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Shortened Telomere Length in White Blood Cells of Patients with Non-Insulin Dependent Diabetes Mellitus (NIDDM)

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ABSTRACT

Type 2 diabetes(TIIDM) aging-related disorder, is caused by a combination of peripheral insulin resistance and β-cell dysfunction .Recent evidence, however, suggests that TIIDM is additionally characterized by impaired β-cell regeneration and reduced β-cell mass .Shortened telomeres have been previously associated with diabetes in several small-scale studies(Zhu *et al*.,2011). Measurement of telomere length in diabetic patients in different age and different duration of disease and compared with control. Also included the comparison telomere length between male and female for both control and diabetic groups and between patients from genetic origin (mother origin or father origin) and patients from non genetic origin. Genomic DNA was prepared from whole blood extraction using genaid kit and was quantified by Nanodrop .Terminal restriction fragment (TRF) lengths were measured using the Southern-blotting technique .This study was conducted between November 2010-November 2012 and, it was carried out at the diabetic Centre / Merjan Teaching Hospital in Babel Province by taking 54 diabetic patients(Type II DM) with disease duration (0-5),(>5-10)and (>10)years ,with age average (35-65 year)and most of them were on oral hypoglycemic drugs. While the study included 18 people apparently healthy that included 9 male and 9 female with age average (35-65 year). The study revealed that telomere length(TL) were differences between males and females of control group. Age- adjusted telomere length were shorter in males than in females of control subjects (13,200 bp) vs. (11,600 bp), (14,200 bp) vs. (13,000 bp) and (15,100 bp) vs. (13,800 bp),and this differences in TL between males and females decreased as aging increased, while this gender differences in TL was not observed among the diabetic patients .In both the controls group and diabetic subjects, the telomere length were shorter in older subjects than younger for both males and females.

Introduction

Telomeres are specialized terminal elements composed of tandem repetitive sequences (TAAGGG)n and specific proteins .In many eukaryotes, telomeric repeat tracts are maintained by a telomere-specific reverse transcriptase, telomerase that can counteract the loss of terminal sequences during DNA replication (Griffith *et al*.,1999 ; Surzycki, 2003).

Epel,(2009); Salpeaa *et al*.,(2010) and Salpea and Humphries (2010) speculate that critically short telomeres contribute to the onset of diabetes by eliciting senescent phenotypes in beta cells.

Also Fernandez-Egea *et al*.,(2009) , Peng *et al*.,(2009) and Salpea *et al*., (2010) mentions that shortened telomere is associated with type 1 and type 2 diabetes ,because abnormal oxidation/reduction balance has also been associated with diabetes .Oxidative stress accelerates aging, increases the amount of telomeric DNA lost during each replication cycle.

The classical perception of adipose tissue as a storage place of fatty acids has been replaced over the last years by the notion that adipocytes and adipose tissue produce a wide range of hormones and cytokines involved in glucose metabolism including adiponectin (Hajer *et al*.,2008 ; Díez and Iglesias, 2009).

Adiponectin was first identified in 1995, also termed Acrp30, AdipoQ ,apM1,or GBP28, was originally identified independently by 4 groups using different approaches. Circulates at relatively high concentration of 2 to 30 µg/ml in blood, accounting for up to 0.01% of total plasma protein in humans (Daimon *et al*.,2003;Shimada *et al*.,2004; Kadowaki *et al*.,2006 and Heidemann *et al*.,2008). Adiponectin encoded by gene APM1 which has been mapped to chromosome 3 q 27 ,consist from 244 amino acid, abundantly synthesized and secreted by the adipose tissue, has structural homology to complement factor C1q and collagen VIII and X (Nedvidkova *et al*.,2005).

Adiponectin exists in 3 major oligomeric forms: alow–molecular weight (LMW), trimer a middle - molecular weight(MMW), hexamer and high-molecular weight (HMW) 12-18-mer adiponectin. Several observations support the hypothesis that HMW adiponectin is the more active form of the protein and has a more relevant role in insulin sensitivity and in protecting against diabetes. Adiponectin is lower in men than women, possibly as a result of suppression by androgens .Moreover, women have higher proportions of high molecular weight adiponectin than men. (Shimada *et al*.,2004; Kadowaki, *et al*.,2006 ; Heidemann *et al*.,2008).

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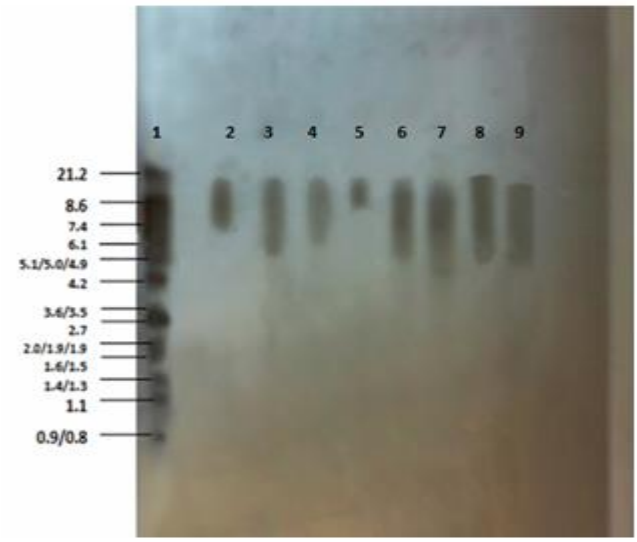
Huerta,(2006) refer that insulin down-regulated adiponectin mRNA expression and it has been proposed that the hyperinsulinemia associated with insulin resistance in T2D may be responsible for the decrease in serum adiponectin.

Materials and Method

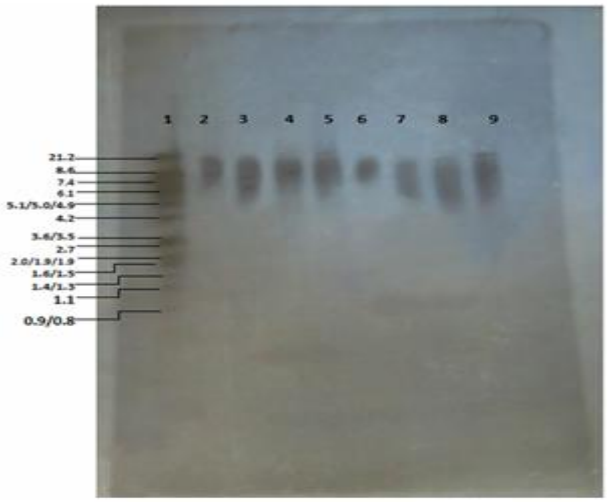
About two milliliters was collected into EDTA containing tubes and used for genetic analysis. Genomic DNA was prepared from whole blood extraction with genaid kit and was quantified by Nanodrop .Terminal restriction fragment (TRF) lengths were measured using the Southern-blotting technique. Briefly, equal amounts of DNA (2 µg) were digested with restriction enzymes HinfI (20 U) and RsaI (20 U) (Roche) for 2 h at 37°C to liberate TRFs, which include both subtelomeric repetitive DNA and telomeric TTAGGG repeats. The TRFs that determines the telomere lengths were separated by electrophoresis on 0.8% agarose gel denatured with 0.5 M NaOH/1.5 M NaCl and neutralized for 30 min in 0.5 M Tris and 1.5 M NaCl. The DNA was transferred overnight to a nylon membrane positively charged using capillary transfer. The membranes were then hybridized with telomeric probe digoxigenin for 3 h in the hybridization solution. Wash the blotting membrane 3 times with 2x SSC.The digoxigenin-labelled probe was detected by the digoxigenin luminescent detection procedure and exposed on X-ray film(Jeanclos *et al*., 1998; Bentos *et al*., 2001; Bentos *et al*., 2004 ; Adaikalakoteswari *et al*., 2005) .

Result:

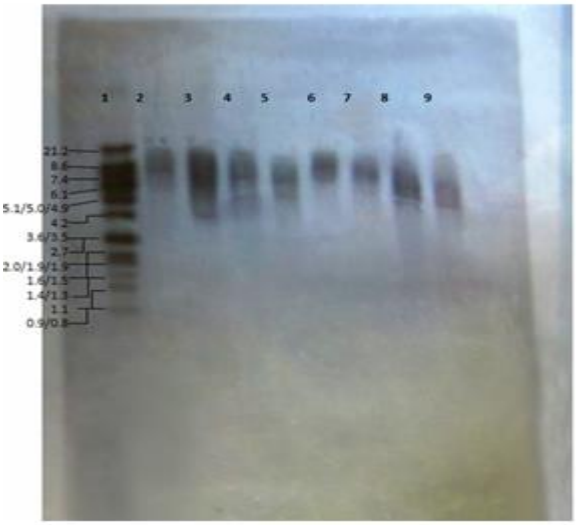
Chemiluminescent detection of Terminal Restriction Fragment (TRFs) of genomic DNA from White Blood Cells (WBCs)of control and diabetic subjects,show in Figure(1),(2) and (3).Determinants of TRF length in the total samples measuring by base pairs (bp) shown in Table(1) and Table(2).



Figure(1): Chemiluminescent detection of Terminal Restriction Fragment (TRFs) of genomic DNA from White Blood Cells (WBCs) .Lanes 1: molecular weight marker (0.8-21.2 Kb);Lanes 2: sample from male control (45 years); Lanes 3: sample from male diabetic subject (45 years, duration of disease (1.5)years); Lanes 4:sample from male diabetic subject (45 years, duration of disease (11) years); Lanes 5: sample from female control (45 years);Lanes 6:sample from male diabetic subject (58 years, duration of disease (8) years, non genetic);Lanes 7: sample from male diabetic subject (58 years, duration of disease (8) years, genetic from father); Lanes 8: sample from female diabetic subject(58 years, duration of disease (8) years, genetic from mother); Lanes 9:sample from female diabetic subject (58 years, duration of disease (8) years, non genetic).



Figure(2): Chemiluminescent detection of Terminal Restriction Fragment (TRFs) of genomic DNA from White Blood Cells (WBCs). Lanes 1: molecular weight marker (0.8-21.2 Kb); Lanes 2: sample from male control (55 years); Lanes 3: sample from male diabetic subject (55 years, duration of disease (1) years); Lanes 4: sample from male diabetic subject (55 years, duration of disease (6) years); Lanes 5:sample from male diabetic subject (55 years, duration of disease (11) years); Lanes 6: sample from female control (55 years); Lanes 7: sample from female diabetic subject (55 years, duration of disease (3) years); Lanes 8: sample from female diabetic subject (55 years, duration of disease (7) years); Lanes 9: sample from female diabetic subject (55 years, duration of disease (11) years).



Figure(3): Chemiluminescent detection of Terminal Restriction Fragment (TRFs) of genomic DNA from White Blood Cells (WBCs) .Lanes 1: molecular weight marker (0.8-21.2 Kb);Lanes 2: sample from male control (65 years); Lanes 3 :sample from male diabetic subject(65 years, duration of disease (2) years); Lanes 4: samplefrom male diabetic subject (65 years, duration of disease (10) years); Lanes 5: sample from male diabetic subject (65 years, duration of disease (26) years); Lanes 6: sample from female control (65 years); Lanes 7: sample from female diabetic subject (65 years, duration of disease (2) years); Lanes 8: sample from female diabetic subject (65 years, duration of disease (8) years); Lanes 9: sample from female diabetic subject(65 years, duration of disease (11) years).

Table(1):Average of Telomere length measured by base pairs (bp) in subjects with and without Type 2 diabetes (N0.= 3)

TL(bp) in control male (45 year)	TL(bp) in male diabetic patients (45 year)			TL(bp) in control female (45 year)	TL(bp) in female diabetic patients (45 year)		
	0-5(1)	>5-10 (2)	>10(3)		0-5(1)	>5-10 (2)	>10(3)
13,200	14,900	14,100	-	11,600	-	-	-
TL(bp) in control male (55 year)	TL in male diabetic patients (55 year)			TL(bp) in control female (55 year)	TL(bp) in female diabetic patients (55 year)		
	0-5(1)	>5-10 (2)	>10(3)		0-5(1)	>5-10 (2)	>10(3)
14,200	16,300	15,100	14,900	13,000	15,200	16,600	15,000
TL(bp) in control male (65 year)	TL (bp) in male diabetic patients (65 year)			TL(bp) in control female (65 year)	TL(bp) in female diabetic patients (65 year)		
	0-5(1)	>5-10 (2)	>10(3)		0-5(1)	>5-10 (2)	>10(3)
15,100	17,000	15,700	15,200	13,800	15,300	16,700	15,500

TL :Telomere length (1): First duration of disease (2): Second duration of disease (3): Third duration of disease

Table(2): Average of Telomere length measured by base pairs(bp) in patients from genetic origin and patients from non-genetic origin (N0.= 3)

TL (bp) in male from non-genetic origin (58 year)(>5-10)	TL (bp) in male from genetic origin (58 year)(>5-10)	TL(bp) in female from non-genetic origin (58 year)(>5-10)	TL(bp) in female from genetic origin (58 year)(>5-10)
15,300	15,700	14,900	15,900

TL :Telomere length

Telomere length analysis in control and diabetic patients according to the gender

The study revealed that telomere length(TL) were differences between males and females of control group (Table 3).

Age- adjusted telomere length were shorter in males than in females of control subjects (13,200 bp) vs. (11,600 bp), (14,200 bp) vs. (13,000 bp) and (15,100 bp) vs. (13,800 bp) and this differences in TL between males and females decreased as aging increased, while this gender differences in TL was not observed among the diabetic patients as shown in Table(4).

Table(3): The difference average in Telomere length between males and females of control group

Male difference from Female
1,600 bp male difference from female (45 year)
1,200 bp male difference from female (55 year)
1,300 bp male difference from female (65 year)

Table (4): The difference average in Telomere length between male and female of diabetic patients

First duration
1,100bp male difference from female (0-5) (55 year)
1,700bp male difference from female (0-5) (65 year)
Second duration
1,500bp female difference from male (5- >10) (55 year)
1000bp female difference from male (5- >10) (65 year)
Third duration
100bp female difference from male (>10) (55 year)
300bp female difference from male (>10) (65 year)

Telomere length analysis in control and diabetic patients according to the aging

Among the controls and diabetic group, the telomere length were shorter in older subjects than younger for both males and females as shown in (Table 5) and (Table 6).

Table(5): The difference average in Telomere length as aging increased for both males and females of control groups

Control male	Control female
1,900bp (65 difference from 45)	2,200bp (65 difference from 45)
1,000bp (55 difference from 45)	1,400bp(55 difference from 45)
900bp (65 from difference 55)	800bp (65 difference from 55)

Telomere length analysis between control and diabetic patients

The result show in Table (7) differences in TL between control and diabetic subjects. TL were shorter in the patients with Type 2diabetes compared with the control subjects ,and this differences in TL in first duration of male diabetic patients(2,100 bp) in male(55 years) and (1,900 bp) in male (65 years) and in second duration of female diabetic patients (3,600 bp) in female (55 years) and (2,900 bp) in female (65 years) were shortest compared with other duration.

Table(6): The difference average in Telomere length of diabetic patients as aging increased

Male	Female
700 (65 difference from 55) (0-5)	100 (65 difference from 55) (0-5)
600 (65 difference from 55) (>5-10)	100 (65 difference from 55) (>5-10)
300 (65 difference from 55) (>10)	500 (65 difference from 55) (>10)

Table (7): The difference average in Telomere length between control and diabetic patients according to the duration of disease

Male (45)	Female (45)
1,700bp (0-5)	-
-	-
900bp (>10)	-
Male (55)	**Female (55)**
2,100bp (0-5)	2,200bp (0-5)
900bp (>5-10)	3,600bp (>5-10)
700 (>10)	2,000 (>10)
Male (65)	**Female (65)**
1,900bp (0-5)	1,500bp (0-5)
600bp (>5-10)	2,900bp (>5-10)
100 (>10)	1,700 (>10)

Telomere length analysis in diabetic patients according to the family history of disease

The differences in TL in patients with family history of disease shown in Table (8).TL in patients with family history of disease were shorter than patients without family history of disease and this differences in TL in patients with family history of disease from mother were shortest than patients with family history of disease from father.

Table (8): The difference average in Telomere length between patients from genetic origin than patients from non-genetic origin

TL in male diabetic patients from father genetic origin	TL in female diabetic patients from mother genetic origin
400bp	1000bp

TL :Telomere length.

Discussion

Age- adjusted telomere length were shorter in males than in females of control subjects and this differences in TL between males and females decreased as aging increased (Table 3),this is because that estrogen may be linked to leukocyte telomere dynamics through its anti-inflammatory property it lowers the production of cytokines including the proinflammatory TNFα caused by oxidative stress, also antioxidant property of estrogen and ability of estrogen to stimulate telomerase a reverse transcriptase that elongates telomere ends(Aviv *et al*.,2006) .

Hanna *et al*.,(2009) refer to the two mechanisms by which estrogen may positively regulate telomere length:(i)estrogen may ameliorate the negative effects of reactive oxygen species which reduce telomere length by inducing single strand breaks and(ii) estrogen may stimulate telomerase activity .

Bekaert *et al*.,(2005) indicate to differences in telomere length between men and women and has been attributed to differences in hormonal status and lifestyle, linked to anti-oxidative defences, also body size differences and different telomere dynamics on the X and Y chromosomes have been proposed.

This protection in telomere length by estrogen decreased with aging because the estrogen level decreased as aging progressed not only the drop in estrogen but also the redistribution of body fat centrally would alter leukocyte telomere attrition because fat is the source of other adipocytokines that impact both inflammation and insulin resistance(Aviv *et al*.,2006).

While this gender differences in TL was not observed among the diabetic patients in which the TL of female were shorter than in males (Table 4),this may be because reduced adiponectin levels in diabetic patients than control and the mean of adiponectin levels lowest in female diabetic patients than male diabetic patients (Snijder *et al*.,2006).

AL-Attas *et al*.,(2010) refer to the significant positive association of adiponectin with TL suggested that adiponectin may be an anti-aging agent by way of improving insulin sensitivity, decreasing inflammation , cell oxidative function, and reversing endothelial dysfunction which is agreement with my study by the lowest of adiponectin levels in female diabetic patients made the TL did not differ from the male diabetic patients.

This result agreement with study of Adaikalakoteswari *et al*.,(2005) that found the gender difference was absent in Type 2 diabetic subjects and it is well known that women with Type 2 diabetes lose their protection from coronary artery disease.

The difference average in TL between males and females of control group was (1,600 bp) in average age (45 years) and decreased as aging increased become (1,200 bp) in average age (55 years) and (1,300 bp) in average age (65 years) (Table 3).In diabetic patients the difference average in TL between males and females (Table 4) was convergence in first duration of disease(1,100 bp and 1,700 bp) to control group in average age (55 and 65 years),while in second duration the difference average of TL in females was shorter than males (1,500 bp and 1000 bp)because the lowest of adiponectin levels in females diabetic patients, but this differences in TL decreased in third duration of disease (100 bp and 300 bp)because the levels of adiponectin increased with duration (Looker *et al*.,2004).

The telomere length were shorter in older subjects than younger for both groups and for both males and females (Table 5 and Table 6) , this may because the telomere length of circulating leukocytes decreases with age (Risques *et al*.,2007). Telomeres progressively shorten with each cell division in cultured primary human cells, until a critically shortened length is achieved, upon which the cells enter replicative senescence , numerous reports suggest that telomere shortening may be associated with organismal aging, with concomitant metabolic decline and increased risk for disease and death ,several cross-sectional studies in humans have shown that telomere length in white blood cells is inversely related to the age of the cell donor (Atzmona *et al*.,2010).

The difference average in TL was (2,200 bp and 1,900 bp between 65 and 45 years of male and female),(1,400 bp and 1,000 bp between 55 and 45 years of male and female) and (800 bp and 900 bp between 65 and 55 years of male and female), this differences because the telomere attrition increased with aging, but this differences was less in females than males because the mechanical protection of females by estrogen .In diabetic patients the difference average in TL was shorter than control group for the same age and was shorter in females than males (100 bp in first duration vs.700 bp and 100 bp in second duration vs. 600 bp), this because that females contain high levels of HMW adiponectin than males which acted active form of this protein (Snijder *et al*.,2006) , so decreased levels of HMW adiponectin in females diabetic patients made her more exposed to the telomere attrition than males but in third duration the average differences in TL in females was less than males(500 bp vs. 300 bp in males) ,this because that the adiponectin increased with duration of disease (Looker *et al*.,2004).

The results show in Table (7) differences in Telomere Length(TL) between control and diabetic subjects. TL were shorter in the patients with Type 2 diabetes compared with the control subjects , this may be due to insulin resistance and oxidative stress are associated with accelerated telomere attrition in leukocytes .Both of these factors are also implicated in the biology of aging and in aging-related disorders, including Type 2 diabetes. Cells are more likely to undergo apoptosis if exposed to increased oxidative insult (Makino *et al*.,2009).

Higher glucose and insulin were associated with shorter telomeres and low telomerase activity , while good glycemic control was associated with more favorable telomere dynamics (Harville *et al*.,2010).

Epel,(2009) show the mechanism in which the glucose and insulin causes the shorting of telomere, glucose and increased glycolysis can decrease sirtuins and autophagy. Sirtuins (protein deacetylases) are important for stabilizing DNA and for longevity. They appear to reduce inflammation and oxidative stress which promote stress resistance, and thus promote stability of the telomere. Autophagy(the breaking down and recycling of damaged molecules) is an important housekeeping function of the cell. It thus allows cells to adapt to their changing environment and might be thought of as a key player in allostasis of the cell. Autophagy,or ‘self-eating’, occurs by enzymatic degradation of intracellular ‘garbage".Autophagy becomes impaired with aging. Insulin may also regulate rate of autophagy, high levels of circulating insulin can impair

autophagy in the kidney, whereas decreased insulin receptor signaling promotes autophagy.

Fernandez-Egea *et al*.,(2009) , Peng *et al*.,(2009) and Salpea *et al*., (2010) mentions that shortened telomere is associated with type 1 and type 2 diabetes ,because abnormal oxidation/reduction balance has also been associated with diabetes .Oxidative stress accelerates aging, increases the amount of telomeric DNA lost during each replication cycle.

AL-Saadi,(2010) and You *et al*.,(2010) also refer that oxygen free radicals are a major cause of telomere shortening and that reduction in oxidative stress reduces the rate of telomere shortening.

Elevated inflammatory activity could accelerate leukocyte telomere shortening by promoting cell turnover and replicative senescence, and by inducing the release of reactive oxygen species that damage telomeric DNA via oxidative stress(O'Donovan *et al*.,2011).

The average of TL in first duration of male diabetic patients and second duration of female diabetic patients were shorter compared with other duration, this may be because the adiponectin level was lowest and the levels of TG was highest in these durations compared with other duration . Adiponectin was decrease in the presence of impaired glucose regulation and early diabetes, whereas long diabetes duration is associated with a significant increase in circulating adiponectin, because the changes in insulin concentration with increasing duration of diabetes (Looker *et al*.,2004 ; Eynatten *et al*.,2009).

Al-Attas *et al*.,(2010) indicate to the significant positive association of adiponectin to TL suggests that adiponectin may be an anti-aging agent by way of improving insulin sensitivity, decreasing inflammation and cell oxidative function, and reversing endothelial dysfunction.

Recent studies by Monickaraj *et al*.,(2012) refer to significant negative relationship between adiposity measure and telomere length and we measure telomere length in adipose tissue from both subcutaneous and visceral locations, and conclude for first time that adipose hypertrophy which connected with decrease of adiponectin secretion and higher levels of pro-inflammatory had shortened of telomere length.

The results show that TL in patients with family history of disease were shorter than patients without family history of disease and TL from mother genetic origin was shorter than TL from father genetic origin (Table 8), this is because the telomeres hold aunique advantage as amarker for biological aging and risk of disease development because they represent both an inherited predisposition to cell senescence as well as acumulative lifelong burden of oxidative stress and several studies have shown that telomere length is familial (Mainous *et al*.,2010) .

Nawrot *et al*., (2004) mention that the X chromosome harbours the DKC1gene encoding the protein dyskerin, which is important for stable accumulation of the hTR component of telomerase. Also nitric oxide activates telomerase, delays cell senescence, react with cellular radicals and reduce oxidative stress resulting in telomerase activation. It is noteworthy that the gene encoding the angiotensin II type 2 receptor (AGTR2), stimulation of which leads to enhanced nitric oxide production also maps to the X chromosome.

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