This disease is affecting all age groups, and children of 8 years or even younger have been reported with this disease. The reason for rise in childhood and adolescent T2DM is linked with obesity which is associated with changes in dietary and lifestyle patterns. Japan and Taiwan are recording more childhood cases of T2DM than type-1 diabetes, similarly in the USA; T2DM is catching up with type-1 diabetes. In 20 years T2DM may account for 60% of the disease burden and mortality in the developing world [6,7].
mitochondria containing enzymes for oxidative phosphorylation compared to the outer side of retina where oxygen supply is comparatively more [7,11,12].

Genetic factors [9], environmental factors along with low birth weight and gestational diabetes are the main reasons for the high prevalence of T2DM in Pakistan [13]. Since gene expression varies in different environmental conditions; therefore, not all diabetics develop DR. The risk of developing severe DR increases in the siblings of affected individuals. The frequency of DR varies with ethnicity and race. Many genes have been studied to determine an association with DR [14].

Over 30 genes have been studied for an association with DR, however, only a fraction of them has shown consistent associations: Aldose reductase (AR2) gene polymorphism, endothelial nitric oxide synthase (eNOS), lymphotoxin-a (LTA), angiotsin converting enzyme (ACE), vascular endothelial growth factor (VEGF), intracellular adhesion molecule 1 (ICAM-1 or CD54), β3-adrenergic receptor gene (ADRB3), endothelin-1 (EDN1) [15], advanced glycation end products receptor, haemochromatosis, and alpha2 beta1 integrin [16].

Integrin α2β1 is a glycoprotein which acts as a receptor for collagen present on the membranes of 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 haemostatic abnormalities [17] and this platelet α2β1 density is genetically determined [18]. Hereditary variation in platelet level of α2β1 integrin is defined by the existence of multiple alleles of α2 gene therefore it could have a significant impact on platelet function, contributing to an increased risk of thrombosis or bleeding in relevant disease states [19]. The same polymorphism has been studied by Matsubara et al. [20] and Shi et al. [21] in Japanese and Suzhou Han population respectively. Polymorphism in introns-7 of a subunit of collagen receptor encoded by α2β1 gene makes retina vulnerable during hyperglycaemia [22].

BglII polymorphism of a subunit of α2β1 integrin has been associated with the extent of platelet adhesion to collagen and DR in Japanese population. The BglII (+) containing platelets can easily interact with non-enzymatically glycosylated collagen and accelerate the process of retinopathy. BglII (+/+) genotypes increased the risk of retinopathy and nephropathy [22]. A higher frequency of BglII (+/-) genotype of gene polymorphism of α2β1 integrin gene was found in Caucasians T2DM with DR compared with patients without DR. In addition to BglII (+/-) polymorphism, age of onset diabetes, duration of diabetes, and insulin therapy were independent risk factor for DR [18] whereas Tsi et al. [23] could not determine an association with DR in the Chinese population with T2DM. A BglII polymorphism has also been detected within prolactin gene region [24].

Diabetic patients with BglII polymorphism (+/-, +/+ develop DR and nephropathy which indicates involvement of platelets, therefore these patients may get benefit from anti-platelet therapy [22]. The precise mechanisms involved in aetiopathogenesis of DR are not known [25] therefore better means for identification, prevention and treatment of DR should be developed before the onset of vision loss [1]. Further studies are required for the analysis of these genes in order to have better understanding of pathophysiology of diabetes [12]. Therefore; a study was designed to evaluate the role of genetic variation in DR. In this study, association of α2β1 Integrin gene polymorphism with DR has been determined.

Material and Methods

Sample collection

In this cross-sectional case-control study, 236 unrelated subjects between 20-75 years were enrolled. Among these subjects, 152 patients had type 2 diabetes mellitus and retinopathy while 30 subjects had T2DM and they did not have retinopathy. Diabetic patients had a history of diabetes for the last 5 years and maximum duration of diabetes was 25 years. In this study, 54 healthy volunteers were included who did not have diabetes mellitus. Patients of T2DM were diagnosed according to World Health Organization criteria (WHO 1999) [2]. Subjects with a history of infection in the last two weeks and suffering from chronic infections like TB, autoimmune disorders, etc. were excluded. Further, to avoid confounding effect of impaired kidney function, patients with obvious nephropathy were not enrolled in the study. The study was approved by the Advanced Study and Research Board and Ethical Committee of the University. Study subjects were recruited from Amin-Hayat Memorial Center for diabetes, Mughal Eye Hospital, Services Hospital and Sheikh Zayed Hospital, Lahore. At each of these centers, diagnosis of retinopathy was made by the consultant ophthalmologist. After explaining details of the study, written informed consent from each participant was obtained.

DNA extraction

Five ml of blood was collected in a sterile vacutainer containing EDTA as anti-coagulant and it was stored at -20°C for 24 hours. In each aliquot, 500 µl of whole blood was mixed with 700 µl of TE buffer, centrifuged at 13500 rpm for 10 minutes. Supernatant was discarded and pellet at the bottom of each aliquot was broken and 700 µl of TE buffer was added. It was centrifuged again at 13500 rpm for 10 minutes. Washing steps were repeated for 3-4 times to get white pellet. Then DNA was extracted after RBC washing by standard protocol as reported earlier [26]. Extracted DNA was quantified by spectrophotometer and was confirmed by Gel Electrophoresis.

Genotyping of BglII polymorphism

For genotyping of BglII polymorphism, restriction fragment length polymorphism (RFLP) technique was used. In this method, a 600 bp DNA fragment that contains BglII site that is located in intron 7 was amplified by PCR with a thermal cycler (BioRad). For PCR, we used DNA=1 µl, DNTPs=2 µl, Primer1=1 µl, Primer2=1 µl (as described by Matsubara et al. [20], Tₐ=30.2 µl, buffer (NH₄), SO₄=2.5 µl, MgCl₂=3 µl, and dH₂O=14.3 µl. The setting for the run of mixture for PCR was 95°C 4 min, 94°C 30 sec, 59°C 30 sec, 72°C 1 min, 72°C 10 min, 40°C and the number of cycles were 35. Sequences of primers are given in table 1. Amplified DNA was digested with BglII at 37°C for 3 hours. Resulting product was electrophoresed on 2% agarose gel. The PCR products containing BglII (+) was cut into fragments of 200 bp and 400 bp whereas those containing BglII(-) were not cut (Figure 1).

Statistical analysis

Data was analysed using SPSS 17.0. Mean ± SD, frequencies and percentages were used for qualitative variables. Tables and figure are presented for both qualitative and quantitative variables. One way ANOVA to observe group mean differences, Post Hoc Tukey test to observe group means differs, Pearson correlation test to observe correlation between quantitative variables and Pearson Chi-Square test was applied to observe associations between qualitative variables. A p value of ≤ 0.05 was considered statistically significant.
Results

The Group I included 34 (63%) males and 20 (37%) females, Group II had 5 (16.66%) males and 25 (83.33%) females and Group III included 51 (33.55%) males and 101 (66.44%) females. The demographic data such as age range, gender distribution, level of HbA1c, and duration of diabetes of the subjects enrolled in this study is given in Table 1. The distribution of frequency of BglIII polymorphism in all the three groups is shown in Table 2.

The frequency of BglIII polymorphism i.e. disease associated homozygous positive (++), disease associated heterozygous positive (+-) and non-disease associated homozygous negative (--) for the allele of α2β1 integrin gene was determined in all the studied population.

In Group I, 3 (5.55%) subjects had ++ polymorphism, there were 33 (61.1%) subjects with + polymorphism and 18 (33.33%) subjects had -- polymorphism. In Group II, there were 4 (13.3%) subjects who had ++ polymorphism, 16 (53.3%) subjects had + polymorphism and 10 (33.33%) subjects had -- polymorphism. In Group III, 29 (19.1%) subjects had ++ polymorphism, 67 (44.7%) subjects had + polymorphism and 56 (36.84%) subjects had -- polymorphism. In total, 33 (15.25%) subjects had ++ polymorphism, 116 (49.15%) had + polymorphism and 84 (35.79%) had -- polymorphism.

On comparison of Bgl II polymorphism between Group I and Group III, higher percentage of ++ polymorphism was found in Group III (29%) compared to Group I (3%) (p value=0.027).

BglII polymorphism (+/+, +/-, -/-) genotypes were compared for association analysis by using all three models i.e. codominant, dominant and recessive model. On comparison, Group I and Group III showed statistically significant value in codominant and recessive models. Association of BglII genotypes was non-significant when Group I was compared with Group II and Group II was compared with Group III. The details are given in Table 3.

The dose of BglII alleles was compared to determine the influence of these alleles on the development of DR. The detail of alleles' comparison is given in Table 4.

Discussion

Various epidemiological and experimental studies show that disease duration of diabetes and glycemic control is major determinants for the development of retinopathy or nephropathy in diabetes patients.

Table 1: Characteristics of individuals included in the study.

| Group                  | BglII polymorphism | Total | HWE   |
|------------------------|--------------------|-------|-------|
|                        | ++ n (%)           | +- n (%) | -/-% |
| Group I (Healthy subjects) | 03 (5.55)         | 33 (61.1) | 18 (33.3) | 54 | 0.017128 |
| Group II (Diabetic without retinopathy) | 4 (13.3)          | 16 (53.3) | 10 (33.3) | 30 | 0.542802 |
| Group III (Diabetic with retinopathy) | 29 (19.07)        | 67 (44.07) | 56 (36.84) | 152 | 0.268781 |
| Total                  | 36 (15.25)         | 116 (49.15) | 84 (35.59) | 236 | 0.695570 |

Table 2: Frequency of BglII polymorphism in Group I, Group II and Group III.

| Codominant model genotype (n%) | BglII (+/+) | BglII (+/-) | BglII (-/-) | p value   |
|--------------------------------|-------------|-------------|-------------|------------|
| Normal individuals vs. Diabetic patients with diabetic retinopathy | 3/5.6 | 33/61.1 | 18/33.3 | 56/36.8 | 0.0272* |
| Recessive model Genotypes (n%) | | | | |
| Normal individuals vs. Diabetic patients with diabetic retinopathy | 51/94.4 | 3/5.6 | 29/19.1 | 0.0167* | 4.01 (1.17, 13.75) |

*Statistically significant ≤ 0.05

Table 3: Association of BglII genotypes with susceptibility to diabetic retinopathy.

| BglII | Normal individuals Genotype (n%) | Diabetic patients with diabetic retinopathy | p value | OR (95% CI) |
|-------|---------------------------------|------------------------------------------|---------|-------------|
| +/-   | 33/61.1 3/5.6                  | 67/44.1 29/19.1                           | 0.0111* | 4.76 (1.35, 16.78) |
| -/+   | 18/33.3 3/5.6                  | 56/36.8 29/19.1                           | 0.1106 | 3.11 (0.85, 11.42) |
| +/   | 33/61.1 18/33.3                | 67/44.1 56/36.8                           | 0.2411 | 1.53 (0.78, 3.01) |

*Statistically significant ≤ 0.05

Table 4: Effect of allele dose on association of BglII genotypes with susceptibility to diabetic retinopathy.
patients which was 49.9 ± 10.8. On comparison of percentage of HbA1c between Group II and Group III, there was no significant difference (Table 1). The reason for this non-significant value could be that in both the groups' distribution of HbA1c percentage was almost similar. The study of Ahmadani et al. [28] documented level of HbA1c as 9.32 ± 2.14 and there was no significant difference in percentage of HbA1c. Tam et al. [31] documented percentage of HbA1c as 8.1 ± 1.5. On comparison of duration of diabetes between Group II and Group III, significant difference was found (Table 1). The possible explanation for this significance in duration of diabetes could be that along with other factors, length of disease could play a role in the development of DR. Regarding duration of diabetes, results of our study are similar with Ahmadani et al. [28] because they included patients of diabetes for 9.32 ± 6.87 years. Similarly, our study is in agreement with the study of Zhang et al. [32] as they included diabetic patients between 6.5-16.5 years.

This study is the first one to demonstrate an association between the Bgl II polymorphism and DR in Pakistani population. It is possible that Bgl II containing platelets can more easily interact with non-enzymatically glycosylated collagen and accelerate the occurrence of retinopathy. In addition, our findings might affect antplatelet drug treatment for DR. For instance, patients with the Bgl II (+/+, +) genotype might benefit more from antplatelet therapies. The frequency of Bgl II polymorphism (+/+, +, +, and ++) was determined in all the three groups (Table 2). The comparison among three groups was made by using codominant, dominant and recessive modes. By using co-dominant mode and recessive mode, comparison between Group I and Group III showed significant difference but comparison between Group II and Group III was not significant (Table 3). The effect of allele dose was assessed for an association of Bgl II genotypes with the susceptibility to develop DR. There was significant difference on comparing ++ and ++ between Group I and Group III. There was no significant difference on comparing -/- with ++, between Group I and Group III and on comparing ++ with ++ between Group I and Group III (Table 4). The percentage of HbA1c was compared with allele dose of Bgl II genotypes to determine the susceptibility to develop DR. On comparing all the variants (+/+, +/-, and -/-) within Group II and Group III, there was no significant difference and p value was 0.556 and 0.924, respectively. Result of Bgl II polymorphism suggests that in our population Bgl II polymorphism (+, +) genotypes play a significant role in the development of DR. The findings of our study are similar with the findings of Funatsu et al. [22] who were the first one to demonstrate an association between Bgl II polymorphism of a subunit of a2β1 integrin and DR among patients of T2DM in Japanese population. They suggested Bgl II (+/+, ++/) genotypes increased the risk of retinopathy and nephropathy. Therefore, they suggested that patients with Bgl II (+/+, ++/) genotypes might benefit from anti-platelet therapies. Similarly, Pavkovic et al. [19] studied polymorphism in GP Ia gene and suggested an association with the variations in platelet a2β1 expression levels. They suggested that platelets from individuals with 807T allele have higher levels of a2β1 integrins as compared to individuals of 807C allele.

This study is also in agreement with the study of Petrovic et al. [18] who determined significantly higher frequency of Bgl II (+/+) genotype of a2β1 integrin gene in patients of DR as compared to patients without DR. They suggested Bgl II (+/+) genotype of a2β1 integrin gene polymorphism, age at the onset of diabetes, duration of diabetes, and insulin therapy as independent risk factors for the development of DR in Caucasians with T2DM. All the variables were compared among three groups and significant difference was observed between gender
distribution, age of subjects, and duration of diabetes. On comparison of Group I and Group II, significant difference was found between the ages of subjects (Table 1). On comparing Group I and Group III, significant difference was noticed between gender, age of subjects, and BglII gene polymorphism (Tables 1 and 3). On comparison between Group II and Group III, significant difference was found between gender of subjects, age, and duration of disease (Table 1). In conclusion, BglII polymorphism of a subunit of α2β1 integrin is associated with the prevalence of retinopathy in patients with type II diabetes mellitus in Pakistani population. The present study may add in early identification of risk and possibly prevention of DR.

Conclusion

In the studied population, frequency of BglII (+/-, +/+ ) genotype of gene polymorphism of α2β1 integrin gene, gender, subject, age at the onset of diabetes, and duration of diabetes contributed towards development of diabetic retinopathy.

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