Relationship between Diabetic Retinopathy and Methylenetetrahydrofolate Reductase Gene Polymorphism

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ABSTRACT

Purpose: To assess MTHFR rs1801133 (C677T) gene polymorphism in diabetic patients as a risk factor for diabetic retinopathy and to establish the changes in Platelet indices & count in diabetic patient as compared to the healthy control group.

Patients and Methods: The study included 40 patients with Type 2 Diabetes Mellitus. They were divided into 2 equal groups, 20 patients with Diabetic Retinopathy, 20 patients without Diabetic Retinopathy. Patients were selected from those attending the outpatient Ophthalmology Unit and Diabetes Clinic of Al-Zahraa University Hospital in the period from June 2014 to June 2015. Their ages ranged between 34 to 66 years old. They were 14 males and 26 females. Twenty cases apparently healthy individuals were selected as a control group. All cases were subjected to full history taking and complete ophthalmological examination. Also laboratory investigations were done including complete blood picture, kidney and liver function tests, coagulation profile, urine analysis, lipid profile, fasting and postprandial blood sugar and Genetic study for detection of MTHFR gene C677T mutation (rs 1801133)by real time PCR.

Results: In all diabetic patients the mutant homozygous TT showed a highly statistically significant increase in FBS (p=0.000), PPBS (p=0.000), HbA1C (p=0.000) and cholesterol (p=0.001) as compared to wild type. Also in all diabetic patients the mutant homozygous TT showed a highly statistically significant increase in FBS (p=0.002), PPBS (p=0.001), HbA1C (p=0.019) and cholesterol (p=0.012) as compared to heterozygous mutant type.

Conclusion: The homozygous mutant type (TT) of rs1801133 was detected in 10% of DR patients group while absent in DWR group and the control group. The heterozygous mutant type (CT) was increased in DR group (50%) as compared to DWR group (35%) and the control group (25%).

Key words: Diabetic retinopathy, Methylenetetrahydrofolate gene, Platelet indices.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia. It is one of the common non-communicable diseases that are increasing globally. Type-2 DM is the most common type[(1)].

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes. Hyperglycemia contributes to the structural and functional changes in the retina [(2)].

Diabetic retinopathy is the most common complication of diabetes mellitus and is a leading cause of blindness. It is characterized by increased vascular permeability, tissue ischemia, and neo-vascularization[(3)].

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that catalyzes the transformation of homocysteine to methionine. The enzyme is coded by the gene with the symbol MTHFR on chromosome 1 location p36.3 in humans [(4)].

Up to 24 numbers of genetic polymorphisms are associated with this gene. The most investigated polymorphisms are C677T (rs1801133) and A1298C (rs1801131) single nucleotide polymorphisms (SNP) [(5)].

A risk factor for vasculopathy in diabetes is elevated homocysteine level which isa consequence of decreased activity of MTHFR. It has been reported that hyperhomocysteinemia in diabetic patients was associated with the prevalence of diabetic retinopathy as it is associated with slight increase in the risk for arterial and venous thrombosis [(6)].

AIM OF THE WORK:

The aim of this work is to study MTHFR rs1801133 (C677T) gene polymorphism in diabetic patients as a risk factor for diabetic retinopathy and to evaluate the changes in platelet indices & count in

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diabetic patients as compared to healthy control group.

PATIENTS AND METHODS

This study is a prospective, non-randomized study. It was carried out on 40 patients with type 2 diabetes mellitus. They were divided into 2 equal groups; 20 patients with diabetic retinopathy, and 20 patients without diabetic retinopathy. Patients were selected from those attending the outpatient Diabetes Clinic and Ophthalmology Unit of Al-Zahraa University Hospital in the period between June 2014 and June 2015. Their ages ranged between 34 and 66 years. They were 14 males (35%) and 26 females (65%). A control group included 20 apparently healthy individuals. They were selected with age and sex matched with patients. Their ages ranged between 38 and 62years. They were 8 males (40%) and12 females (60%).

Inclusion criteria: Patients with fasting plasma glucose ≥126 mg/dl, Hemoglobin A1c ≥6.5% and/or treatment for diabetes including diet and/or oral antidiabetic drugs to achieve the glycemic control.

Exclusion criteria: The study excluded any patient with hypertension, renal or liver failure, cardiovascular disease, malignancy and autoimmune disease.

All cases were subjected to the following:

- **Full History:** Demographic data collection was taken including age, sex, full medical history regarding duration of diabetes mellitus and the type of treatment.
- **Clinical examination:** Assessment for diabetic retinopathy was done by testing the best corrected visual acuity, slit-lamp biomicroscopic examination and indirect ophthalmoscopic examination.

- **Sampling form patients and healthy control subjects:** Venous blood samples were collected aseptically from each subject in five separate test tubes:

I- One sterile serum separator tube without anticoagulants for biochemical analysis.

II- Three sterile tubes containing EDTA for hemoglobin A1C, complete blood picture, and DNA extraction.

III- One sterile tube containing sodium citrate for PT, PTT.

- **Laboratory investigations included:**

I- Complete blood count.

II- Biochemical analysis: Serum urea, Creatinine, AST,ALT, Cholesterol, Triglyceride, Fasting, Postprandial blood sugar, HbA1C, Prothrombin time, Partial thromboplastin time, Complete urine analysis.

III- Real time PCR for the MTHFR gene C677T mutation**:** amplification was done by Rotor - Gene Q Real Time PCR instruments from QIAGEN USA. Using real time PCR kit (Lot Number 1091352 Rev. E APPLIED BIOSYSTEMS.

Genetic study (Thermo Scientific) for detection of MTHFR (rs1801133):

A- DNA extraction.

B- Testing DNA extract integrity.

C- Detection of MTHFR gene SNPs (rs1801133) using Quantitative Real Time RT-PCR.

The study was done after approval of ethical board of Al-Azhar university and an informed written consent was taken from each participant in the study.

Statistics: Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 17 and the following were done:

Qualitative data were presented as number and percentages while quantitative data were presented as mean, standard deviation and ranges. The comparison between groups with qualitative data was done by using *Chi-square test.* The comparison between two groups with quantitative data and parametric distribution were done by using *Independent t-test.*

Pearson correlation coefficients were used to assess the significant relation between two quantitative parameters.

The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant as the following: **P > 0.05:** Non significant, **P < 0.05:** Significant, **P < 0.01:** Highly significant.

RESULTS

Demographic data of patients: The age of all studied patients ranged from 34 to 66 years old (Mean±SD was 54.98 ± 8.31 years). They were 14 males (35%) and 26 females (65%) with male to female ratio (1:1.9). Twenty cases apparently healthy individuals were selected as control group and they were age and sex matched with patients. Their ages ranged from 38 to 62

years old (Mean±SD was 52.55 ± 7.21 years). They were 8 males (40%)and12 females(60%).The differences between age and sex of all patients compared to the control group were statistically insignificant.
As regards the CBC, there was a highly significant decrease in Hb and a highly significant increase in MPV and PDW in all patients compared to the control group.

There were statistically insignificant differences in PT, PTT, coagulation profile, liver and kidney function in all patients group compared to the control group. But there was a statistically highly significant increase in HbA1C, cholesterol, TG, FBS and PPBS in all patients compared to the control group as shown in table (1).
The homozygous mutant type (TT) was detected in 5% of all diabetic patients group while absent in control group. The heterozygous mutant type (CT) was increased (42.5%) in all patients group as compared to the control group (25%) but did not reach statistically significant difference. There was high significant increase in the duration of DM in DR group as compared to DWR group. There was highly significant decrease in Hb in DR and DWR in comparison to the control group. There was significant decrease in platelet count in DR in comparison to the control group. There was a highly significant increase in MPV and PDW in DR than DWR and the control group.
There was highly significant increase in FBS, PPBS, HbA1C, cholesterol and TG in DR than DWR and the control group. There was no significant difference in liver and kidney functions between DR, DWR and the control group.

Table (1): Frequency of distribution of SNP rs 1801133 genotypes in DR, DWR patients compared to the control group

PCR	Control group		DR group		DWR group		P2	P3	P4
	No.	%	No.	%	No.	%			
CC	15	75%	8	40%	13	65%	0.055	0.490	0.156
CT	5	25%	10	50%	7	35%			
TT	0	0%	2	10%	0	0%			

P > 0.05: NS P < 0.05: S P < 0.01: HS

P2: Comparison between the Control group and DR group.P3: Comparison between the Control group and DWR group.P4: Comparison between DR group and DWR group.

The homozygous mutant type (TT) was detected in 10% of DR patients group while it was absent in DWR group and the control group. The heterozygous mutant type (CT) was increased in DR group (50%) as compared to DWR group (35%) and the control group (25%) but did not reach statistically significant difference.

Table (2): Number of alleles of SNP rs 1801133 genotypes in DR, DWR patients compared to control group

Number of cases	CC	CT	TT	T allele	C allele
D.R	8	10	2	14	26
D. W.R	13	7	0	7	33
Control	15	5	0	5	35

The number of T allele was increased in patients with diabetic retinopathy (14) as compared with (7) T alleles in patients without diabetic retinopathy and (5) T alleles in the control group.
Comparison in Control group: There was a statistically significant increase in wild type in female controls than males and there was a statistically insignificant difference between wild type and heterozygous type in controls regarding age.
There was a statistically insignificant difference between wild type (CC) and heterozygous mutant (CT) rs 1801133 in the control group as regard CBC results, coagulation profile and chemical parameters.
Comparison between the 2 patients groups:
There was a highly significant increase in homozygous mutant type (TT) rs1801133than

wild type (CC) regarding duration of DM, FBS, PPBS, HbA1C, AST, ALT and cholesterol.

DR patients with mutant homozygous TT showed a highly significant increase in FBS, cholesterol, HbA1C and PPBS than wild type, heterozygous type. But there was a significant increase in AST in heterozygous type than wild type.

The DWR patients group with heterozygous mutant type showed a highly significant increase in HbA1C and FBS than wild group. There was positive correlation between HbA1C, FBS, PPBS and Cholesterol in all patients, with diabetic retinopathy and diabetic patients without retinopathy. There was a positive correlation between PDW and HbA1C in heterozygous type in patients without DR. There was a positive correlation between FBS, PPBS and HbA1C in wild type in patients without DR.

DISCUSSION

Diabetic retinopathy is the most common complication and is a leading cause of blindness. A risk factor for vasculopathy in diabetes is elevated homocysteine levels which are consequence of decreased activity of MTHFR [(7)].

In the present study, there was a highly significant increase in FBS (p=0.001), PPBS (p=0.001) and HbA1C (p= 0.001) in all diabetic patients compared to the control group. There was a highly significant increase in the duration of DM in patients with DR compared to patients without DR (p=0.001). In the present study, there was a highly significant increase in FBS (p=0.001), PPBS (p=0.001) and HbA1C (p=0.001) in DR patients compared to control group. In the present study, there was a highly significant increase in FBS (p=0.001), PPBS (p=0.001) and HbA1C (p=0.001) in DWR patients compared to control group- In the present study, there was highly significant increase in FBS (p=0.003), PPBS (p=0.017) and HbA1C (p=0.000) in DR patients compared to DWR patients group. These findings were in agreement with[(8)].

Our results showed that the mean platelet count in the diabetic group with retinopathy was lower than that of the control group with p value (p=0.047) which is significant. Other study done by ***Hekimosy et al.*** has observed the same finding with lower platelet count in diabetic group compared to healthy subjects. Our study disagreed with results obtained by ***Demirtunc et al.*** and ***Zuberi et al.*** who represented higher platelet count. Hence the platelet count could be dependent on several variables as mean platelet survival, platelet production rate, and turnover rate in DM [(9-11)].

In the present study, there was a highly significant increase in MPV (p=0.000) in all diabetic patients compared to the control group. There was a highly significant increase in MPV (p=0.001) in DR patients compared to the control group. There was a highly significant increase in MPV (p=0.001) in DWR patients compared to control group. There was a highly significant increase in MPV (p=0.001) in DR patients compared to DWR patients group -

In agreement with the present study results ***Ayhan Tuzcu et al.*** reported that there was a correlation between MPV values and DR stage in their study with 192 individuals [(12)].

Also the results of the present study were in accordance with ***Zhong et al.*** who found that MPV was significantly higher in patients with proliferative DR and proposed that MPV is a risk factor for retinal neovascularization [(13)].

In contrary to our study ***Ateş et al.*** reported that there was no significant difference in MPV value between patients who had background retinopathy and those who developed retinopathy later. ***Citirik et al***. also reported similar results[(14-15)].

In the present study, there was a highly significant increase in PDW (p=0.001) in all diabetic patients compared to the control group. In the present study, there was a highly significant increase in PDW (p=0.001) in DR patients compared to the control group. In the present study, there was a highly significant increase in PDW (p=0.001) in DWR patients compared to the control group. In the present study, there was a highly significant increase in PDW (p=0.001) in DR patients compared to DWR patients group.

In concordance with the present study, the results obtained by ***Jindal et al.*** found that PDW was significantly increased in patients with T2DM and they reported that it was higher in patients who developed microvascular complications [(16)].

In contrast to our study ***Citirik et al.*** reported that there was insignificant difference in PDW value between patients who had background retinopathy and those who developed retinopathy later [(15)].

Kakouros et al. suggested that hyperglycemia (both fasting and post prandial) causes to generate larger platelets which is parallel to our results [(17)].

In our study we used P-values and Coefficients (r). Correlation between MPV and variant parameters (FBG, PDW and duration of DM) and we found highly significant results (p=0.018, p=0.005, p=0.00 7) respectively. In agreement with our results those obtained by ***Gokce et al.*** and***Kodiatte et al.*** [(18-19)].

But ***Hekimsoy, et al.*** did not find any correlation between MPV and FBS in patients with type 2 diabetes mellitus. In the present study, there was positive correlation between PDW and FBS in all diabetic patients with p value =0.05[(9)].

Results obtained by ***Demirtunc et al.***and ***Jindal et al.*** agreed with our results that PDW is correlated with hyperglycemia. In the opposite to our study ***Khan et al.*** reported that there is no relation between blood glucose level and PDW [(10-16-20)].

In our results there was highly significant increase in duration of DM in patients with DR (p=0.001) compared to patients without DR. It was showed that there is positive correlation between HbA1C and duration of DM. Also, there is positive correlation between duration of diabetes in all diabetic patients and MPV (p=0.007), and PDW (p=0.001).

In agreement with our results ***Bavbeket al.*** reported that MPV&PDW are correlated with the duration of diabetes mellitus [(21)].

In contrast with our present study results obtained by ***Hekimsoy et al.*** and ***Kodiatte et al.*** suggested a relation between MPV and retinopathy but not with diabetes duration [(9-19)].

In our present study there is no correlation between HbA1c and MPV, PDW and this was agreed with studies done by ***Unubol et al.*** [(22)].

In contrast to our study ***Shah et al.*** showed that HbA1c and diabetes duration has individually induces MPV and PDW changes in adolescents with type 2 diabetes mellitus. Also studies done by ***Dalamagaet al.*** disagreed with our results which revealed that elevated HbA1c concentration was directly correlated with increased MPV and PDW [(23-24)].

Our present study revealed that there was no correlation between MPV and platelet count. While ***Giovanetti et al.*** showed an inverse relation between MPV and the platelet count [(25)].

The association between increased platelet volume and decreased platelet count could be a result of small platelets being consumed in order to maintain a constant platelet functional mass [(26)].

In the present study, the frequency `of SNP rs 1801133 in all diabetic patients as compared to the control group showed that the homozygous mutant type (TT) was detected in 5% of all diabetic patients group while absent in the control group. The heterozygous mutant type (CT) was increased (42.5%) in all patients groups as compared to the control group (25%).

In agreement with our present study, **Naglaa *et al.*** reported a significant difference in the distribution of MTHFR C677T genotypes (p< 0.001) and mutant T allele (p= 0.015) between diabetic patients and the control subjects. MTHFR TT homotype C677T/ was found to be significantly higher in T2DM patients compared to controls, and conferred an almost 3.5-fold increased risk for T2DM (OR: 3.5, 95% CI: 1.06–11.57, p-value: 0.032) [(27)].

Movva et al. found fourfold risk for developing T2DM with MTHFR TT homotype C677T in Indian population (OR: 4.0423; 95% CI: 1.8753, 8.7133) which was found to be significantly higher in T2DM patients compared to controls [(28)].

In our present study the homozygous mutant type (TT) was detected in 10% of DR patients group while absent in DWR group and the control group. The heterozygous mutant type (CT) was increased in DR group (50%) as compared to DWR group (35%) and control group (25%).

The number of T allele was increased in patients with diabetic retinopathy (14) as compared with (7) T alleles in patients without diabetic retinopathy and (5) T alleles in the control group.

In our present study as regard fundus changes, T/T (2out of 20) of diabetic patients

with retinopathy with fundus changes and the C/C subgroup was (8out of 20) of patients when compared with C/T (10 out of 20).

In agreement with our results, studies done by ***Abo El Asrar et al.*** reported that the T/T subgroup and C/T subgroup showed a significantly higher number of patients with fundus changes when compared with the C/C subgroup. This implies that the C677T mutation (rs1801133) in the MTHFR gene could be an independent risk factor for retinopathy[(29)].

In agreement with our results, ***Maeda et al.*** and ***Wiltshire et al. (2008)*** found that patients with (T/T) genotype have reduced survival free of retinopathy [(30-31)].

Marginal association between C677T transition and the risk of developing DR was reported by ***Zintzraras et al.*** [(32)].

In contrary to our results, ***Ukinc et al.*** did not find any association between diabetic retinopathy and MTHFR gene polymorphism [(33)].

On contrary to our results, ***Abo El Asrar et al.*** revealed that as regards duration of DM no significant differences were found when the C/C subgroup was compared with C/T and with T/T subgroups [(29)].

In our present study, in all diabetic patients the mutant homozygous TT shows highly statistically significant increase in FBS (p=0.001), PPBS (p=0.001), HbA1C (p=0.001) and cholesterol (p=0.001) as compared to wild type. Also, in all diabetic patients the mutant homozygous TT shows a statistically highly significant increase in FBS (p=0.002), PPBS (p=0.001), HbA1C (p=0.019) and cholesterol (p=0.012) as compared to heterozygous mutant type.

In agreement with our results ***Abo El Asrar et al.*** reported that the TT subroup showed significantly higher HbA1c when compared with the C/T subgroup[(29)].

On contrary to our results, no significant associations between lipid/glucose metabolic indexes with MTHFR genotypes among diabetic patients were observed by ***Abo El Asrar et al.*** and ***Chang et al.*** [(29-34)].

CONCLUSION

The homozygous mutant type (TT) of rs1801133 was detected in 10% of DR patients group while absent in DWR group and the control group. The heterozygous mutant type (CT) was increased in DR group (50%) as compared to DWR group (35%) and the control group (25%).

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