Bull. Egypt. Soc. Physiol. Sci. 38(1),77-88

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Plasma Circular RNA (0054633) Expression as a Biomarker for Pre-diabetes and Type 2 diabetes mellitus

***Sally M. El-Hefnway[1], Nevein M. Al-sheikh[1], Heba E .Kasem[2], Fatma A.El-Esrigy[3]**

[1] Medical Biochemistry Department, Faculty of Medicine- Menoufia University, Egypt.
[2] Internal Medicine Department, Faculty of Medicine – Menoufia University, Egypt.
[3] Family Medicine Department, Faculty of Medicine - Menoufia University, Egypt.

Received: 5 July 2017
Accepted: 25 August 2017
Available online: 1 Jan 2018

Keywords

- Circular RNA
- pre-diabetes
- T2DM

Abstract

Background: Circular RNAs are a type of closed non-coding RNAs, lack the terminating 5'-cap and 3'-polyadenylated tail structures that present in linear RNAs. They have higher biological stability because of their resistance to RNA exonucleases and in some tissues; their expression levels are ten times higher than other types of RNA. CircRNAs act as biomarkers for various diseases including diabetes mellitus as they have been found to affect insulin secretion and β-cell renewal. **Objective:** to investigate the role of circRNA (0054633) as a biomarker for pre-diabetes and T2DM. **Subjects & Methods:** A total number of 149 subjects, selected from endocrinology unit of Internal Medicine Department, and Munshaat Sultan Family Medicine Clinic Menoufia university hospital, classified into three groups: group I were 55 T2DM patients, group II were 44 pre-diabetics and group III including 50 healthy subjects. Expression profile of circRNA (0054633) in plasma of the studied subjects was analyzed by quantitative real- time polymerase chain reaction (Q-PCR). **Results:** CircRNA (0054633) expression was significantly increased gradually from controls to the pre-diabetes group to the T2DM group ($p<0.05$).The sensitivity of CircRNA (0054633) expression for prediction of diabetes and prediabetes at cutoff point (2.95 ,1.95) was (92.7%, 77.3%) respectivily and the specificity was (98% and 98.6%) respectivily. The most significant pedictors for pre-diabetes were CircRNA (0054633) expression and BMI, OR (59.8, 24.9 respectivily). **Conclusion:** Plasma CircRNA (0054633) expression could be considered as a predictive and diagnostic biomarker for pre-diabetes and T2DM.

Corresponding author: Sally M. El-Hefnway Medical Biochemistry Department, Faculty of Medicine- Menoufia University, Egypt, Tel: +201009896870. Email: doctor_sally@rocketmail.com

Introduction

Circular RNAs (circRNAs) are a novel type of RNA, differing from linear one, in having covalently closed loops in their structure and in being highly represented in the eukaryotic transcriptome (1). CircRNAs are a type of endogenous closed non-coding RNAs, formed by an exon, an intron, or by reverse splicing of the two (2). Because of their closed loop structure, they cannot be mapped directly to the genome, which is one of the reasons for the late discovery of these new RNA species (3).CircRNAs are distributed widely in the nucleus and cytoplasm (4). Genome-wide analyses indicated that circRNAs are abundant, conserved across species and often dynamically expressed in a tissue-specific manner, suggesting their potential regulatory roles (5). As circular RNAs do not have 5' or 3' ends, they are resistant to exonuclease-mediated degradation and are more stable and have reduced rates of turnover than that of linear RNAs in cells (6). In 2015, Enuka et al. measured the half-lives of about 60 of circRNAs (over 48 h) and their linear counterparts (<20 h) expressed from the same host gene and found that the median half-life of circRNAs of mammary cells is at least 2.5 times longer than that of their linear counterparts (7). CircRNAs can function as microRNA (miRNA) sponges, regulators of splicing and transcription, playing a role in posttranscriptional regulation by engaging in competitive combination with miRNA and modifiers of parental gene expression. Similar to other types of non- coding RNA, as miRNAs and long noncoding RNAs (lncRNAs), circRNAs are becoming a new research hotspot in the field of RNA and could be widely involved in the processes of life (8). Impaired glucose tolerance (IGT) is an intermediate category between normal glucose tolerance and overt diabetes. Individuals with IGT are now referred to have “pre-diabetes” indicating the high risk for diabetes mellitus development (9). Nearly 410 million diabetic patients exist worldwide, 46.5% of them have not been diagnosed. The International diabetes federation predicts that nearly 7.6 million Egyptians will have the disease by 2025, making it one of the top 10 countries in the world in relation to diabetes incidence, by 2040, the number of patients with diabetes worldwide may increase to 642 million (10). In the advanced stages of T2DM, patients often experience various complications. Therefore, early diagnosis and intervention are urgently needed (11). Large scale investigations have revealed that intensive glucose lowing therapy in the early phases of T2DM can reduce the incidence of macro-vascular and micro-vascular complications (12). CircRNAs act as biomarkers for various diseases including diabetes mellitus, cancer, atherosclerosis, osteoarthritis, pulmonary fibrosis and Alzheimer’s disease (13). Abnormal expression of several circRNAs has been linked to cancers of the liver, bladder and stomach, also linked to cardiovascular disease and neurological disorders such as Parkinson’s disease (14). CircRNAs have been found to affect insulin secretion and β-cell renewal (15). The aim of present study was to study expression profile of circRNA (0054633) in the plasma and to investigate its role in pre-diabetes and T2DM.

Subjects and methods:

This study was carried out by collaboration between Medical Biochemistry, Internal Medicine and Family Medicine Departments, Faculty of Medicine, Menoufia University. A total of 149 subjects aged (41- 67 years old) were included in the present study: 50 known patients with T2DM during follow up visits, 44 pre-diabetics with IFG and IGT , and 50 healthy volunteers, age and gender matched as the control group. Both patients and controls were selected from Endocrinology Unit at department of Internal Medicine, Munshaat Sultan family Medicine Clinic, Menoufia university hospital during the period between August 2015 and December 2016). Patients were diagnosed according to the 2010 Standards of The American Diabetes Association (ADA) criteria for the diagnosis of diabetes. So, cases have either of the following criteria were diagnosed as having T2DM: (i) 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, (ii) a 2-hours plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher during a 75-g oral glucose 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 and (iii) a hemoglobin A1c (HbA1c) level of 6.5% or higher. Additionally, patients have either of the following criteria were diagnosed as pre-diabetes: (i) FPG 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l) [IFG], (ii) 2-h PG in the 75-g OGTT 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l) [IGT] and a hemoglobin A1c (HbA1c) level 5.7–6.4% (16).

Subjects with any of the following criteria were excluded from our study: liver or kidney dysfunction, inflammatory diseases, malignancies, untreated hypertension, autoimmune diseases and any endocrine disease other than T2DM. All studied groups were subjected to complete history taking, full clinical examination including anthropometric measurements. Weight was measured in kilograms with light clothes and height in meters without shoes. Estimation of body mass index (BMI) was done by dividing body weight in kilograms by (height in square meters) (17). Laboratory investigations including fasting and 2 hours post prandial blood glucose levels, glycated hemoglobin (HbA1c%), lipid profile [serum total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-c) and calculation of low density lipoprotein cholesterol (LDL-c)]. Expression profile of circRNA (0054633) in plasma was analyzed by quantitative real- time polymerase chain reaction (Q-PCR). Before collection of blood samples, written consent approved by the Committee of Human Rights in Research at Menoufia University was obtained from all studied cases and controls. After 12 hours of overnight fasting, 10 ml of venous blood were withdrawn from every subject by sterile vein-puncture and divided into three samples: The first sample was 4 ml of blood and subdivided into 2 EDTA tubes: one of them was used for quantitative colorimetric determination of glycated hemoglobin as percent of total hemoglobin using kits supplied by Teco diagnostics, USA (18), the other EDTA

tube was centrifuged for 15 min at 4000 r.p.m. The plasma obtained was used for total RNA extraction. The second sample was one ml of blood and transferred into sodium flouride tube, another sample of blood was taken after 2 hours for determination of blood glucose by enzymatic colorimetric method using Spinreact kit, SPAIN (19). The third sample was 5 ml of blood and transferred into a plain tube, allowed to clot at 37°C, centrifuged for 15 minutes at 4000 r.p.m. The clear supernatant serum was separated from the clot and kept frozen at -80°C until determination of serum TC (20), HDL-c (21) and TG (22). Serum TC and TG were measured by enzymatic colorimetric test, using Spinreact kit, SPAIN. Serum HDL-c was measured by colorimetric method, using Human kit, GERMANY. LDL-c was calculated from TC concentration, HDL-c and TG according to (23). Total RNA was extracted from fresh plasma samples using miRNeasy plasma kit, QIAGEN, USA according to the manufacturer's instructions. The yield and purity of RNA were measured by Nano-Drop instrument (Thermo Scientific, USA). RNA extract was stored in -80ºC till analysis. QuantiTect Reverse Transcription Kit, Qiagen, USA was used for reverse transcription step and production of complementary DNA (cDNA). Each reaction was carried out on ice with a total volume of 20 µl, containing 1 µl of Quantiscript Reverse Transcriptase, 4µl of Quantiscript RT Buffer, 5µl of template RNA and 10 µl of nuclease free water. Incubation was done using Applied Bio systems 2720 thermal cycler (Singapore) for only one cycle: 10 min at 42°C then, 5 min at 95°C to inactivate Reverse Transcriptase and finally for 5 min at 4°C. The reverse-transcription reactions were stored at –20°C till real-time PCR step. Quantitative or real-time PCR was performed by SensiFAST™ SYBR Lo-ROX Kit, USA. Real-time PCR was carried out to a total volume of 20 µl, containing 10 µl of SYBR green Master Mix with low ROX; 4 µl of Nuclease-free water, 4 µl of Template cDNA and 1 µl of each primer (forward & reverse). The following designed primers (Midland, Texas) were used: CircRNA (0054633): Forward primer sequence: 5`TTGCTTTCTACACTTTCAGGTGAC3`,Reverse primer5`GCTTTTTGTCTGTAGTCAACCACCA3`and GAPDH Forward primer sequence: 5`GAAGGTGAAGGTCGGAGTC3`,Reverse primer:5`GAAGATGGTGATGGGATTTC 3`.The PCR condition for CircRNA (0054633) amplification consisted of three phases: initial activation phase at 95°C for 5 minutes followed by 45 cycles at 95°C for 30 sec; 60°C for 30 sec; 72°C for 1 minute; and a final extension phase at 72°C for 10 minutes. Finally, fluorescence detection and data analysis were performed using the ABI PRISM 7500 (Applied Biosystems, USA) version 2.0.1. The relative quantification (RQ) of CircRNA (0054633) gene expression was performed using comparative ΔΔCt method ***(24)*** **Figure (1)**, where the amount of the target gene is normalized to an endogenous reference gene *(GAPDH)* and relative to a control.

Statistical analysis

Results were collected, tabulated and statistically analyzed by IBM personal computer and statistical package (SPSS version 22, Inc., Chicago, Illinois, USA). Data was expressed into two phases: I-Descriptive: Mean value and Standard Deviation [SD]: for quantitative data, frequency and

percentage for qualitative date. II- Analytic: F test (one way ANOVA): for comparison of more than two independent quantitative variables normally distributed.K (kruskal wallis): for comparison of more than two independent quantitative variables not normally distributed. ROC-curve: Receiver operating characteristic Curve analysis. Sensitivity :Probability that the test results will be positive when the disease is present (true positive rate, expressed as percentage. Specificity : Probability that the test results will be negative when the disease is abscent, true negative rate, expressed as percentage. PPV :Positive Predictive Value: probability that the disease is present when the test is positive. NPV: Negative Predictive Value: probability that the disease is present when the test is negative.

Table (1): Demographic, clinical and laboratory data of studied groups

	Group I N= 55	**Group II N= 44**	**Group III N= 50**	**F test**	**Post-hoc test**
Age (years)	53.9±7.5	52.4±6.7	52.6±7.3	0.514	---
Gender **Females** **Males**	 26(47.3%) 29(52.7%)	 21(47.7%) 23(52.3%)	 22(44%) 28(56%)	χ^2 0.922	
BMI (kg/m^2)	27.5±2.5	22.7±0.7	22.3±2.4	<0.001*	I&II:<0.001 I&III:<0.001 II&III:0.403
FPG (mg/dl)	258.9±70.4	118.9±4	88.8±9.1	<0.001*	I&II:<0.001 I&III:<0.001 II&III:0.001
2HPPG (mg/dl)	289.1±74.9	157.3±14.2	88.6±8.6	<0.001*	I&II:<0.001 I&III:<0.001 II&III:<0.001
HbA1c (%)	10.3±1.3	5.97±0.2	5.2±0.8	<0.001*	I&II:<0.001 I&III:<0.001 II&III:<0.001
HDL-c (mg/dl)	32.1±1.3	38.3±1.3	48.6±1.2	<0.001*	I&II:<0.001 I&III:<0.001 II&III:<0.001
T.Cholest. (mg/dl)	210.2±26.9	213.6±11.9	172.3±9.2	<0.001*	I&II:0.361 I&III:<0.001 II&III:<0.001
TGs. (mg/dl)	164.1±10.1	112.2±3.5	93.2±4.9	<0.001*	I&II:<0.001 I&III:<0.001 II&III:<0.001
LDLc (mg/dl)	145.3±26.8	149.9±9.5	105±8.8	<0.001*	I&II:0.212 I&III:<0.001 II&III:<0.001
CircRNA (0054633) expression fold change	4.9±1.2	2.8±1.04	1±0.5	**K test** <0.001*	I&II:<0.001 I&III:<0.001 II&III:<0.001

F test: one way ANOVA, **K test:** kruskal wallis, χ^2: **Chi square test**, *: Statistically significant at p value ≤ 0.05 . BMI= body mass index, FPG= fasting plasma glucose, 2 HPPG= 2 hour post prandial glucose, HbA1c= glycated hemoglobin, HDL-c = high density lipoprotein cholesterol, T.Cholest. = total cholesterol, TGs= triglycerides, LDLc = low density lipoprotein cholesterol and CircRNA = circular ribonucleic acid.

Accuracy: the ratio of the true positive and true negative on all patients. Regression analysis calculates the effects of risk factors as independent Odds ratios with the effects of other confounders removed. P value > 0.05 was considered statistically non-significant. P value < 0.05 was considered statistically significant.

Results:

Our results revealed that, there were no significant statistical differences as regards age and gender among the three studied groups, indicating matching (P>0.05).There was a significant statistical increase of BMI in group I (diabetic patients) when compared to both of group II,III (p<0.05). On the other hand, non- significant differences regarding BMI in group II when compared to group III (P>0.05). There was a significant statistical increase of fasting and 2 hours post prandial plasma glucose & HbA1c (%) and TGs in group I when compared to both of group II,III and in group II when compared to group III (p<0.05). Significant statistical decrease was detected as regards HDL-c in group I when compared to both of group II,III and in group II when compared to group III (p<0.05). Non-significant differences regarding total cholesterol (TC) in in group I when compared to group II (P>0.05) , while there was a significant statistical increase of TC in group I when compared to group III, in group II compared to group III (p<0.05). There was a significant increase regarding LDL-c in group I compared to group III, and in group II compared to group III (p<0.05). Non - significant differences regarding LDL-c detected between group I and group III (P>0.05) as demonstrated in (Table 1).

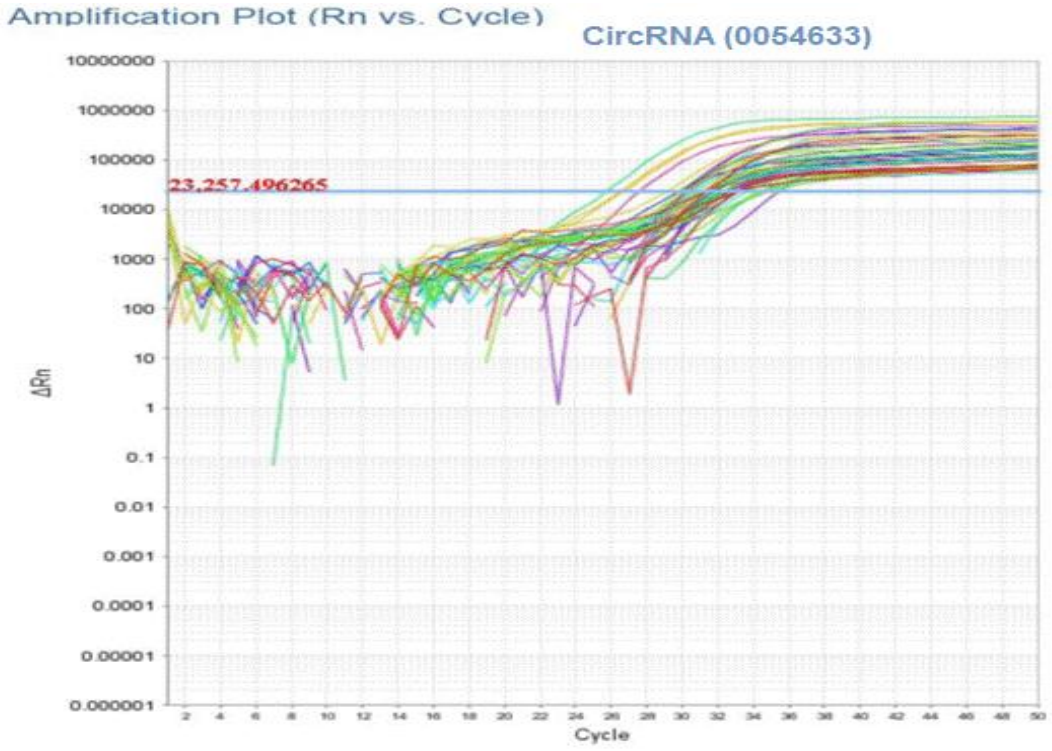

Figure (1): Amplification plot of plasma circRNA (0054633) expression (normalized fluorescence signal (ΔRn) plotted versus cycle number.

Table (2): Validity of CircRNA (0054633) for differentiation between of diabetics & pre-diabetics and controls

	AUC	P value	Cutoff	Sensitivity	Specificity	PPV	NPPV	Accuracy
T2DM patients	0.97	<0.001	2.95	92.7	98%	98.1	92.4	95.2
Pre-diabetics	0.95	<0.001	1.95	77.3	98.6	97.1	83	88.3

*: Statistically significant at p value ≤ 0.05, AUC= area under curve, PPV= positive predictive value and NPV= negative predictive value

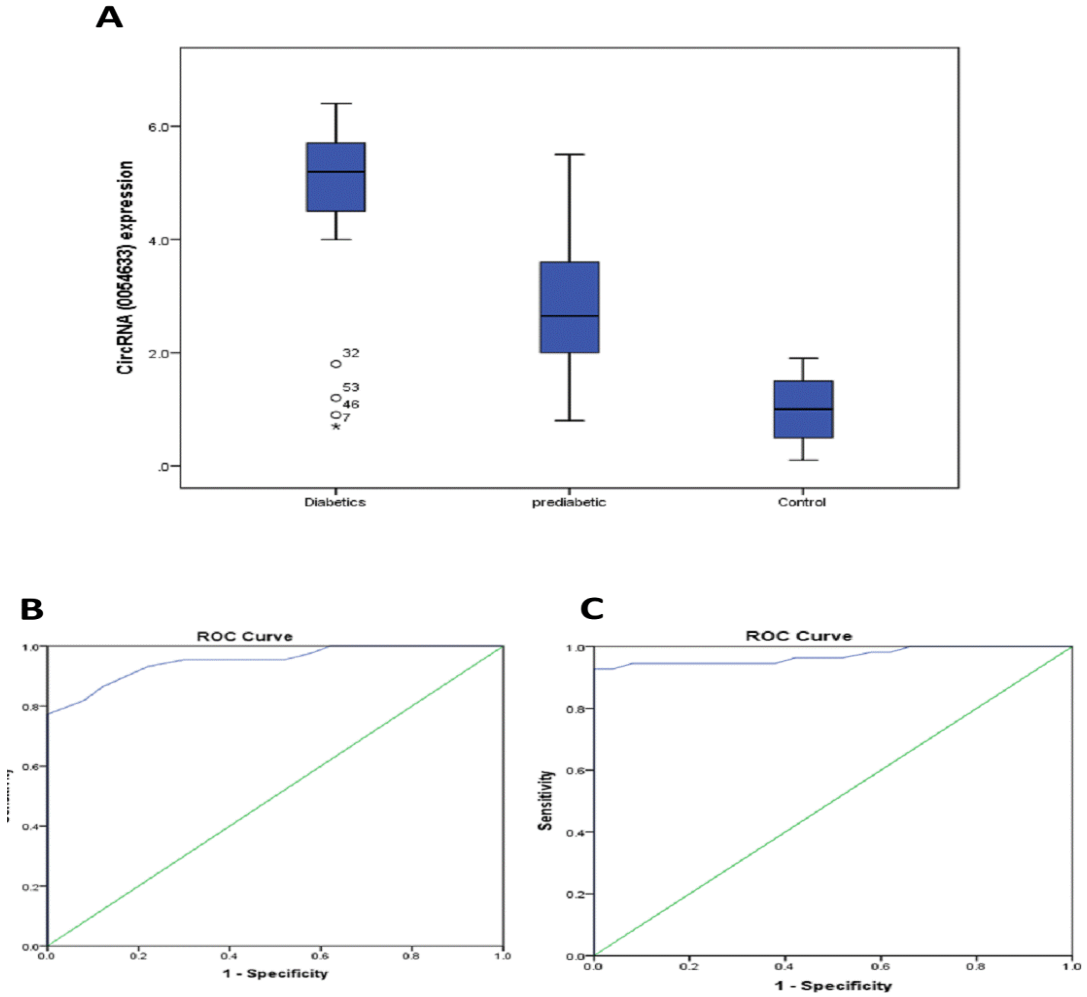

Figure (2): (a) Relative expression of plasma CircRNA (0054633) in the three studied groups**, (b)** Receiver operating characteristic Curve (R0C) analysis of CircRNA (0054633) for diagnosis of pre-diabetes**, (c)** Receiver operating characteristic Curve (R0C) analysis of CircRNA (0054633) for diagnosis of type 2 diabetes mellitus**.**

CircRNA (0054633) expression profile showed significantly gradual increase from group III to group I (p<0.05) (table 1) & (Figure 2a). In an attempt to investigate whether plasma circRNA (0054633) expression can predict future development of pre-diabetes and T2DM, our study revealed that, plasma circRNA (0054633) expression at cutoff value of 1.95 gives a sensitivity of 77.3% , a specificity of 98.6%, PPV

of 97.1 %, NPV of 83% and accuracy of 88.3 %for differentiation between controls and pre-diabetics. On the other hand, a cutoff value of 2.95 gives a sensitivity of 92.7% and a specificity of 98% PPV of 98.1%, NPV of 92.4% and accuracy of 95.2 %for differentiation between pre-diabetics and diabetic patients (Table 2) & (Figure 2b &2c). The AUC of circRNA (0054633) for the diagnosis of pre-diabetes and T2DM were 0.95 and 0.97, respectively, ($p<0.05$) (Table 2) & (Figure 2b &2c).

Stepwise binary regression analysis revealed that, the most significant variable for prediction of pre-diabetes was CircRNA (0054633) expression fold change above 1.95 (p value 0.001), (OR 59.8); CI 95% (10.1-66.9) followed by BMI >25 (p value 0.013) ,(OR 24.9) ;CI 95% (1.9-31.5), while the most protective variable was HDL-c <40 (p value 0.002), OR (0.008) CI 95% (0-0.2) (Table 3)

Table (3): Stepwise binary regression for prediction of pre-diabetes

	B coefficient	P value	OR	CI (95%)
BMI (kg/m^2)	3.2	0.013	24.9	1.9-31.5
T. cholesterol (mg/dl)	0.68	0.69	1.98	0.1-6.2
LDL-c (mg/dl)	2.3	0.135	9.9	0.5-20.6
HDL-c (mg/dl)	-4.8	0.002	0.008	0-0.2
CircRNA (0054633) expression	5.6	0.001	59.8	10.1-66.9

OR: odds ratio, CI: confidence interval, *P<0.05: significant, BMI: body mass index, T.cholesterol: total cholesterol, HDL-c: high density lipoprotein cholesterol, LDL-c: low density lipoprotein cholesterol

Discussion:

OGTT is the gold standard for diagnosis of pre-diabetes and T2DM. However, this test is time consuming, inconvenient and complicated. Fasting plasma glucose is convenient tool for T2DM diagnosis, but the rate of missed diagnosis is high (25). HbA1c% seems likely to be related to changes in red blood cell life rather than glycaemia or glycation rates. So it is affected by haemoglobinopathies, as HbS (sickle cell) and persistent HbF (fetal) (26).Current methods show various insufficiencies for the early diagnosis and prediction of pre-diabetes and T2DM (11).So, numerous studies have tried to identify convenient, highly specific and sensitive marker for T2DM at early stages (27). Pre-diabetics are asymptomatic, so they rarely visit hospitals to seek diagnosis and therapy (28). Nearly all pre-diabetics become frankly diabetics after a variable period emphasizing the importance of strategies for this category to allow early detection and hence prevention or delay the development of T2DM and its complications (9). American Diabetes Association Consensus Development Panel (ADA-CDP) reported that, it is mandatory for pre-diabetics to follow interventions that significantly decrease the rate of onset of diabetes including, intensive lifestyle modification programs like, 5–

10% weight loss and moderate physical activity as brisk walking for 30 min / day, leading to 58% reduction of risk of T2DM, after 3 years of interventions and use of the pharmacologic agents as metformin (28). The aim of present study was to study expression profile of circRNA (0054633) in the plasma and to investigate its role in pre-diabetes and T2DM.In this study, fasting, 2hours post prandial blood glucose levels and HbA1c% were significantly higher in both pre-diabetics and T2DM groups compared with the control group. This is in agreement with the results obtained by Cho et al., (29) and Graham et al., (30).

The present study reported that total cholesterol, triglycerides and LDL-c were significantly higher in T2DM group compared with controls and pre-diabetics, whereas serum HDL-c was significantly lower. This comes in line with Songa et al., (32) who reported that in patients with T2DM, triglycerides are often elevated, HDL-c is generally decreased, and LDL-c may be elevated, borderline, or normal. As insulin resistance in diabetics increase the expression of hepatic lipase which acts on HDL-c, resulting in smaller HDL particles that are more rapidly catabolized by the kidney leading to, lower plasma HDL-c.

As RNA researchers continue to explore circRNAs possible functions, multiple labs have discovered that circRNA expression levels vary with disease, leading to growing interest in how these molecules might be related to diagnosis and treatment (14). The present study revealed significant differences between the plasma circRNA (0054633) expression levels of type 2 diabetics and that of pre-diabetics and controls. The level of circRNA (0054633) expression increased gradually from the control group to the pre-diabetics to the T2DM group, with a fold change of 1.8 between the first two groups and 2.1 between the latter two groups. These results are in agreement with (28) who reported that; circRNA (0054633) had the highest diagnostic value for pre-diabetes and T2DM among other five candidate biomarkers showing highest AUC and lowest P values than other circular RNAs. Prior to Q-PCR step, Zhao et al., (28) performed microarray analysis for expression profile of circRNAs in peripheral blood. There were marked differences in the expression profiles of 489 circRNAs between the diabetic and healthy groups. There were 78 circRNAs up-regulated and 411 were down-regulated in the diabetics. In order to choose the biomarker that would be most applicable clinically, the candidate biomarkers were selected from the 78 up-regulated circRNAs utilizing stricter criteria: fold change [$\geq$2.4 and P$\leq$ 0.01. Only five circRNAs met these criteria: hsa_circ_0068087, hsa_circ_0054633, hsa_circ_0124636, hsa_circ_0139110 and hsa_circ_0018508. These circRNAs were used as candidate biomarkers in a subsequent validation in a larger cohort using Q-PCR technique.

Gene ontology (GO) analysis revealed that circRNA (0054633) participates in biological processes, such as cell cycle & mitotic cell cycle arrest, as it is involved in maintaining homeostasis, regulating cell growth and death, also strongly correlated with molecular catabolism (33). The cell cycle is the basic process of cellular life activities. The proliferation of β-cells is regulated by cell cycle progress, and decreased β-cell proliferation is the major cause of insufficient insulin secretion and hence incidence of T2DM (34). Zhao et al.,

(28) hypothesize that circRNA (0054633) may participate in the pathogenesis of T2DM by influencing the cellular metabolism and cell cycle. Because of convenience of sampling of circRNA (0054633) and its stability and higher rate of expression than linear RNAs and high specificity and sensitivity it could be considered as potentially highly useful tool for the diagnosis of pre-diabetes and prediction of T2DM.

Xu et al., (15) found that, some circRNA can regulate insulin transcription and secretion in islet cells of transgenic mouse through interaction with over expressed microRNA that cause diabetes, which in turn improves insulin secretion and could become a new target for improving β cell function in diabetes.

CONCLUSION

Plasma circRNA (0054633) expression could be considered as a predictive biomarker for pre-diabetes and hence it is a useful convenient tool for early detection of T2DM. We recommend further researches to evaluate circRNA as new target to decrease the progression of pre-diabetes into diabetes and to delay the incidence of diabetes mellitus complication.

Financial support and sponsorship: Nil.

REFERENCES

1. **Qu S, Yang X, Li X, Wang J.et al** :Circular RNA: A new star of noncoding RNAs, *Cancer Letters*. **365**:141-148, 2015

2. Barrett S, Wang P, Salzman J: Circular RNA biogenesis can proceed through an exon-containing lariat precursor. Elife4:e07540. doi:10.7554/eLife.07540, 2015

3. **Wang, P Bao Y, Yee M., Barrett S, Hogan G, Olsen M, Dinneny J, Brown P and Salzman J:** Circular RNA Is Expressed across the Eukaryotic Tree of Life. PLoS One, 9, e95116. http://dx.doi.org/10.1371/journal.pone.0090859, 2014

4. **Jeck W and Sharpless N:** Detecting and characterizing circular RNAs. Nat Biotechnol 32:453–461. doi:10.1038/nbt. 2890, 2014

5. **Meng X, Li X, Zhang P, Wang J, Zhou Y and Chen M**: Circular RNA: an emerging key player in RNA world, *Briefings in Bioinformatics*, 1–11. doi: 10.1093/bib/bbw045, 2016

6. **Guo X, He C and Wang Y et al:** Circular RNA Profiling and Bioinformatic Modeling Identify Its Regulatory Role in Hepatic Steatosis. *Hindawi BioMed Research International*, https://doi.org/10.1155/2017/5936171, 2017

7. **Enuka, Y.; Lauriola, M; Feldman, M.; Sas-Chen A.; Ulitsky, I and Yarden, Y:** Circular RNAs are long-lived and display only minimal early alterations in response to a growth factor". *Nucleic Acids Research:* gkv1367. PMID 26657629. doi:10.1093/nar/gkv1367, 2015

8. **Hansen T, Jensen T, Clausen B, Bramsen J, Finsen B et al:** Natural RNA circles function as efficient microRNA sponges. *Nature* **495**:384–388., 2013

9. **Govers E:** Obesity and Insulin Resistance Are the Central Issues in Prevention of and Care for Comorbidities Healthcare, **3**, 408-416; 2015

10. **Rahelic D**: 7th edition of idf diabetes ATLAS–CALL for immediate action. *Lijec Vjesn*. **138**:57–58, 2016

11. **Ghasemi A, Tohidi M, Derakhshan A, Hasheminia M, Azizi F et al** : Cut-off points of homeostasis model assessment of insulin

resistance, beta-cell function, and fasting serum insulin to identify future type 2 diabetes: Tehran Lipid and Glucose Study. *Acta Diabetol* , **52**:905–915, 2015.

12**. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA**: 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med* **359**:1577–1589, 2008

13**. Liu Q, Zhang X, Hu X, Dai L, Fu X et al :** Circular RNA related to the chondrocyte ECM regulates MMP13 Expression by functioning as a MiR-136 'Sponge' in human cartilage degradation. Sci Rep 6:22572, 2016

14. Offord C: Uncovering Functions of Circular RNAs. *The scientist*, July 17, 2017.

15. Xu H, Li W, and Guo S et al.: The circular RNA Cdr1as, via miR-7 and its targets, regulates insulin transcription and secretion in islet cells. DOI: 10.1038/srep12453, 2015

16.American Diabetes Association: Standards of Medical Care in Diabetes—2010 *Diabetes care* **33**, supplement1, 2010

17. Enyioma O; Michael PT; Abd-Ishakur A; Mustapha S and Mona A: Leptin, lipid and lipid metabolism related hormones in chronic renal failure in Arabia. *Nephrology*. ; **7:**115-119, 2002

18. Gonen B and Rubenstien AH: Determination of glycohemoglobin. *Diabetologia;* **15**: 1 5, 1978

19.Trinder P: Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *J Ann Clin Biochem*; **6**: 24-25,1969

20. Rifai N and Warnick R.: Lipids, lipoproteins, apolipoproteins and other cardiovascular risk factors. In: Tietz Textbook of Clinical Chemistry and Molecular Diagnosis. Carl, A. B, Edward, R. A and David, E. B [edrs.]. Saunders. [4th edition]; Ch 26. 2006, PP. 918-922.

21.Gordon T, Zidek W and Amer M. Determination of highdensity lipoprotein cholesterol. *J. Med* ; **42:** 707:710, 1979

22. Fossati P and Prenciphe L. Determination of serum triglyceride. *Clin Chem ;* **28**: 207-210, 1982

23.Friedewald W, Levy R and Fredrickson D. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem;* **18**:499- 502, 1972

24. Dorak M : Real-time PCR. *Clinical Chemistry*.50:1680-1682, 2004

25. Gholap N, Davies M, Mostafa S, Squire I, Khunti K.: A simple strategy for screening for glucose intolerance using glycated haemoglobin, in individuals admitted with acute coronary syndrome. Diabetic Medicine March. DOI: 10.1111/j.1464-5491.2012.03643, 2012

26. Kobold U; Jeppsson J and Dulffer T: Candidate reference methods for hemoglobin A1c based on peptide mapping. *Clin. Chem.* **43**:1944 - 1951, 2007

27. Chidambaram M, Liju S, Saboo B, Sathyavani K, Viswanathan V et al Replication of genome-wide association signals in Asian Indians with early-onset type 2 diabetes. *Acta Diabetol*. doi:10.1007/s00592-016-0889-2, 2016

28. Zhao Z, Li X, Jian D, Hao P, Rao L and Li M: Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker of pre-diabetes and type 2 diabetes mellitus. *Acta Diabetol*, **54**: 237–245, 2016.

29.Goodman M; Thurston H and Penman J: Efficacy and tolerability of vildagliptin in patients with type 2 diabetes inadequately controlled with metformin monotherapy. *Horm Metab Res*. **41**: 368−373,2009

30. Cho YM ; Youn BS ; Lee H ; Lee N ; Min SS ; Kwak SH ; Lee H and Park K: Plasma retinol binding protein 4 concentrations are elevated inhuman subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care.* **29**:2457–2461, 2006

31. Graham TE ; Yang Q ; Bluher M;Hammarstedt A ; Ciaraldi TP; Henry RR ; Wason CJ ; Oberbach A ; Jansson PA ; U and Kahn BB:Retinol binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med*. **354** :2552-2563, 2006

32. Songa R, Viswabarathi N and Siddhartha K: Comparative study of lipid profile in obese type 2 diabetes mellitus and obese non diabetes. IOSR Journal of Dental and Medical Sciences (*IOSR-JDMS*) **14**, 87-91, 2015

33. Fischer J and Leung A: CircRNAs: a regulator of cellular stress. critical reviews in biochemistry and molecular biology, http://dx.doi.org/10.1080/10409238.2016.1276882 , 2017

34. Antezana E, Egaña M, Blondé W,et al: The Cell Cycle Ontology: an application ontology for the representation and integrated analysis of the cell cycle process Genome Biology, 10:R58, https://doi.org/10.1186/gb-2009-10-5-r58, 2009