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Full Length Research Paper

Telomere dysfunction-related serological markers in patients with type 2 diabetes; Correlation with methylene tetrahydrofolate reductase (C677T) gene polymorphism and diabetic complications

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Recent studies have identified a set of serological markers for telomere dysfunction and DNA damage. In the present study, the levels of 2 serological markers of telomere dysfunction namely chitinase and N-acetylglucosaminidase (NAG) were studied in type2 diabetic mellitus (T2DM) patients. The possibility that genomic damage, accumulation of reactive oxygen species and shorter telomeres may be linked to the onset and progression of diabetes and it is complications A total of 38 patients with T2DM together with 15 healthy persons comparable in age and sex with patients were included, the serum samples were used for determination of chitinase, NAG and lipid peroxide (LPER) by colorimetric methods, and homocysteine by ELISA. methylene tetrahydrofolate reductase (MTHFR) C677T gene polymorphism was determined by polymerase chain reaction(PCR). Serological chitinase, NAG, LPER and homocysteine were significantly increased in T2DM compared with controls and correlated significantly with age. Moreover, in T2DM showed that the genotype frequencies were CC (36.48%), CT (39.47%) and TT (23.68%). Patients with mutant gene, CT, TT showed significantly elevated indices compared to CC type. Serological chitinase and NAG were the recent markers of telomere dysfunction and DNA damage were found to be markedly increased in T2DM.

Key words: T2DM, Telomere dysfunction, MTHFR, Gene polymorphism.

INTRODUCTION

Diabetes mellitus affects 200 million people worldwide (Lefebvre, 2005).Diabetes mellitus is a complex metabolic disorders resulting from progressive impairment of insulin secretion and insulin resistance. Type 2 diabetes is the most common form of diabetes (Kahn, 2003 and Karunakaran and Park, 2013). Premature cell senescence has recently been postulated as an important cause and consequence of type 2 diabetes and its complications (Sampson and Hughes, 2006).The telomere shortening hypothesis is a widely accepted mechanism leading to senescence (Allsopp and Harely, 1995). Telomeres are specialized DNA protein structures at the end of all chromosomes, which preserve chromosome stability and integrity. In human they consist of thousands of tandem repeats of the TTAGGG sequence (Blackburn, 1991). Telomeres shorten with cell division and cells are triggered into senescence once mean length reduces below a critical value (Allsopp and Harely, 1995).

Telomere attrition is mainly caused by the “end – replication ”problem generated by the incapability of DNA polymerase to fully copy the very end of the lagging strand (Olovnikov,1973).Another factor contributing to telomere attrition involves the processing of telomere ends to reconstitute single- strand overhangs. Moreover telomere loss due to the fact that DNA repair mechanisms, particularly for single–stranded DNA damage, are less efficient in telomeric DNA than else-

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where in the genome (Salpea et al.,). The resulting accumulation of single-strand breaks along the telomeres lead to DNA damage-dependent shortening during replication (Richter and Von Zglinicki, 2007). Hence, telomere shortening could serve as an indicator of replicative history and cumulative genomic damage of somatic cells. Telomeric DNA is particularly prone to oxidative damage at the GGG sequence. Moreover, exposure to free radicals or oxidants causes DNA damage including single- strand breaks and telomere erosion as shown with in vitro experiments (Mattews et al., 2006).

Therefore, it is speculated that the rate of telomere shortening will be dependent on the balance between intracellular oxidative stress and antioxidant defense (Salpea et al., 2010). It is well established that hyperglycaemia elicits an increase in reactive oxygen species (ROS) production, due to increased input of reducing equivalent into the mitochondrial electron transport chain. ROS overproduction is a trigger for pathways responsible for hyperglycaemia- induced cell damage (Nishikawa et al., 2000). Recently, several cross-sectional studies suggested that shorter telomeres correlate not only with the risk of type II diabetes but also with its complications, such as micro-albuminuria and myocardial infarction (Tentolouris et al., 2007 and Olivieri et al., 2009). Recent studies identified a set of biomarkers including chitinase and N –acetyl-glucosaminidase (NAG) that are induced by telomere dysfunction or γ irradiation (Jiang et al., 2008 and Xiao et al., 2011).

Due to the limited studies investigating the telomere dysfunction –related serological markers in type 2 diabetes and its complications, the present study was designed to investigate serum levels of chitinase and NAG in patients with type 2 diabetes, complicated and uncomplicated. Meanwhile, due to the involvement of ROS in this process the levels of these 2 biomarkers would be correlated with lipid peroxides (LPER), as an index of increased oxidant stress. Moreover, the relationship of serum indices of telomere shortening and homocysteine blood levels with genetic variation in the enzyme Methylene tetrahydofolate reductase would be evaluate methylene tetrahydrofolate reductase reduces 5,10- methyl tetrahydrofolate to produce 5- methyl tetrahydrofolate, which acts as a carbon donor in the conversion of homocysteine to methionine (Bova et al ., 1999).

C to T transition at position 677 produces an alanine to valine substitution, increasing the thermolability of the enzyme and reducing its activity (Spence et al., 1999). Homocysteine levels tend to be higher in persons homozygous for the thermolabile variant; particularly in the setting of dietary folate deficiency (Jacques et al., 1996) Homocysteine may promote atherosclerosis and thrombosis by enhancing vascular cell proliferation and promoting prothrombotic activity in the vessel wall (Bova et al., 1999).

The effect of patient's criteria in the form of age, sex, blood sugar, body mass index (BMI), duration of disease, glcosylated hemoglobin (HBA_1c) and lipid profile as well as type of complication on telomere dysfunction indices would be studied.

PATIENTS AND METHODS

The present study included 38 type2 diabetic (T2DM) patients recruited among the attendants of out and in patients of Internal Medicine department, Sohag University Hospital. They included 17 males and 21 females. Their mean ±S.D ages was 51.53±9.27 years. Patient data were evaluated anonymously. The study also included 15 completely healthy persons representing control group. They included 9males and 6 females .Their mean ±S.D ages was 48.93±11.35 years. All participants underwent complete and thorough clinical examination in the form of full history taking, concentrating on duration of disease as well as history of diabetic complications. They were also subjected to complete physical examination including height and weight measurement in all participants, and body mass index (BMI) was calculated as weight (Kg) divided by height (m) square. Blood pressure was measured using a standard mercury sphygmomanometer and recorded as mean value of three measurements taken in the sitting position after the subject had rested supine for 5min. Examination of heart, chest and abdomen was done beside instrumental examination in the form of ultrasonography or computed tomography (C.T) when required. Diagnosis of retinopathy was made by experienced ophthalmologist using ophthalmoscopy and fluorescein angiography .The diagnosis of diabetic nephropathy was depended of measurement of the urinary albumin –to- creatinine ratio. At least three measurement of urinary albumin to –creatinine ratio were used. The patients were classified as either normoalbuminuric (without nephropathy) or albuminuric (with nephropathy).

Methods

Venous blood was obtained from antecubital vein from all participants after a 10-12hours overnights fast for the measurement of blood indices and DNA extraction. Blood specimens were centrifuged immediately; serum and plasma samples were stored at -80˚C without repeated freeze thaw cycles until they were analyzed. Blood concentrations of total, HDL and LDL cholesterol, triglycerides and fasting blood glucose were measured by routine laboratory techniques.

hemoglobin A_1C is determined by chromatographic –spectrophotometric ion exchange methods using kits supplied by Bio Systems S.A , Barcelona, Spain, Cat. N.11045.

Homocysteine was determined by ELISA kits supplied by WKEA MED supplies corp. USA .

N-acetyl glucosaminidase activity was determined by colorimetric method using N_acetyl-p-nitrophenyl—β-glucosaminidase as substrate using the method described by Skrha et al (1987). The results are expressed as µmol of substrate liberated / 60min per ml serum.

Chitinase activity was measured with colloidal chitin as substrate. Colloidal chitin 1% (w/v) was prepared and modified according to the method of Lee et al (2009): 1gm chitin powder was added slowly to 20 ml of concentrated HCL and left at 4°C overnight with vigorous stirring. The mixture was then added to 200 ml of ice-cold 95% ethanol with rapid stirring and kept overnight at 4°C. The precipitate was collected by centrifugation at 5,000xg for20min at 4˚C and then washed with sterile distilled water until the colloidal chitin became neutral (pH7.0). Subsequently, the final volume was raised to 100ml by adding 50 mM pH 6.5 sodium phosphate buffer. The reaction mixture consisted of 1ml % (w/v) colloidal chitin and 1ml enzyme solution. After incubation at 60˚C for 45min, the reaction was stopped by heating in boiling water for 20min. Then the mixture was centrifuged immediately at 10,000 xg˚ for 5 min. The supernatant was used for determination of N-acetyl-D-glucosamine released as described by Reissig et al (1955). One unit (U) of chitinase activity was defined as amount of enzyme required to release 1µmol N-actyl-D-glucosamine per minute.

Lipid peroxides were determined by colorimetric method according to Buege and Aust. (1978).

Detection of Methylene Tetrahydrofolate Reductatase (MTHFR) Gene Polymorphism

Genotyping was based on the method described by Frosset et al., (1995). Genomic DNA was extracted from leucocytes using commercially available Qiagen DNA extraction Kit. After extraction, MTHFR polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) technique. A pair of primers were used ,a forward primer ¯5-TGAA GGA GAA GGT GTC TGC GGG A-3 and reverse primer¯5-AGG ACG GTG CGG TGA GAG TGT G-3 , 50µM each dNTP, standard PCR amplification products were obtained using 25µL reactions (0.5 µg genomic DNA, 200 Pmol of each primer, 0.5 mM each of deoxy-ATP,GTP,CTP and TTP nucleotides,3 mM Mg Cl_2, 1unit of Taq DNA polymerase(Promega,UK) and 2.5ML 10x PCR buffer(50mmol/ L KCL,0.001% gelatin and 10mmol / L Tris HCl , PH 8.3). The amplification was carried by thermal cycling included one cycle of denaturation for 5 min at 95˚ C followed by 35Cycles of denaturation (1min at 95˚C) primer annealing (50sec. at 55˚C) and extension at 72˚C for 30sec .The reaction was terminated at 72˚C for7min (one cycle). The thermal cycler used is Hybaid limited equipment class I made in UK, HBPX200.The PCR product is a 198-bp fragment. The MTHFR polymorphism, a C to T substitution at bp 677, creates Hinf1 recognition sequence. If the mutation is present, Hinf1 digests the 198 bp fragments into a 175 -bp and a 23- bp fragment. Ten µL of the 198 bp PCR products were digested with the restriction enzyme Hinf1 at 37˚c for 3-4 hours in the buffer. Hinf1 can recognize the C-T substitution in the fragments that causing a conversion of Ala to Val in the MTHFR coding region. The two different alleles were digested T (Val) and C (Ala).The 198-bp fragment derived from the C allele is not digested by Hinf1 producing 198bp fragment, subjects of CC allele showed a DNA fragment of 198-bp. Whereas subjects with TT allele mutation showed the two DNA fragments of 175-bp and 23-bp as it was digested by Hinf1. Subjects with TC showed three DNA fragments of 198-bp 175-bp and 23- bp. The Hinf1 –treated PCR fragments were detected using 3% agarose gel including 5 mg /ml ethidium bromide. To every 5µl of digest DNA products 5 µl of 6X gel loading dye (prepared by bromophenol blue 0.25% and sucrose 40% in 50Mm EDTA). Running condition at 85 w for 6-8 hours. One hundred bp ladders was 0.2 mg /ml in 10 Mm Tris (pH8.0), 1mM EDTA. Electrophoresis was done by Hybaid apparatus, P250.

Statistical Analysis

Results were statistically analyzed using SPSS11.5 for windows. The statistical data were calculated for mean, standard deviation (SD). Data were examined for distribution: differences between values of normally distributed data were analyzed by student's t- test, while in those with skewed distribution; the differences between mean values were analyzed by Mann-Whitney test. Analysis of variance (ANOVA) was used to compare the results. The association between MTHFR (C677) polymorphism and the development of T2DM was examined by calculating Odds ratios (OR).Correlation between indices was done by Pearson's correlation. Results were considered significant or non significant at P< or > 0.0 5 respectively (Kirkwood ., 1989).

Table (1) shows clinical and laboratory data of type2 diabetic patients and controls. Diabetic patients showed significantly increased serum risk factors TC, LDLc, TG and significantly decreased HDL -c compared with healthy controls (P< 0.001 for each). Mean while , homocysteine levels and the two indices of telomere shortening (Chitinase and NAG) as well as well as lipid peroxides exhibited also significantly increased levels in sera of diabetic patients in comparison with corresponding levels in controls.

Table (2) shows the effect of age of patients on indices studied.

Patients with age more than 50 years, showed significantly higher, TC, LDL-C, TG, homocysteine, Chitinase, NAG and LPER and significantly lower HDL- c compared with patients less than 50 years. Also indices

Table 1. Clinical and laboratory data of study participants T2DM versus control.

Characteristics	T2DM (n=38)	Control (n-15)	P VALUE
Age(years)	51.53±9.27	48.93±11.35	P=0.393
BMI(kg/m^2)	29.18±3.05	28.20±3.32	P=0.311
Duration(years)	5.58±1.84	-	-
FBG(mg/dl)	229.7±64.91	93.3±9.82	P<0.0001***
TC(mg/dl)	235.1±67.56	149.4±17.78	P<0.0001***
HDLc(mg/dl)	31.07±7.96	45.17±7.66	P<0.0001***
LDLc (mg/dl)	169.4±61.84	88.26±16.68	P<0.0001***
TG(mg/dl)	335.7±100.2	172.3±12.18	P<0.0001***
HBA1c(%)	7.44±1.13	5.8±0.44	P<0.0001***
Homocysteine(µmol/L)	15.43±5.59	11.83±3.38	P=0.042*
Chitinase(U/ml)	106.4±34.51	57.78±12.0	P<0.0001***
NAG(mIU/ml)	10.96±3.46	6.53±1.07	P<0.0001***

Table 2. Clinical and laboratory data of study participants T2DM according to the age

Characteristics	More than50 years (n=20)	Less than 50 years (n=18)	Value
BMI(kg/m2)	29.04±2.96	29.33±3.32	P=0.776
Duration(years)	6.85±1.30	4.189±1.23	P<0.0001***
FBG(mg/dl)	260.2±59.46	195.9±53.99	P=0.0014**
TC(mg/dl)	269.2±60.99	197.3±43.75	P=0.0005***
HDLc(mg/dl)	26.77±6.23	35.86±6.46	P=0.0001***
LDLc (mg/dl)	203.1±55.76	131.9±44.97	P=0.0001***
TG(mg/dl)	357.6±64.94	311.4±126.3	P=0.0013**
HBA1c(%)	7.94±1.11	6.93±0.92	P=0.0048**
Homocysteine(µmol/L)	18.59±4.38	11.92±4.66	P<0.0001***
Chitinase(U/ml)	123.5±31.33	87.26±27.61	P=0.0006***
NAG(mIU/ml)	12.80±3.27	8.90±2.39	P=0.0002***
Lipid peroxids(µmol/L)	8.27±1.04	7.31±0.89	P=0.0045**

of diabetic control in the form of fasting blood glucose and HbA_1c were significantly increased in patients with more than 50 years age compared with those with less than 50 years age. Table (3) clarifies the effect of disease duration on indices studied with prolonged duration of disease (more than 5 years), the levels of indices evaluated showed significant increase except HDL-c which showed significantly decreased levels compared with patients with disease duration less than 5 years. Mean while, indices of diabetic controls were significantly higher in patients with prolonged disease duration.

The effects of diabetic complications on indices studied are shown in Tables (4) and (5). Patients with diabetic complications as evident in table (4) exhibited significantly increased levels of TC, LDL-c, TG, homocysteine, Chitinase, NAG and LPER but significan-

Table 3. Clinical and laboratory data of study participants T2DM according to the duration

Characteristics	More than5 years (n=24)	Less than 5 years (n=14)	P VALUE
Age(years)	55.8±8.52	44.00±5.61	P<0.0001***
BMI(kg/m2)	28.86±3.22	29.42±2.62	P=0.597
Duration(years)	6.68±1.22	3.59±0.90	P<0.0001***
FBG(mg/dl)	257.5±59.55	184.4±43.87	P=0.0004***
TC(mg/dl)	267.4±60.81	178.2±35.81	P<0.0001***
HDLc(mg/dl)	27.32±6.76	38.06±5.18	P<0.0001***
LDLc (mg/dl)	197.6±56.69	118.7±35.17	P<0.0001***
TG(mg/dl)	378.5±102.7	260.0±27.10	P=0.0003***
HBA1c(%)	7.88±1.13	6.73±0.75	P=0.0023**
Homocysteine(µmol/L)	17.98±4.87	11.00±3.93	P<0.0001***
Chitinase(U/ml)	121.3±29.07	80.03±28.90	P=0.0002***
NAG(mIU/ml)	12.71±3.13	7.92±1.23	P<0.0001***
Lipid peroxids(µmol/L)	8.25±1.03	7.13±0.69	P=0.0013**

Table 4. Clinical and laboratory data of study participants T2DM (complicated and non complicated).

Characteristics	Retinopathy (n=9)	Nephropathy (n=7)	nephropathy and retinopathy (n=7)	Pvalue
Age(years)	54.89±6.11	52.29±11.19	60.14±8.050	P_1=0.159 P_2=0.566 P_3=0.157
BMI(kg/m^2)	29.71±3.04	29.76±4.04	29.3±2.44	P_1=0.782 P_2=0.979 P_3=0.80
Duration(years)	5.90±1.01	6.08±0.81	8.10±1.08	P_1=0.0009*** P_2=0.698 P_3=0.002**
FBG(mg/dl)	253.2±38.84	253.5±31.76	311.9±49.64	P_1=0.018* P_2=0.98 P_3=0.022
TG(mg/dl)	248.6±48.02	299.3±50.09	291.6±23.70	P_1=0.048 P_2=0.059 P_3=0.72
HDLc(mg/dl)	27.70±6.01	25.61±3.06	23.70±3.64	P_1=0.144 P_2=o.41 P_3=0.30
LDLc (mg/dl)	195.0±48.66	214.3±33.19	214.5±40.05	P_1=0.406 P_2=0.462 P_3=0.994
TC(mg/dl)	386.7±156.4	382.7±53.19	379.3±44.13	P_1=0.516 P_2=0.335 P_3=0.898
HBA1c(%)	7.633±0.67	7.91±0.74	8.88±1.078	P_1=0.012* P_2=0.44 P_3=0.072
Homocysteine(µmol/L)	17.51±2.31	18.11±4.35	22.26±3.159	P_1=0.003** P_2=0.72 P_3=0.064
Chitinase(U/ml)	122.2±23.38	120.6±24.48	140.3±21.41	P_1=0.133 P_2=0.898 P_3=0.135
NAG(mIU/ml)	11.74±2.38	12.44±2.69	15.39±2.00	P_1=0.005** P_2=0.59 P_3=0.038
Lipid peroxids(µmol/L)	8.16±.519	8.00±0.81	9.27±0.70	P_1=0.199 P_2=0.236 P_3=0.00898**

P_1=comparison between **Retinopathy and Nephropathy**
P_2= comparison between **Retinopathy and nephropathy and retinopathy**
P_3= comparison between **Nephropathy and nephropathy and retinopathy.**

Table 5. Clinical and laboratory data of study participants T2DM according to complication.

Characteristics	Complicated (n=23)	Non complicated (15)	Value
Age(years)	55.70±8.69	45.13±5.99	0.002***
BMI(kg/m2)	29.60±3.08	28.52±3.00	0.291
Duration(years)	6.60±1.36	4.00±1.24	P<0.0001
FBG(mg/dl)	271.1±47.49	166.2±21.33	P<0.0001
TC(mg/dl)	277.1±47.49	170.8±34.82	P<0.0001
HDLc(mg/dl)	25.85±4.71	39.09±4.37	P<0.0001
LDLc (mg/dl)	206.8±46.63	112.0±29.30	P<0.0001
TG(mg/dl)	383.2±101.0	263.0±33.27	P<0.0001
HBA1c(%)	8.10±0.96	6.48±0.51	P<0.0001
Homocysteine(µmol/L)	19.14±3.79	9.73±1.62	P<0.0001
Chitinase(U/ml)	127.2±23.79	74.39±21.21	P<0.0001
NAG(mIU/ml)	13.07±2.77	7.72±1.06	P<0.0001
Lipid peroxids(µmol/L)	8.45±0.853	6.85±0.50	P<0.0001

Table 6. Clinical and laboratory data in patients with retinopathy compared with non Complicated cases

Characteristics	Nephropathy (n=7)	Non complicated (n=15)	P value
Age(years)	52.29±11.19	45.13±5.99	0.0625
BMI(kg/m^2)	29.76±4.04	28.52±3.00	0.429
Duration(years)	6.08±0.81	4.00±1.24	0.0007***
FBG(mg/dl)	253.5±31.76	166.2±21.33	P<0.0001***
TG(mg/dl)	299.3±50.09	170.8±34.82	P<0.0001***
HDLc(mg/dl)	25.61±3.06	39.09±4.37	P<0.0001***
LDLc (mg/dl)	214.3±33.19	112.0±29.30	P<0.0001***
TC(mg/dl)	382.7±53.19	263.0±33.27	P<0.0001***
HBA1c(%)	7.91±0.74	6.48±0.51	P<0.0001***
Homocysteine(µmol/L)	18.11±4.35	9.73±1.62	P<0.0001***
Chitinase(U/ml)	120.6±24.48	74.39±21.21	0.0002***
NAG(mIU/ml)	12.44±2.69	7.72±1.06	P<0.0001***
Lipid peroxids(µmol/L)	8.00±0.81	6.85±0.50	0.0006***

tly lower HDL-c compared to those with uncomplicated disease. However regarding type of complications (Table 5), it was found that, the levels of TG, homocyteine and NAG were significantly higher in patients with nephropathy and retinopathy in comparison with patients suffering from retinopathy only. However, complicated cases, whether with nephropathy, neuropathy or both showed significantly higher levels of indices compared with uncomplicated cases (tables 6,7,8).

The C677T polymorphism of the MTHFR gene in controls and T2DM is shown in table (9, 10, 11) and figure (1). In T2DM 14 persons (36.84%) had CC genotype, 15 persons (39.47%) had CT genotype and 9 persons (23.68%) had TT genotype .In healthy controls, 8 (53.3%) person had CC genotype,4 (26.66%) had CT genotype , 3 (20%) had TT genotype. The C allele frequency in patients and control was 43 (56.58%) and 20 (66.67%) respectively, while the T allele frequency in T2DM and controls was 33(43.42%) and 10(33.33%) respectively. Thus, CT genotype is the most prominent in T2DM patients (39.47%) while in controls CC genotype is the most prominent (53.33%).The association between MTHFR genotype and the development of T2DM was examined by calculating Odds ratio (OR). It revealed that

Table 7. Clinical and laboratory data in patients with nephropathy compared with non Complicated cases.

Characteristics	Retinopathy (n=9)	Non complicated (n=15)	P value
Age(years)	54.89±6.11	45.13±5.99	0.0009***
BMI(kg/m^2)	29.71±3.04	28.52±3.00	0.359
Duration(years)	5.90±1.01	4.00±1.24	0.0008***
FBG(mg/dl)	253.2±38.84	166.2±21.33	P<0.0001***
TG(mg/dl)	248.6±48.02	170.8±34.82	0.0001 ***
HDLc(mg/dl)	27.70±6.01	39.09±4.37	P<0.0001***
LDLc (mg/dl)	195.0±48.66	112.0±29.30	P<0.0001***
TC(mg/dl)	386.7±156.4	263.0±33.27	0.0067**
HBA1c(%)	7.633±0.67	6.48±0.51	0.0001***
Homocysteine(µmol/L)	17.51±2.31	9.73±1.62	P<0.0001***
Chitinase(U/ml)	122.2±23.38	74.39±21.21	P<0.0001***
NAG(mIU/ml)	11.74±2.38	7.72±1.06	P<0.0001***
Lipid peroxids(µmol/L)	8.16±.519	6.85±0.50	P<0.0001***

Table 8. Clinical and laboratory data in patients with retinopathy and nephropathy compared with non Complicated cases.

Characteristics	Retinopathy &neph (n=7)	Non complicated (n=15)	P value
Age(years)	60.14±8.050	45.13±5.99	P<0.0001***
BMI(kg/m^2)	29.3±2.44	28.52±3.00	0.5491
Duration(years)	8.10±1.08	4.00±1.24	0.0008***
FBG(mg/dl)	311.9±49.64	166.2±21.33	P<0.0001***
TG(mg/dl)	291.6±23.70	170.8±34.82	P<0.0001***
HDLc(mg/dl)	23.70±3.64	39.09±4.37	P<0.0001***
LDLc (mg/dl)	214.5±40.05	112.0±29.30	P<0.0001***
TC(mg/dl)	379.3±44.13	263.0±33.27	P<0.0001***
HBA1c(%)	8.88±1.078	6.48±0.51	P<0.0001***
Homocysteine(µmol/L)	22.26±3.159	9.73±1.62	P<0.0001***
Chitinase(U/ml)	140.3±21.41	74.39±21.21	P<0.0001***
NAG(mIU/ml)	15.39±2.00	7.72±1.06	P<0.0001***
Lipid peroxids(µmol/L)	9.27±0.70	6.85±0.50	P<0.0001***

comparing the frequency of different genotypes among patients and controls evaluation of MTHFR CT677 gene mutations (CT, TT compared to the wild type CC) conferred increased T2DM risk (OR=1.79 and 1.24 respectively).

Table (9) shows the effect of MTHFR genotype on clinical and biochemical data evaluated. T2DM patients with either CT or TT genotyping showed significantly increased TC, LDL-c, homocysteine, chitinase, NAG and LPER compared with CC genotype . Also FBG was significantly higher in CT and TT genotypes compared to CC genotypes.

Matrix correlations between various indices are shown in Table (12).

DISCUSSION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secre-

Table 9. Clinical and laboratory data of study participants T2DM of different genotypes.

Characteristics	CC (n=14)	CT (n=15)	TT (n=9)	Pvalue
Age(years)	46.29±5.62	54.07±8.86	55.44±11.47	P_1=0.0093** P_2=0.0179* P_3=0.744
BMI(kg/m2)	28.52±3.20	29.61±3.20	29.48±2.71	P_1=0.3703 P_2=0.468 P_3=0.92
Duration(years)	4.47±1.39	6.18±1.53	6.32±2.22	P_1=0.004** P_2=0.022* P_3=0.86
FBG(mg/dl)	174.0±34.29	260.3±49.67	265.4±68.11	P_1<0.0001*** P_2=0.0003*** P_3=0.833
TC(mg/dl)	189.1±60.17	272.7±58.80	244.1±52.24	P_1=0.0008*** P_2=0.035* P_3=0.241
HDLc(mg/dl)	35.29±7.23	27.91±6.60	29.80±8.98	P_1=0.0078** P_2=o.121 P_3=0.558
LDLc (mg/dl)	129.29±52.27	198.2±58.7	182.8±52.14	P_1=0.002** P_2=0.272* P_3=0.525
TG(mg/dl)	317.5±146.3	356.3±60.74	329.8±61.33	P_1=0.352 P_2=0.813 P_3=0.314
HBA1c(%)	7.07±1.07	7.73±1.33	7.55±0.72	P_1=0.154 P_2=0.248 P_3=0.717
Homocysteine(µmol/L)	11.23±3.46	17.54±4.95	18.43±5.72	P_1=0.0005*** P_2=0.0011** P_3=0.690
Chitinase(U/ml)	76.44±21.55	123.6±25.42	124.1±33.81	P_1<0.0001*** P_2=0.0005*** P_3=0.971
NAG(mIU/ml)	8.34±1.76	12.35±2.85	12.70±4.15	P_1=0.0001*** P_2=0.002** P_3=0.806
Lipid peroxids(µmol/L)	6.94±0.64	8.26±0.80	8.45±1.16	P_1<0.0001*** P_2=0.0006*** P_3<0.0001***

P_1=comparison between **CC and CT**
P_2= comparison between **CC and TT**
P_3= comparison between **CT and TT**

tion, insulin action or both .The chronic hyperglycemia of diabetes is associated with long – term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart and blood vessels. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories, type I diabetes and type 2diabetes. The latter accounts for 90-95% of those with diabetes. Premature cell senescence has recently been postulated as an important cause and consequence of T2DM and its complications (Sampson and Hughes, 2006).Telomere length is a tantalizing biomarker for the aging process, it is positively correlated with life expectancy and negatively correlated with stress and disease. If telomere shortening is a biomarker of aging, then the measurable consequences of telomere shortening should also function as biomarkers, i.e. aging bodies should contain high levels of factors secreted by cells with dysfunctional or critically short telomeres. Xiao et al (2011)have recently identified a set of biomarkers (cathelin- related antimicrobial peptide (CRAMP), stathmin, elongation factor 1 α (EF-1 α), chitinase and N acetyl glucosaminidase) that are induced by telomere dysfunction. Due to the complexity of the assays for telomere length, in the present study both chitinase and N –acetyl glucosaminidase were evaluated in patients with T2DM compared with controls. The levels of both biomarkers showed significant increase in T2DM patients compared with controls (table1). The indices were significantly higher in patients with more than 50 years age compared with patients with age less than 50 years

Table 10. MTHFR C677T polymorphisms, odds ratio in T2DM and controls

MTHFR Genotype	T2DM	Control	Odd ratios	P value
MTHFR 677 (CC)	14(36.84%)	8 (53.33%)	0.51	0.776
MTHFR 677 (CT)	15 (39.47%)	4 (26.66%)	1.79	0.799
MTHFR 677 (TT)	9 (23.68%)	3(20%)	1,24	0.154

Table 11. Association between MTHFR C677 genotype versus clinical and biochemical parameters in T2DM patients.

Characteristics	CT and TT (n=24)	CC(n=14)	P value
Age(years)	54.58±9.69	46.29±5.62	0.0060**
BMI(kg/m^2)	29.56±2.96	28.52±3.20	0.319
Duration(years)	6.238±1.78	4.479±1.39	0.0031**
FBG(mg/dl)	262.2±55.87	174.0±34.29	P<0.0001***
TC(mg/dl)	262.0±57.05	189.1±60.17	0.0007***
HDLc(mg/dl)	28.62±7.44	35.29±7.23	0.0107*
LDLc (mg/dl)	192.4±55.96	129.4±52.27	0.0016**
TG(mg/dl)	346.4±61.04	281.8±64.71	0.004**
HBA1c(%)	7.66±1.129	7.07±1.07	0.119
Homocysteine(µmol/L)	17.88±5.150	11.23±3.4	P=0.0001***
Chitinase(U/ml)	123.8±28.12	76.44±21.55	P<0.0001***
NAG(mIU/ml)	12.48±3.316	8.34±1.76	P=0.0001***
Lipid peroxids(µmol/L)	8.33±0.937	6.94±0.64	P<0.0001***

(table 2). With prolonged diseased duration and development of complications the levels of indices of telomere shortening exhibited significant increase (Table 4,5). However, the increase was present in all types of diabetic complications despite lack of significance between different types (Tables 6, 7, 8) but with significant differences with uncomplicated cases.

The present findings are in agreement with the previous limited studies on this subject .Thus, Jiang et al (2008) reported a significant increase in the blood level of telomere dysfunction secreted proteins including chitinase enzyme, in patients with chronic diseases that are associated with increased rates of cell turnover and telomere shortening. According to these investigators, there was a further increase of these proteins in blood plasma of aging humans and shows a further increase in geriatric patients with aging- associated diseases. In their study, analysis of blinded test samples validated the effectiveness of the biomarkers to discriminate between young and old, and between disease groups and healthy controls. This is also clear in the present study, where a significant positive correlation was observed between ages and both chitinase and NAG (Table12) .These two proteins also discriminated between healthy controls and T2DM group (Table1). In another study, Song et al (2010) indicated that these biomarkers represent the first protein markers of DNA damage and telomere dysfunction that can easily be detected in serum and show a strong correlation with markers of cellular senescence. The technical difficulties in the in vivo determination of senescence, telomere dysfunction and DNA damage have hampered its use in clinical trials and lifestyle intervention studies. The studies conducted by Jiang et al (2008) and Song et al (2010) besides the present study, indicate that the use of these novel serum markers of telomere dysfunction and DNA damage could prevent age-related declines in cellular and organism functions (Song et al., 2010).

In their recent study Xiao et al., (2011), reported that increased serological NAG levels correlate with aging. AS

Table(12); Matrix correlation between various indices

	age	BMI	Duration	FBG	TC	HDLc	LDLc	TG	HB1c	homocystein	Chitinase	NAG	Lipid peroxides
age		-0.10 Ns	0.84 P<0.000 ***	0.70 P<0.000 ***	0.54 P=0.0004 ***	-0.616 P<0.000 ***	0.550 P=0.0003 ***	0.2725 ns	0.644 P<0.0001 ***	0.721 p<0.0001 ***	0.694 P<0.0001 ***	0.700 P<0.0001 ***	0.66 P<0.0001 ***
BMI	-0.10 ns		-0.004 ns	0.220 ns	0.109 ns	-0.77 P<0.000 ***	0.045 ns	-0.152 ns	0.206 ns	0.167 ns	0.159 ns	0.159 ns	0.157 ns
Duration	0.84 P<0.0001 ***	- 0.00 4 Ns		0.77 P<0.0001 ***	0.655 P<0.0001 ***	-0.725 P<0.0001 ***	0.661 P<0.0001 ***	0.441 P=0.0055 **	0.70 P<0.0001 ***	0.759 P<0.0001 ***	0.711 P<0.0001 ***	0.72 P<0.000 ***	0.721 P<0.0001 ***
FBG	0.70 P<0.0001 ***	0.22 0 Ns	0.77 P<0.0001 ***		0.75 P<0.0001 ***	-0.77 P<0.0001 ***	0.73 P<0.0001 ***	0.32 P=0.0447 *	0.926 P<0.0001 ***	0.91 P<0.000 ***	0.88 P<0.0001 ***	0.86 P<0.000 ***	0.93 P<0.0001 ***
TC	0.54 P=0.0004 ***	0.10 9 Ns	0.655 P<0.0001 ***	0.75 P<0.000 ***		0.88- P<0.0001 ***	0.93 P<0.0001 ***	0.55 P<0.0001 ***	0.73 P<0.0001 ***	0.74 P<0.0001 ***	0.72 P<0.0001 ***	0.7 P<0.000 ***	0.72 P<0.0001 ***
HDLc	-0.616 P<0.0001 ***	-0.17 Ns	-0.725 P<0.0001 ***	-0.77 P<0.0001 ***	-0.88 P<0.0001 ***		-0.889 P<0.0001 ***	-0.49 P=0.0015 **	-0.76 P<0.0001 ***	-0.81 P<0.0001 ***	-0.75 P<0.0001 ***	-0.78 P<0.000 ***	-0.73 P<0.0001 ***
LDLc	0.550 P=0.0003 ***	0.04 5 Ns	0.661 P<0.0001 ***	0.73 P<0.000 ***	0.93 P<0.000 ***	-0.889 P<0.0001 ***		0.532 P=0.0006 ***	0.716 P<0.0001 ***	0.70 P<0.0001 ***	0.71 P<0.0001 ***	0.74 P<0.000 ***	0.67 P<0.0001 ***
TG	0.2725 ns	- 0.15 2 Ns	0.441 P=0.0055 **	0.32 P=0.0447 *	0.55 P=0.0003 ***	-0.49 P=0.0015 **	0.532 P=0.0006 ***		0.31 ns	0.37 P=0.021 *	0.37 P=0.0213 *	0.46 p=0.0035 **	0.37 P=0.018 *
HBA1c	0.644 P<0.0001 ***	0.20 6 Ns	0.70 P<0.0001 ***	0.926 P<0.0001 ***	0.73 P<0.0001 ***	-0.76 P<0.0001 ***	0.716 P<0.0001 ***	0.31 ns		0.90 P<0.000 ***	0.85 P<0.0001 ***	0.79 P<0.000 ***	0.90 P<0.0001 ***
Homocystei n	0.721 p<0.0001 ***	0.16 7 Ns	0.759 P<0.0001 ***	0.91 P<0.0001 ***	0.74 P<0.0001 ***	-0.81 P<0.0001 ***	0.70 P<0.0001 ***	0.37 P=0.021 *	0.90 p<0.0001 ***		0.85 P<0.0001 ***	0,82 P<0.000 ***	0.87 P<0.0001 ***
Chitinase	0.694 P<0.0001 ***	0.15 9 Ns	0.711 P<0.0001 ***	0.88 P<0.0001 ***	0.72 P<0.0001 ***	-0.75 P<0.0001 ***	0.711 P<0.0001 ***	0.37 P=0.021 *	0.85 P<0.0001 ***	0.85 P<0.0001 ***		0.73 P<0.000 ***	0.80 P<0.0001 ***
NAG	0.700 P<0.0001 ***	0.06 8 Ns	0.823 P<0.0001 ***	0.86 P<0.0001 ***	0.75 P<0.0001 ***	-0.78 P<0.0001 ***	0.74 P<0.0001 ***	0.46 P=0.0035 **	0.79 P<0.0001 ***	0.82 P<0.0001 ***	0.73 P<0.0001 ***		0.86 P<0.0001 ***
Lipid peroxides	0.66 P<0.0001***	0.15 7 Ns	0.721 P<0.0001***	0.932 P<0.000***	0.72 P<0.0001***	-0.73 P<0.0001***	0.67 P<0.0001***	0.37 P=0.018*	0.90 P<0.0001***	0.87 P<0.0001***	0.80 P<0.0001***	0.86 P<0.000**	

also, found in the present study, an age dependent increase of serological NAG and chitinase here, and stathmin in the study of Xiao et al (2011) were found. The levels of these markers of telomere dysfunction correlated with T2DM, where their levels were significantly higher in T2DM compared with controls (Table1) Previous studies indicated that telomere length seems like a useful marker for T2DM since it is associated with its progression. Thus, Adaikalakoteswar et al., (2007) reported that telomeres were shorter in patients with only impaired glucose tolerance compared to controls and even shorter in T2DM patients. In addition, telomere shortening has been linked to diabetes complications, such as diabetic nephropathy (Verzola et al., 2008), microalbuminuria (Tentolouris et al., 2007), while telomere shortening seems to be attenuated in patients with well controlled diabetes (Uziel et al ., 2007). Also, shorter telomeres in T2DM had been reported by Olivieri et al., (2009) and Salpea (2010).In a recent study, Hovatta et al., (2012) reported that telomere length may be linked to the progression from impaired glucose tolerance to T2DM. According to these investigations it seems that individuals telomere length is a dynamic feature and it can vary in both directions during life time. Since overweight and IGT had an increased risk of T2DM, Hovatta and associates (2012) did not found a stronger association between telomere shortening and T2DM diagnosis. Obesity and IGT have themselves been associated with shortened telomere (Kim et al., 2009).

In the present study , indices of telomere shortening, namely NAG and chitinase, showed significant positive correlation with indices of blood glucose control namely, FBG and HBA_1C (Table12) Moreover, they were significantly higher in complicated in comparison with uncomplicated cases (Table 4,6,7,8).

The biological relevance of increased sereological NAG to the etiology of T2DM is still obscure. Increased activity of NAG has been related to inflammation (Zhu et al., 2004) and diabetes (Belfiore et al., 1974).Research done in mouse models suggested that insulin resistance and the resultant T2DM can be prevented by disabling the macroghage inflammatory pathway (McDuffie et al., 2008) In human, insulin resistance is closely related to the presence of inflammatory cells in fatty tissue, where they produce cytokines (Wentworth et al., 2010). These cytokines cause the neighboring liver, muscle, or fat cells to become insulin resistant ,which in turn can lead to T2DM (Xiao and associates., 2011). Recent experimental evidence suggested that telomere attrition provokes senescence associated secretion of inflammatory cytokines (Coppe et al., 2008). Experimental evidence from mouse models demonstrated that shorter telomeres induce the overproduction of cytokines leading to tissue stem-cell aging (Ju et al., 2007) and abnormal hematopoiesis (impaired lymphopoiesis and accelerated myelopoiesis) in telomerase knockout mice (Song et al., 2010). The accelerated myelopoiesis induced by telomere dysfunction and aging could in turn, enhance the activation of the macrophage inflammatory pathway in T2DM(Xiao et al., 2011). However, while NAG, after adjustment for age, sex and conventional risk factors for T2DM ,a higher level of serological NAG seems to be independent risk factor for T2DM while no significant elevation of stathmin or EF-1α in patients with T2DM (Xiao et al ., 2011) could be observed.

Telomere length was also found by Chen et al., (2009) to correlate positively with HDL-c levels in T2DM. Also, in the present work, a significant negative correlation was observed between NAG, chitinase and HDL-c level in T2DM.Such correlations suggest that low telomere length probably reflects the life long accumulating burden of increased oxidative stress and inflammation whereas instant markers of these are not as representative (Chen et al., 2009). In the present study, we approached the problem of increased oxidant stress in T2DM by determination of LPER, which showed significant increased levels in comparison with healthy controls . Moreover, their levels correlated negatively with HDL-c (Table12). According to Chen et al., (2009), as HDL-c exerts antioxidant and anti-inflammatory effects and short telomere length registers the accruing burden of oxidative stress and inflammation, the significant positive correlations between age and LPER as well as chitinase and NAG (table 12) further supports this view.Rates of telomere shortening and therefore telomere length are highly dependent on oxidatively induced strand breaks in telomeric DNA and on cellular oxidant balance (Minamino et al, 2002). According to Andreassi et al. (2005) and Sampson and Hughes (2006) lymphocyte DNA from subjects with T2DM is characterized by increased susceptibility to oxidative damage. Telomeric DNA is particularly prone to oxidative damage at the GGG sequence (Serra et al ,2000) and it is probable that oxidatively induced single – and double – strand DNA breaks in people with T2DM (Andreassi et al ., 2005) would translate into accelerated telomere shortening and a progression to replicative senescence (Von Zglinicki et al., 2001). It has also been suggested that in utero programming of telomere length contributes to later risk of T2DM. Moreover, in their study, Salpea et al. (2010) provided evidence for a link between systemic oxidative stress and mitochondrial production of ROS with the shorter telomeres in T2DM. This agrees with the present findings where serological indices of short telomeres correlated strongly in a positive manner with LPER (table12).

In the study of Richards et al. (2008), telomere length correlated significantly in a negative manner with homocysteine level, in T2DM patients. The present study also showed a significant correlation in a positive manner between the 2 indices of telomere shortening and homocysteine blood levels in T2DM (Table 12).

Classical homocystinuria is a rare disease characterized by a marked increase in plasma homocysteine levels and

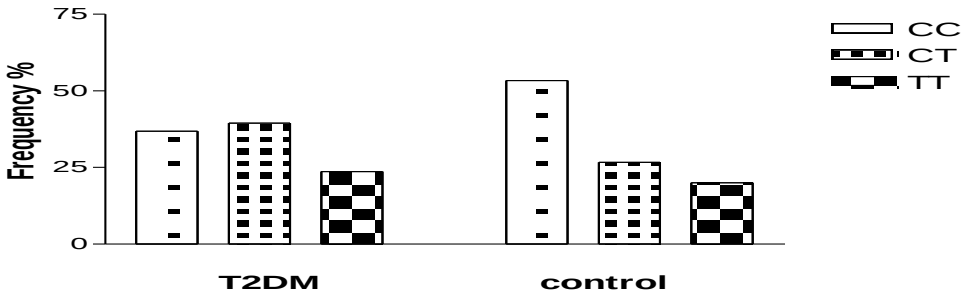

Figure 1. Frequency of MTHFR in T2DM versus control

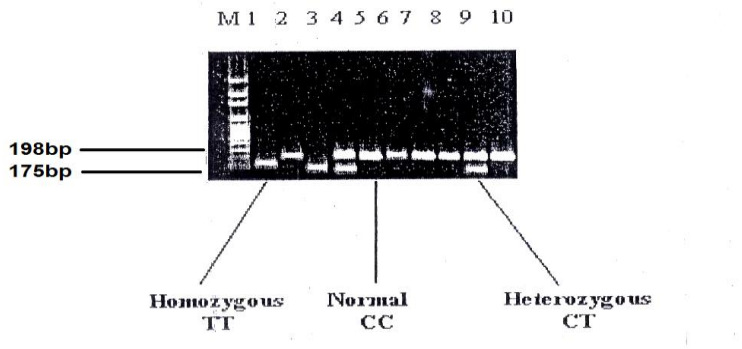

Figure 2. An agarose gel illustrating the different genotypes of the C677T mutation in patients of T2DM .the wild type CC showed a single band at the 198Bp.The heterozygote CT showed two bands at 198BP,175BP, The homozygote TT showed one band at 175.Lane M was 100Bp DNA molecular weight marker.

early onset of many age – related diseases such as severe premature atherosclerosis, thromoembolic disease and osteoporotic fractures (Rezvani et al., 2004).

In normal subjects without homocystinuria, plasma homocysteine levels are increased in essential hypertension (Nygard et al., 1995), cardiovascular disease (Mclean et al., 2004) and dementia (Seshadri et al., 2002). Shortened leucocyte telomere length (LTL) has been observed in a similar spectrum of diseases and conditions marked by increased oxidative stress and inflammation including among others insulin resistance (Aviv et al., 2006). The common threads that link elevated levels of plasma homocysteine with shortened LTL may be oxidative stress and inflammation. In a study conducted on a large number of healthy persons, Richards et al., 2008, suggested that shortened LTL is independently associated with high plasma homocysteine. In the present study, serological indices of telomere shortening showed significant positive correlations with serum homocysteine levels. Also, the serum levels of homocysteine showed significant positive correlation with LPER (Table 12), in T2DM. The present data are consistent with previous observations that shortened LTL is associated with circumstances and disorders marked by increased oxidative stress and inflammation (Minamino and Komurg 2007), a condition present in T2DM.

In order to further clarify the causes of homocystinemia in T2DM whether it is a result of the disease or related to MTHFR gene polymorphisms, therefore, MTHFRC677 polymorphism was genotyped by polymerase chain reaction, in T2DM patients, aiming also to clarify the association between this polymorphism and risk of T2DM and its complications. As evident in Table (9, 10,11)and figure(1). The total number of mutation (CT+TT) in patients was 63.16% .Thus; CT genotype is the most prominent in T2DM patients (39.47%) while CC genotype is the most prominent in healthy controls. Calculation of odds ratio (OD) revealed that these two mutant genotypes conferred increased T2DM risk. The present data agree with Movva et al., (2011), who found fourfold risk for developing T2DM in Indian and in Egyptian populations by AbdRaboh et al., (2013).

In the present study, as evident in table (9,10), significant relation existed between lipid /glucose indices as well as serological markers of telomere length, homocysteine and LPER, with MTHFR genotype among diabetic patients. Thus, it could be assumed that MTHFR polymorphisms may play some roles in the pathogenesis and complications in our patients, a finding previously re-

ported in Caucasians T2DM patients (Chang et al., 2011).

However, in the study of AbdRaboh et al., (2013), no significant relation could be observed between clinical and biochemical criteria with MTHFR polymorphisms. It should be considered that a given population may have elements in it its genetic reservoir that are protective against certain disease despite the high prevalence of disease susceptible alleles (Chang et al., 2011), Furthermore, the process of linkage disequilibrium of the mutant allele with a nearby non causative polymorphism may underlie the findings (AbdRaboh et al., 2013).

The C677T polymorphism of the MTHFR gene has been reported to cause reduced enzyme activity and impaired homocysteine /folate metabolism, leading to moderate hyperhomocysteinemia (Fletcher and Kessling.,1998). However, reviewing the literature, no data directly associate the MTHFR-linked homocysteinemia could be involved in various diabetic complications.

In conclusion, our results suggest that the C677T polymorphism of MTHFR gene is a leading cause of hyperhomocysteinemia and identify who are at high risk of diabetes. Further studies are needed to investigate in more depth what is the mechanism of telomeres in the development of diabetes.

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