ASSOCIATION OF INTRINSIC AND INDUCED GENOMIC INSTABILITY IN PERIPHERAL BLOOD LYMPHOCYTES OF TYPE 2 DIABETES PATIENTS WITH EXPRESSION LEVEL OF GENES *PRKCB* AND *SP1*) AND MICRORNAS (MIR-126 AND MIR-15A-3P)

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ABSTRACT

***Background**: Type 2 diabetes mellitus (T2D) is a metabolic disorder that its prevalence has been increasing all over the world so the number of people affected expected to double in the next decade. In this study we aim to investigate the expression level of genes (SP1 and PRKCB) and microRNAs (miR-126 and miR-15a-3p) and genomic instability in T2D patients.*

***Methods**: Blood samples from 20 Iranian T2D patients and 20 Iranian normal individuals during 2016-2017 were obtained for measuring the expression level of SP1, PRKCB, miR-126 and miR-15a-3p by Real time RT-PCR and evaluating genomic instability by G2 assay technique.*

Statistical analyses were performed with the SPSS software ver. 23 and P values <0.05 was considered as statistically significant. The Kolmogorov-smirnov test, Independent-Samples t-test, One Way ANOVA and correlation test were used.

***Results**: The expression level of the SP1 and PRKCB genes showed a significant increase in T2D patients compared to normal individuals, miR-126 and miR-15a-3p showed a significant decrease in T2D patients compared to normal individuals Furthermore, the genomic instability was correlated with expression level of these genes and microRNAs.*

***Conclusions**: Our study shows that these genes and microRNAs are involved in genomic instability of T2D and may be also involved in the pathogenesis of T2D.*

***Keywords**: Type 2 diabetes, Peripheral blood, genomic instability, Gene expression, microRNA.*

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Introduction

Type 2 diabetes mellitus (T2D) is a chronic metabolic disorder and critical health problem worldwide and is associated with severe complications[(1)]. T2D (formerly known as non-insulin dependent DM) is the most common form of DM and the main characteristics of this disease are Hyper-glycaemia and insulin resistance, and result in increased risk for cardiovascular disease. T2D results from interaction between genetic, environmental and behavioral risk factors[(2)].

Diabetes mellitus (DM) was first reported in Egyptian manuscript about 3000 years ago[(3)]. T2D was first described as a component of metabolic syndrome in 1988[(4)]. It is estimated that 366 million people had DM all over the world in 2011 and it's estimated that by 2030 this would have risen to 552 million[(5)].

The incidence of T2D varies substantially from one geographical region to the other as a result of environmental and lifestyle risk factors[(6)]. There is a strong inheritable genetic connection in T2D, having relatives (especially first degree) with type 2 DM increases the risks of developing T2D substantially[(7)]. Lifestyle factors and genetics are both involved in the incidence of T2D. A number of lifestyle factors are known to be important to the development of T2D. These are physical inactivity, sedentary lifestyle, cigarette smoking and ETC[(8)]. The American Diabetes Association (ADA) criteria for the diagnosis of T2D are any of the four criteria including: A hemoglobin A1c (HbA1c) level of 6.5% or higher, or A fasting plasma glucose (FPG) level of 126 mg/dL (7 mmol/L) or higher; fasting is defined as no caloric intake for at least 8 hours, or A 2-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher during a 75-g oral glu-

cose tolerance test (OGTT), or a random plasma glucose of 200 mg/dL (11.1 mmol/L) or higher in a patient with classic symptoms of hyperglycemia (ie, polyuria, polydipsia, polyphagia, weight loss) or hyperglycemic crisis[9]. According to this fact that postponing the age onset of the T2D, we can reduce conflicts and problems associated with this disease, it is not surprising that studies have been initiated in the last decade to determine strategies to prevent or delay the onset of T2D. T2D can be prevented through proper nutrition and regular exercise or delayed its incidence[10, 11].

Sp1 gene encodes a zinc finger transcription factor that binds to GC-rich motifs of many promoters. The encoded protein is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling[12] Protein kinase C beta type is an enzyme that in humans is encoded by the *PRKCB* gene. This enzyme involved in many different cellular functions, such as B cell activation, apoptosis induction, endothelial cell proliferation, and intestinal sugar absorption[13]. miR-126 is a human microRNA that is expressed only in endothelial cells, throughout capillaries as well as larger blood vessels and acts upon various transcripts to control angiogenesis[14, 15].

miR-15a-3p affecting anti-apoptotic gene *bcl2* and is involved in apoptosis regulation also its down regulation correlated with *CHEK1* up-regulation so miR-15a-3p is a key factor in occurring genome instability[16]. *Sp1*, *PRKCB* genes, miR-126 and miR-15a-3p all have a key role in genome instability and it is the reason that in this study we are evaluating the expression level of these genes and micoRNAs.

The aim of this study was therefore to evaluate the expression level of genes (*SP1-PRKCB*) and microRNAs (miR-126 and miR-15a-3p) in repair mechanism of DNA damage and their correlation to genomic instability in the lymphocytes of T2D patients compared to normal individuals.

Methods

Participants: The case-control study was performed on blood samples of 20 Iranian T2D patient as the case group and the blood samples of 20 Iranian matched healthy control women (matched from age and ethnicity) during 2016-2017. The protocols of the study were approved by the Ethical Committee of the Tarbiat Modares University. All study subjects gave their written informed consent before entering the study and blood donation.

T2D patients with the age <45 years were chosen based on the American Diabetes Association (ADA) criteria for T2D and were not taking any special drugs including Metformin and Glibenclamide for treatment as well as antibiotics. Matched age control group individuals had to be free of diabetes according to ADA. All samples had been screened to exclude individuals with smoking habits, infections, hepatitis, and radiation exposure. Demographic information including age, bio mass index (BMI) and fasting blood sugar (FBS) level had been evaluated in this study.

RNA extraction and cDNA synthesis: RNA was extracted from plasma and whole blood of samples obtained from patients with venipuncture in tubes containing ethylene diamine tetra acetic acid (EDTA) as anti-coagulant, by the TRIzol® Reagent (Invitrogen, USA) protocol. cDNA was synthetized using Universal cDNA Synthesis kit II (Exiqon, Denmark).

Real time RT-PCR: Evaluation of the expression level of genes and microRNAs was performed by real-time quantitative PCR using the system Step One PLUS (ABI, Massachusetts, USA) and Master SYBR-Green I kit (Exiqon, Denmark). LNATM primers were used for amplifying miR-126 and miR-15a-3p. The primers that were used for evaluating gene expression are shown in table 1. *GAPDH* was selected as the housekeeping gene for assessment of expression[17]. miR-93-5p was selected as the housekeeping microRNA for assessment of expression.

Gene name	Forward primer	Reverse primer	Annealing Temp.	PCR product size
GAPDH	GCAGGG-GGGAGC-CAAAAG-GGT	TGGGTGG-CAGTGAT-GGCATGG	60	219
PRKCB	TTACT-GAGCCAG-GAGGAAGG	TGGAGA-CAGTGTTG-GTCGTC	55	151
Sp1	TTGAAAAA-GGAGTTG-GTGGC	TGCTGGT-TCTGTAA-GTTGGG	53	283

Tab. 1: The sequence of primers used for Real-Time-PCR.

The melt curves and the gel pictures for all three genes and three microRNAs are shown in figure 1a-f.

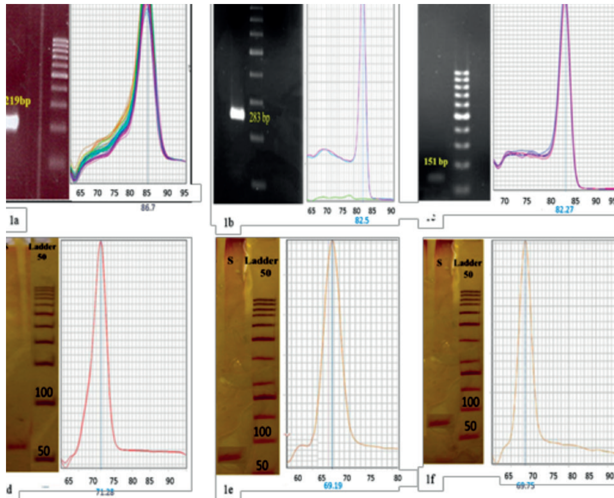

Fig. 1: The melt curves and the gels for genes and microRNAs.

1a (melt curve and the agarose gel for GAPDH gene; 1b (melt curve and the agarose gel for Sp1 gene); 1c (melt curve and the agarose gel for PRKCB gene); 1d (melt curve and the acrylamide gel for miR-93-5p)

1e (melt curve and the acrylamide gel for miR-126); 1f (melt curve and the acrylamide gel for miR-15a-3p)

Genomic instability: Human peripheral blood lymphocytes (PBL) drawn in heparinized tubes were cultured in two culture vessels by adding 500μl heparinized whole blood to 4 ml RPMI 1640 medium (Gibco, USA), supplemented with 20% fetal bovine serum (FBS) (Gibco, USA), 1.9% phytohemagglutinin (PHA) (Gibco, USA) and antibiotics (100 iu/ml penicillin/ 100 μg/ml streptomycin) (Gibco, USA), and then incubated at 37 ºC for 72h. On day 3 of incubation the cultures were treated for 5h with bleomycin 4 μg/ml. Cells were treated with 0.04 μg/ml colcemid one hour before harvesting to arrest mitotic cells. At 72h incubation the cells were harvested by centrifugation and treated with a 0.075 M KCl hypotonic solution for 20 min at 37 ºC, then the cells were fixed and washed with Carnoy's fixative (3:1 methanol: acetic acid) (Merck, Darmstadt, Germany). Metaphase spreads were prepared by dropping the cells on wet slides. After air drying, the slides were stained with a 5% Giemsa solution. For each sample 100 metaphases were scored for chromatid breaks.

Statistical analysis

Statistical analyses were performed with the SPSS software ver. 23 and P values <0.05 were considered statistically significant. The Kolmogrove-Smirnov test, Independent-Samples t-test, One Way ANOVA and correlation test were used.

Results

The blood samples of 20 T2D patients and 20 healthy women as controls were obtained. The mean age (SD) of the participants in T2D and control group was 44.80 + 1.20 and 37.35 + 8.97 years respectively that was not statistically significant different (P =0.52).

Demographic findings in patients and control individuals are shown in table 2. As seen, BMI of the T2D group (25.98 ± 2.16) compared to control group (24.5 ± 5.60) was not statistically significant different (P=0.43). However, FBS level in T2D and control group (201.5 ± 5.8 and 74.18 ± 13.01 respectively) was statistically different (P=0.03). Our results show that expression level of *Sp1* and *PRKCB* had been increased in T2D patient group compared with control group (P=0.001), (Figure 2a and 2b) and expression level of miR-126 and miR-15a-3p was decreased in T2D patient group compared with control group (P=0.001), (Figure 3a and 3b).

age	BMI*	FBS**	FBS**
T2D group	44.80 ± 1.20	25.98 ± 2.16	201.5 ± 5.8
control Group	37.35 ± 8.97	24.5 ± 5.60	74.18 ± 13.01

Tab. 2: Demographic findings in patients and control individuals.

**BMI: Bio Mass Index and **FBS: Fasting Blood Sugar*

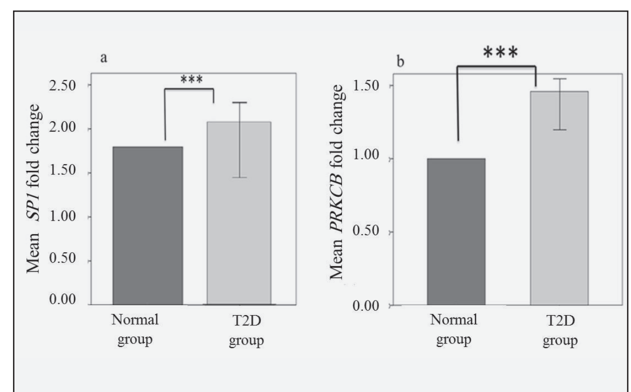

Fig. 2: The mean fold change of *Sp1* gene expression in normal and T2D patient group (a) and the mean fold change of *PRKCB* gene expression in normal and T2D patient group (b).

*Note: Significance between the groups is indicated (***means P<0.001)*

In the other hand the expression level of *Sp1* was significantly different regarding BMI index of the participants, higher expression level of *Sp1* was observed in persons with high BMI index (P=0.04). No correlation observed between age, BMI and FBS level of participants and expression level of *PRKCB* gene.

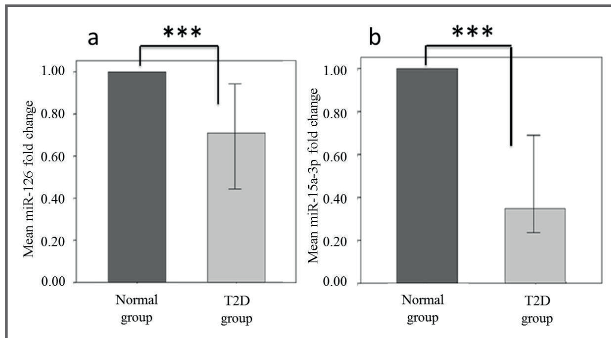

Fig. 3: The mean fold change of miR-126 expression in normal and T2D patient group (a) and the mean fold change of miR-15a-3p expression in normal and T2D patient group (b).
*Note: Significance between the groups is indicated (***means P<0.001)*

Analyzing the correlation between expressions of these two genres showed that there is a significant correlation between *Sp*1 and *PRKC*B expression (P=0.001, PC=0.878).

Genomic instability analysis showed that in T2D patients and normal individuals, induced genomic instability with Bleomycin drug is significantly higher compared to intrinsic genomic instability (P=0.01) (Figure 4).

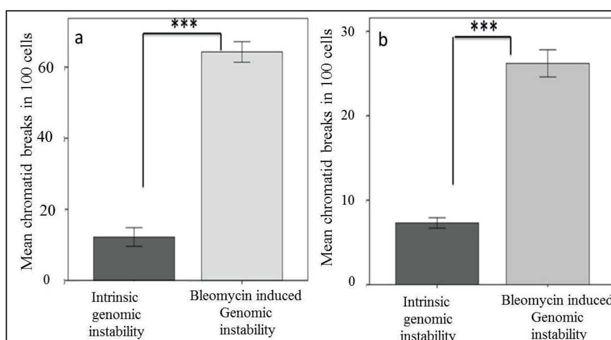

Fig. 4: Induced genomic instability VS intrinsic genomic instability in T2D group (a) and induced genomic instability VS intrinsic genomic instability in normal group (b).
*Note: Significance between the groups is indicated (***means P<0.001)*

In this study we observed positive correlation between the expression level of *SP1* and *PRKCB* genes and both intrinsic genomic instability and induced genomic instability. Also there was a positive correlation between the expression level of miR-126 and miR-15a-3p and both intrinsic genomic instability and induced genomic instability (P=0.001).

Discussion

In this study we evaluate the expression level of genes (*Sp1* and *PRKC*B) and microRNAs (miR-126 and miR-15a-3p) in 20 T2D patients and 20 healthy individuals as controls. Our results revealed that expression level of *Sp1* was significantly higher in T2D patient group compared to normal ones, in the other hand there was a significant positive correlation between expression level of *Sp1* and BMI index of the participants.

Chen et al. (2013) evaluate the expression level of *SP1* in T2D and reported that *Sp1* gene expression was up-regulated in blood; they also showed that *SP1* not only is located within ±1Mb of obesity SNP, but also has the highest differential expression percentage(2).

Different studies have focused on Sp1 role in insulin regulation of gene expression and revealed that there are several mechanisms by which Sp1 alters gene activity in response to insulin(18-22). Pathway analysis also showed that SP1 in involved in adipogenesis(2). These roles of *Sp1* in this process may be the reason of its increased expression in T2D patients compared to normal ones. Concerning *PRKCB* gene expression, the results show that expression level of this gene was significantly higher in T2D patient group compared to normal individuals. We observed no correlation between age, BMI and FBS level of participants and expression level of *PRKCB* gene. In Chen et al. (2013) results of this research showed that *PRKCB* is up-regulated in blood of T2D individuals (2). Pathway analyses revealed that *PRKCB* is involved in insulin signaling pathway networks and mainly correlated with T2D.

Our results in figure 3 show that expression level of miR-126 and miR-15a-3p is significantly lower in T2D patients compared to normal ones. Zampetaki et al. (2010) revealed a plasma microRNA signature for T2D that includes loss of endothelial miR-126 and miR-15a-5p(23).

There are some reports that monocytes of patients with DM show impaired responsiveness to VEGF contributing to defects in collateral vessel development, so according to our results low plasma levels of miR-126 in T2D patients group might

result in reduced delivery of this microRNA to monocytes and contribute to VEGF resistance and endothelial dysfunction.

Our results as shown in figure 4 with G2 assay technique showed that induced genomic instability is higher than intrinsic genomic instability in our two target groups, but in T2D group this difference was very remarkable

It has been shown that ROS are produced in various tissues under diabetic conditions through many sources including nonenzymatic glycosylation reaction. The electron transport chain in mitochondria and membrane-bound NADPH oxidase[(23)].

There is a fact that T2D and hyperglycemia can be sources of DNA damage by oxidation of DNA bases and sugar-phosphate binding sites. The occurrence of base oxidation can result in mutagenic effects and/or DNA replication arrest[(24)]. In T2D electron transport chain is activated and glycation reaction is observed in various tissues and, during the process, ROS are also produced.

Increase genomic instability in T2D patients have been reported using micronuclei and comet assay techniques. For example, Palazzo et al. (2012) reported that the frequencies of micronuclei and nuclear buds were higher in T2D patients than in controls[(25)]. Grindel et al. (2016) showed that the occurrence of micronuclei was significantly increased in T2D patients compared to healthy controls[(26)].

In this study we also observed positive correlation between the expression level of *SP1* and *PRKCB* genes and both intrinsic genomic instability and induced genomic instability in our two target groups. But according to these facts that Sp1 is a ubiquitously expressed transcription factor that also translocate directly to DNA lesions and ATM kinase phosphorylates this transcription factor in response to ionizing radiation and oxidative stress[(12)], so this correlation observed between this gene expression and genomic instability can be reasonable.

To our knowledge there are limited study has ever analyzed the link between genomic instability measured and expression level of genes *Sp1* and *PRKCB*.

As shown in figure 3 we also observed positive correlation between the expression level of miR-126 and miR-15a-3p and both intrinsic genomic instability and induced genomic instability in our target groups. Pathway analysis showed that miR-126 is involved in many cellular processes including cell cycle[(23)].

miR-15a-3p down regulation correlated with CHEK1 up-regulation so this microRNA is a key factor in occurring genome instability[(16)].

To our knowledge there are limited studies has ever analyzed the link between genomic instability measured and expression level of microRNAs (miR-126 and miR-15a-3p).

Conclusion

Over all the results of this study show that genomic instability in T2D patients is higher than normal persons and different genes and microRNAs such as *PRKCB*, *Sp*1, miR-126 and miR-15a-3p are involved in this phenomenon.

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