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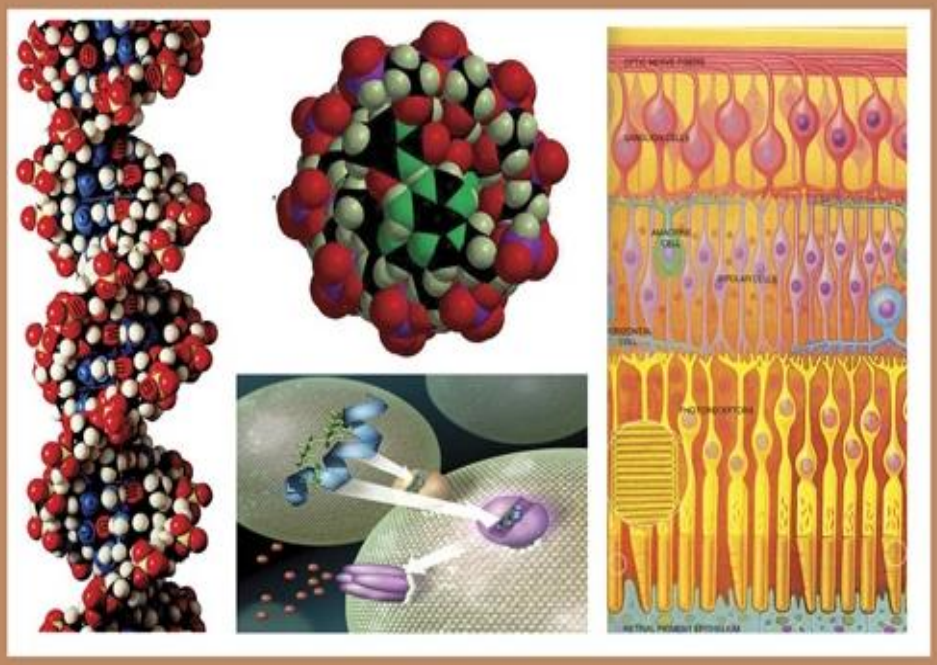

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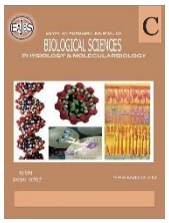

A possible Correlation between the SNP rs10830963 of the MTNR1B Gene and Early-onset Type 2 Diabetes mellitus (EOD) in A Sample of the Egyptian Population

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ABSTRACT

Type 2 diabetes (T2DM) is a prime public health condition in Egypt. Along with the global development of T2DM, the frequency of T2DM diagnosed in adults < 40 years old has increased dramatically. Pancreatic B-cells express the MTNR1B gene, which expresses the receptor 1B of melatonin. Multiple large-scale genome-wide association studies and meta-analyses revealed a strong connection between the rs10830963 of the MTNR1B gene with fasting glucose and T2DM in many ethnic groups. This study aims to detect the link between rs10830963 and early-onset type 2 diabetes (EOD) in the Egyptian population. The rs10830963 was identified using polymerase chain reaction (PCR) followed by DNA sequencing technology on 12 T2DM patients and 18 healthy controls group with an age range of 30 to 39 years. Fasting blood sugar (FBS), glycated hemoglobin (HbA1c), serum creatinine (CREAT), triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C) were determined for both healthy and diabetic groups. Four T2DM patients (one male and three females) in addition to four healthy individuals (one male and three females) were found to contain the risk G allele when compared to the NCBI reference sequence using the T-Coffee online tool for multiple sequence alignment. Furthermore, the RNA secondary structure for reference and sample sequences with the rs10830963 was predicted in terms of minimum free energy (MFE) using the Vienna online tool, finding that there is a slight difference in MFE between the compared sequences, which may explain the upregulation of mRNA of the MTNR1B gene in presence of rs10830963. This study reveals a possible association between the MTNR1B gene variant rs10830963 and EOD in our Egyptian population sample.

INTRODUCTION

Over the past few decades, the frequency of type 2 diabetes mellitus (T2DM) has risen at a shocking rate globally (Persaud *et al.,* 2016). T2DM, the most widespread form of diabetes, is a chronic and diverse illness with genetic and environmental causes (Stumvoll *et al.*, 2005).

It is known to occur either when β-cells in the pancreas cannot produce an adequate amount of insulin or when insulin receptors fail to respond adequately to insulin, preventing glucose from entering cells (Reinehr 2013; Reaven 1980). Melatonin, a pleiotropic hormone that regulates the circadian as well as seasonal rhythms, sleep, the function of the retina, and the body's immune system, is produced by the pineal gland in high concentrations during the night and low concentrations during the daytime (Karamitri and Jockers 2019). Melatonin binds with a potent affinity to MT1 and MT2 receptors (Jockers *et al.,* 2016), which belong to the G-protein coupled receptors (GPCR) superfamily and are primarily associated with Gi/o and Gq/11 subclasses of G proteins (Dubocovich *et al.,* 2010), hence activating several downstream signaling pathways.

Over 150 risk alleles, involving a prevalent variant in the gene expressing MTNR1B, have been informed by numerous Genome-wide association studies (GWAS) to increase the possibility of developing T2DM (Persaud *et al.,* 2016; Wang *et al.,* 2019). The MT2 receptor for melatonin is encoded by the MTNR1B gene and expressed in different body tissues like pancreatic islet cells (Lyssenko *et al.,* 2009). Several human and animal studies have clarified the major influence of the MTNR1B gene on the control of glucose homeostasis (Tuomi *et al.,* 2016; Pate *et al.,* 2018; Karamitri and Jockers 2019).

The MTNR1B gene contains two widespread variants (rs1387153 and rs10830963) that have been linked to high plasma glucose concentrations, a decrease in the first phase of insulin response to oral as well as intravenous glucose, and a quicker decline in insulin production over time (Bouatia-Naji *et al.,* 2009; Prokopenko *et al.,* 2009; Kan *et al.,* 2010). These studies have revealed a linkage between the MTNR1B gene and T2DM. However, the mechanism underlying this relationship is still unknown. The purpose of the current study is to investigate the linkage between the common intronic SNP rs10830963 with early-onset T2DM in some Egyptian participants (< 40 years old).

MATERIALS AND METHODS

Study Population:

This study recruited 30 participants, including 12 patients with T2DM including 5 males and 7 females admitted to Benha University Hospital, and 18 healthy controls including 6 males and 12 females aged 30 to 39 years. Participants were divided into case and control groups in accordance with the American Diabetes Association (ADA) recommendation (Marathe *et al*., 2017). Those having a fasting blood sugar greater than 126 mg/dL were categorized as diabetic patients. The study excluded patients diagnosed with T1DM and gestational diabetes (GDM). As a control group, individuals whose glycated hemoglobin (HbA1c) was less than 6, from the same area, were chosen. Through interviews with the participants, the primary characteristics of the participants, like gender, age, diabetes family history, and ethnicity, were determined. All applicants provided written informed agreement, and the study was permitted by the Scientific Research Ethics Committee of the Faculty of Medicine at Benha University (ID research ethics: RC:5-6-2022).

Anthropometric (clinical) and Biochemical Measurements:

The current study selected height (in centimeters) and weight (in kilograms) as the general anthropometric factors. Blood glucose was tested using the SPINREACT kit. Using a Diamond Diagnostics kit, serum creatinine was determined. Triacylglycerol (TG) was determined using the SPINREACT kit. Total cholesterol (TC) was tested using the SPINREACT kit, and high-density lipoprotein cholesterol (HDL-C) was assessed with the SPECTRUM kit. (Friedewald *et al.,* 1972) equations for determination of the low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were used as listed below:

- LDL-C = Total C – [HDL-C + (Triacylglycerol/5)].
- VLDL-C = Triacylglycerol/5.

Molecular Biology Techniques:

In this investigation, 5 mL of venous blood was taken out from every participant in an EDTA tube. According to the manufacturer's instructions, DNA was extracted using a complete DNA purification kit (Qiagen, QIAamp DNA Mini Kit). The Thermo Scientific NanoDrop 2000 was utilized to quantify and evaluate the purity of all DNA samples to ensure that the concentrations were pure enough for Conventional PCR. The MTNR1B Gene was amplified utilizing COSMO PCR RED Master Mix, Willowfort, with the following primers: a forward primer: 5'GGCTGTCT GGGAGGTTTAGT3'and a reverse primer: 5'TTGGAAGAGAGGAGGCAAGG3',
under the following thermocycler conditions: an initial polymerase activation step at 95 C° for 2 min, followed by 25 cycles of 95 C° for 15 sec, 57 C° for 40 sec and final extension at 72 C° for 1 min. 2% agarose gel and 100bp DNA Ladder were utilized to visualize the PCR results. PCR products were cleaned of unincorporated PCR primers and dNTPs using the Millipore Montage PCR Clean-up Kit. As indicated previously, the purified PCR products were sequenced using two primers. Big Dye terminator cycle sequencing kit v.3.1 was utilized to perform the sequencing (Applied BioSystems, USA). Macrogen, Inc., Seoul, Korea, resolved sequencing products on an Applied Biosystems model 3730XL automated DNA sequencing device (Applied Biosystems, USA). T coffee (http://tcoffee.crg.eu/) was used to create a multiple sequence alignment (MSA) of the partial sequence of the MTNR1B gene in all samples from the Normal and T2DM groups, as well as the NCBI reference sequence (NG_028160.1). The Vienna online tool (http://rna.tbi.univie.act.at/) was utilized to predict the secondary structure of RNA for each sample in both groups.

Statistical Analysis:

Version 20 of SPSS was used for data analysis. All biochemical parameters were presented as means ± standard error (S.E). Using T-test, a comparison of quantitative variables was conducted. A *p*-value < 0.05 was considered statistically significant.

RESULTS

Totally, 30 participants, healthy control (n=18) and T2DM (n=12), with a mean (S.E.) age of 34.67 (3.37) and 35.17 (3.46), respectively, were successfully sequenced for MTNR1B gene variants.

The main features of participants in normal and T2DM studied groups were shown in (Table 1**)**. Age (*P* = 0.723), weight (*P* = 0.053), height (*P* = 0.581), and body mass index (BMI) (*P* = 0.065) were not significantly different from the normal group.

Normal and T2DM groups had significantly different HbA1c, FBS, TC, TG, and VLDL values. The levels of HDL, LDL, and CREAT did not differ significantly between the normal and T2DM groups (Fig. 1).

Table 1: Baseline characteristics of normal and T2DM groups**.**

Parameters	Normal Group (n=18)	T2DM Group (n=12)	*P*-value
Age (Years)	34.67 ± 3.37	35.17 ± 3.46	0.723
Weight (kg)	81.29 ± 14.62	101.79 ±31.51	0.053
Height (cm)	164.75 ± 10.91	166.92 ± 7.79	0.581
BMI (kg/m^2)	29.42± 4.79	36.65 ± 11.60	0.065

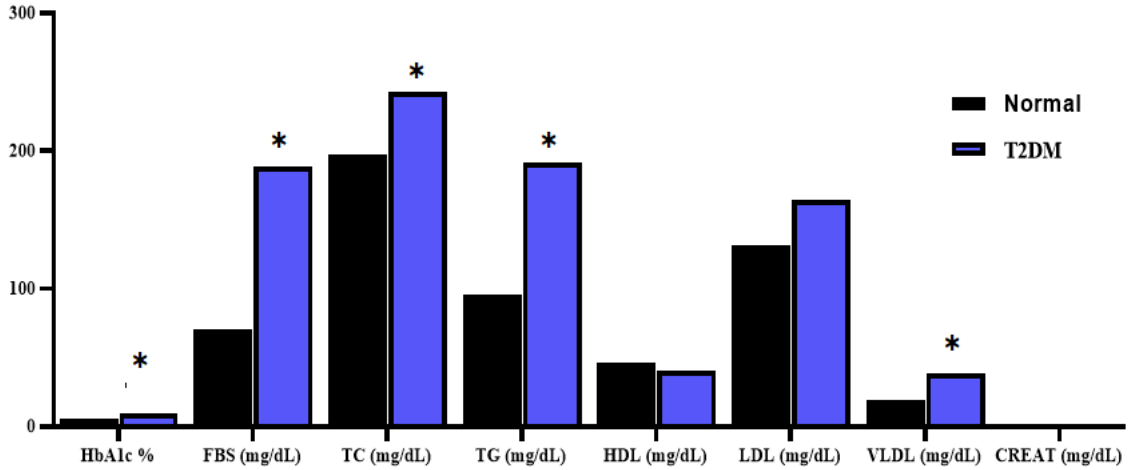

Fig. 1: Biochemical parameters for normal and T2DM Groups

After PCR products were extracted from the agarose gel (Fig. 2), and purified, they were sequenced. In addition to the NCBI reference sequence (Fig. 3), the MTNR1B (partial) consensus sequences of the Normal and T2DM groups were 261-265 nucleotides.

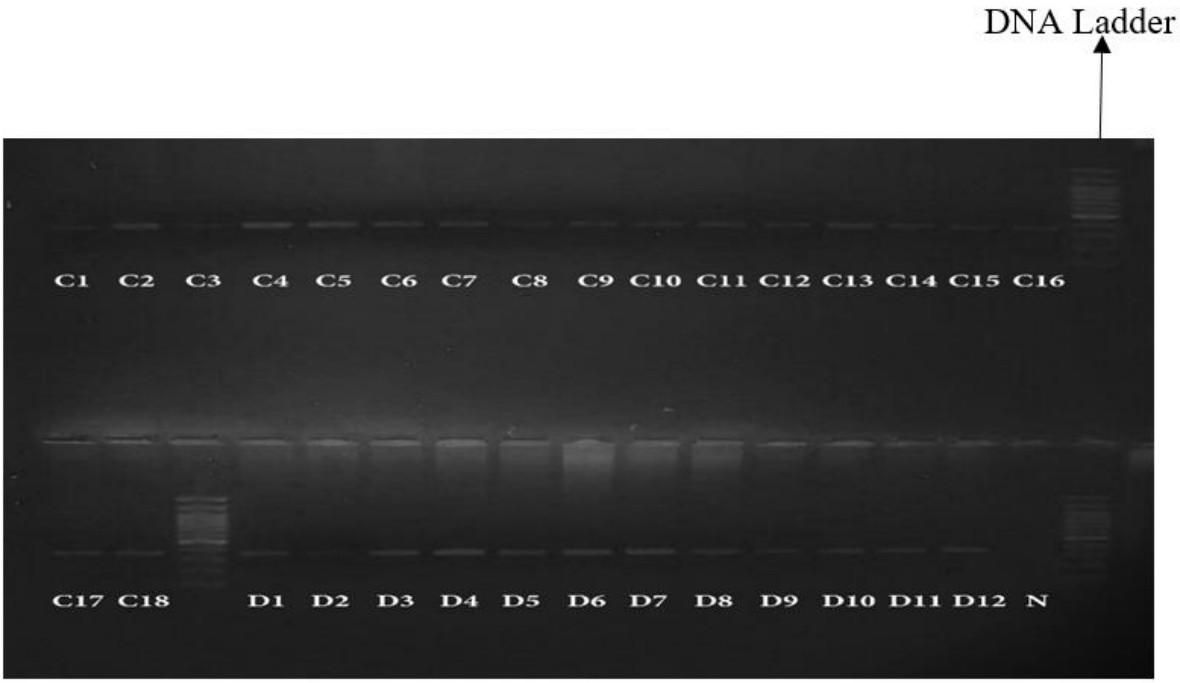

Fig. 2: The separated DNA fragments of intronic MTNR1B gene on agarose gel electrophoresis for both normal (C1-C18) and T2DM (D1-D12) groups

In the normal samples C5, C11, and diabetes sample D10, there is an additional T nucleotide at position 31. In addition, normal samples C5 and C11 contain an extra T nucleotide at positions 41 and 44. Moreover, the normal C11 sample contains an additional T nucleotide at position 64. The diabetic D7 sample sequence contains an additional T nucleotide at locations 40 and 43 (Fig. 4).

Also, alignment results have detected the presence of the rs10830963 C>G in both normal and T2DM samples, where normal samples C1, C10, C15, C16, and diabetic samples D1, D3, D9, D12 sequences have the minor allele (G allele) instead of the major allele (C allele) (Fig. 5). At position 249 in the D1 sample, a T nucleotide has replaced a C nucleotide (Fig. 6).

ATTTGATGTTAAGATTGCCTAGGAAAACATGGAGGATTTGCTTGCTGAACACACA
GATCTTTGTCCAAGGTCACTCTGTCTATGCTGGCAAAGCTGCCCCTCCTCCAGGC
CCCCAGTGATGCTAAGAATTCACACCATCTCCTATCCAGAACCAGTAACTGCCTG
GGAGGTTCCTGATGGGAATATTCTGCCTATGCAGGTGCTTTGCTTCCCCGGTTAG
ATTAGAAAACAGTCTGCACTGTGGCCCCTGTTCCTTGCCTC

Fig. 3: The NCBI partial consensus sequence of MTNR1 gene (261 bp)

```
reference   AACAT-GGAGGATTT-GC-TTGCTGAACACACAGATCT-TTGTC
C5          AACATTGGAGGATTTTGCTTTGCTGAACACACAGATCT-TTGTC
C11         AACATTGGAGGATTTTGCTTTGCTGAACACACAGATCTTTTGTC
D7          AACAT-GGAGGATTTTGCTTTGCTGAACACACAGATCT-TTGTC
D10         AACATTGGAGGATTT-GC-TTGCTGAACACACAGATCT-TTGTC

cons        ***** ********* ** ****************** *****
```

Fig. 4: T Coffee alignment of MTNR1B intronic DNA partial consensus sequence of NCBI reference sequence and the partial DNA sequence of control samples (C5, C11) and diabetic samples (D7, D10).

```
reference   CATCTCCTATC
C1          CATCTGCTATC
C10         CATCTGCTATC
C15         CATCTGCTATC
C16         CATCTGCTATC
D1          CATCTGCTATC
D3          CATCTGCTATC
D9          CATCTGCTATC
D12         CATCTGCTATC

cons        ***** *****
```

Fig. 5: T Coffee alignment of MTNR1B intronic DNA partial consensus sequence of NCBI reference sequence and the partial DNA sequence of control samples (C1, C10, C15, C16) and diabetic samples (D1, D3, D9, D12).

```
reference   GCCCCTGTTCC
D1          GCCCCCGTTCC

cons        ***** *****
```

Fig. 6: T Coffee alignment of MTNR1B intronic DNA partial consensus sequence of NCBI reference sequence and the partial DNA sequence of diabetic sample D1.

The predicted RNA secondary structure of the NCBI reference partial intronic sequence of the MTNR1B gene differed from the sequence derived from normal and diabetic samples containing the G risk allele as the only mutation in this partial region. As depicted in (Figs. 7, 8), the minimum free energy (MFE) of the RNA secondary structure predicted for diabetes samples with the G risk allele was somewhat less than that expected for the NCBI reference partial intronic sequence of the MTNR1B gene.

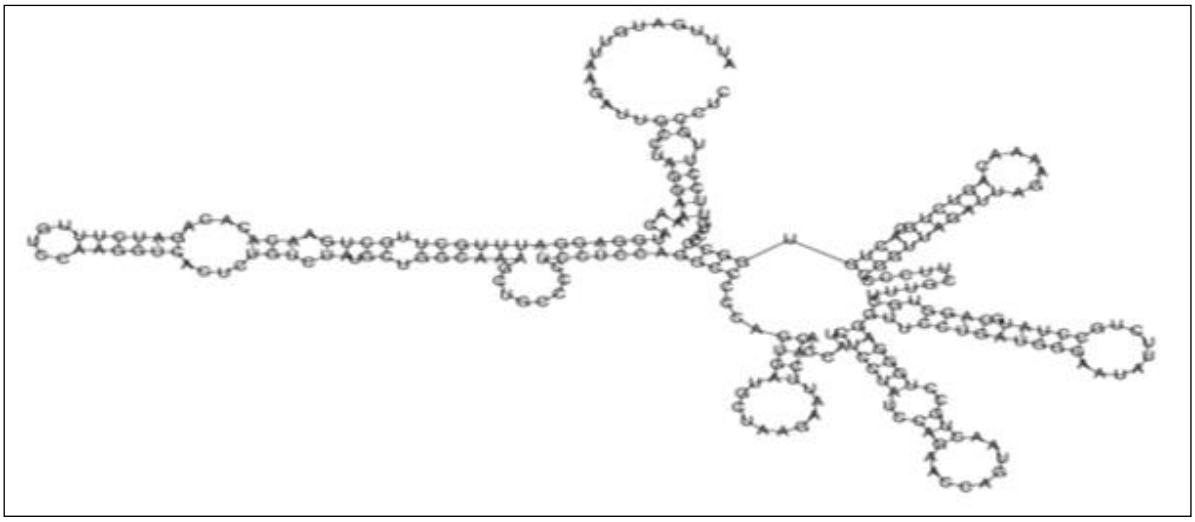

Fig. 7: The predicted RNA secondary structure for NCBI Ref Sequence. MFE = -69.72 kcal/mol.

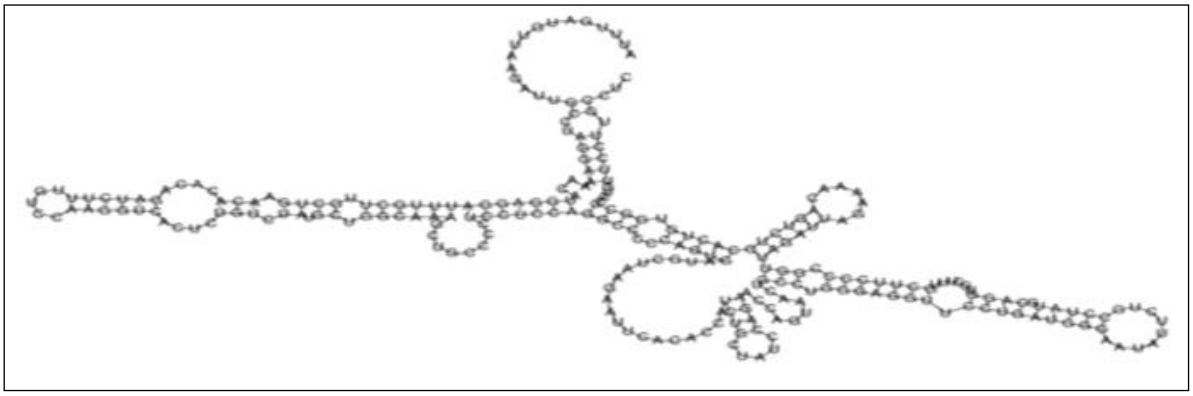

Fig. 8: The predicted RNA secondary structure for normal and diabetic sequences with rs1083096 only. MFE = - 70.94 kcal/mol

DISCUSSION

Diabetes mellitus has progressed at alarming rates and is one of the most pressing and significant diseases affecting human health. This disease is characterized by abnormalities in the metabolism of carbs, lipids, and proteins that result in hyperglycemia or an increase in blood sugar levels **(**Dilworth *et al.*, 2021**).** 90 % of diabetic cases worldwide fall under the category of T2DM making it the most predominant type of the disease. Multiple interacting factors, including genetic and epigenetic factors, in addition to environmental factors, can participate in the development of T2DM **(**Song *et al.*, 2022). Along with the global growth in T2DM, there is a massive progression in the expansion of early-onset type 2 (EOD) diabetes mellitus which is diagnosed in people under the age of 40 **(**Kong *et al.*, 2020). EOD is linked to a higher risk of several diabetes-related complications **(**Lascar *et al.*, 2018), and patients with EOD were found to experience a higher risk for macrovascular and microvascular complications **(**Dart *et al.*, 2014), cardiovascular disease, and microalbuminuria **(**Piko *et al.*, 2020), and pancreatic beta cell loss than patients with the typical onset of the disease **(**Kong *et al.*, 2020).

In mammals, melatonin has been found in various investigations to suppress insulin release **(**Tuomi *et al.*, 2016). Melatonin has multiple functions in mammals, including the regulation of 24-hour behavioral rhythms (such as the sleep-wake cycle), reproductive cycles in seasonally

breeding animals, retinal function, thermoregulation, and sleep (Nikolaev *et al.*, 2021). The MTNR1B gene on human chromosome 11q21–22 encodes for the MT2 receptor (Nikolaev *et al.*, 2021). Several genome-wide association studies (GWAS) have linked the rs10930963 variant in the intron of the MTNR1B gene to higher fasting glucose levels and T2DM (Bouatia-Naji *et al.*, 2009; Prokopenko *et al.*, 2009; Patel *et al.*, 2018; Arikoglu *et al.*, 2021). According to reports, the intronic SNP rs10830963 of the MTNR1B gene induces upregulation of the MTNR1B gene, resulting in an increase in mRNA levels and expression of the MT2 receptor in pancreatic beta cells, which further inhibits insulin release (Saki *et al.*, 2020). The MTNR1B gene locus contains a large number of forkhead box A2 (FOXA2) binding sites, which suggests that FOXA2 and/or other transcription factor proteins can bind to an enhancer region (Gaulton *et al.*, 2015). Moreover, it was also clarified that the rs10830963 favorably binds the transcription factor protein NEUROD1 in the EndoC-BH1 human fetal beta-cell line, whereas the rs10830963 establishes a consensus binding site for NEUROD1 (Andersson *et al.*, 2015; Ravassard *et al.*, 2011). Consequently, in EndoC-BH1 cells, the area surrounding the rs10830963 and embracing the G allele displays enhanced enhancer activity. The initial studies performed to investigate the association of the rs10830963 and T2DM recruited participants of the population with younger age.

In the current study, the common risk G allele was identified in four diabetic individuals, one male and three females, out of a total of 12 diabetic participants with an average age of 35.17, suggesting that the G allele may be a genetic cause in these individuals who are<40 years old and are therefore classified as EOD patients. Another SNP was identified at position 249, where a C nucleotide replaces a T nucleotide in one of the people with the risk G allele. Therefore, it is possible that rs10830963 may not be the only genetic cause for EOD in this individual. Consequently, a whole intron sequencing of the MTNR1B gene should be performed to detect additional SNPs that may or may not interact with the common SNP rs10830963, and which in turn may play a role in T2DM or EOD.

The link between the common SNP rs10830963 of the MTNR1B gene and EOD had not been previously examined in Egyptian adults under 40 years of age. We were unable to conduct the statistical analysis required to support or refute our hypothesis regarding the relationship between rs10830963 and EOD because of the small sample size. Therefore, larger-scale replication studies involving the Egyptian population should be conducted in the future. Nonetheless, a prior investigation on a sample of the Egyptian population, where the average age of diabetics was 52.90 years, supported the link between the G allele and T2DM, where the risk G allele was positively correlated with FBS and HbA1c levels (Heshmat *et al.*, 2014).

The sequence of either the diabetes or control sample including the risk G allele has an RNA secondary structure with a lower MFE (-70.94 kcal/mol) than the reference sequence lacking the risk G allele (-69.72 kcal/mol). RNA secondary structures with low ΔG-calculated free energy exhibit more base-pairing interactions than sequences with the same length and higher ΔG values (Soemedi *et al.*, 2017). Therefore, more stable RNA sequences possess lower ΔG values. Therefore, we hypothesize that the stability of RNA transcripts encoded by a sequence including the risk G allele is greater than that of transcripts without the risk allele.

RNA secondary structures with a greater MFE are clearly less thermodynamically stable and splice more efficiently than those with a lower MFG (Soemedi *et al.*, 2017). This suggests that the RNA transcript encoded from the DNA sequence of MTNR1B gene having the risk G allele splice less efficiently than the RNA transcript encoded from the DNA sequence with the non-risk C allele. Consequently, the correlation between the risk G allele and T2DM may be attributable to differences in

splicing efficiency. However, we recommend additional research on the splicing efficiency of the MTNR1B gene to establish the relevance between the rs10830963 and T2DM.

Conclusion

This study reveals a probable linkage between the MTNR1B gene variant rs10830963 and EOD in our sample of the Egyptian population.

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