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Explore the role of miRNA155 and IL-2 level in type-1 diabetic disease

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Abstract

Type 1 Diabetes Mellitus (T1DM) is a chronic progressive autoimmune disease characterized by destruction of insulin-producing beta cells in the pancreas. The immune system plays a critical role in this illness, particularly regarding micro ribonucleic acid (miRNA) expression and cytokines levels. This study aimed to investigate the role of miRNA-155 and interleukin-2 (IL-2) in diagnosis of T1DM with related to antibodies against glutamic acid decarboxylase 65 (anti-GAD65) and Connecting peptide (C-peptide). This case-control study involved 120 participants, of whom 80 were T1DM patients and 40 apparently healthy subjects as controls. The patients' age ranged from 3 to 17 years of both sexes, collected from the Department of Diabetic in Al-Sader medical city in AL- Najaf Al-Ashraf province during October 2023 till February 2024. Their diagnoses were made based on clinical and serological parameters. Blood samples were collected from all participants to detect IL-2 serum level by an enzyme linked immunosorbent assay and miRNA155 by the reverse transcription polymerase chain reaction (RT-PCR), whereas anti-GAD65 and C-peptide diagnosis by a chemiluminescence immunoassay. The results showed that serum IL-2 was significantly lower in T1DM patients (330.66 ±129.92 ng/ml) compared to the control group (960.67 ±188.05 ng/ml). The expression of miR-155 was significantly higher in the patients' group (2.61 ± 1.17) versus the control group (1.0 ± 0.71) ($p< 0.001$). The serum level of anti-GAD antibodies among patients with T1DM was significantly higher than in controls (375.01 U/ml vs 5.66 U/ml). While the serum level of C-peptide in the patients was lower than in controls. In conclusion, the elevated expression of miRNA-155, along with significantly reduced blood IL-2 levels in T1DM patients indicates their potential as valid biomarkers for the diagnosis and progress of T1DM.

Keywords: T1-DM, miRNA155, IL-2, anti-GAD65, C-peptide.

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Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune endocrine breakdown of pancreatic β-cells that produce insulin, which lowers insulin production and causes metabolic dysregulation leading to hyperglycemia. This causes long life insulin dependent injection with increased risk of complication as vascular damage throughout the body leading to nephropathy, retinopathy and neuropathy.[1,2] Diabetes mellitus (DM) emerged as a major global public health concern with more than 463 million people worldwide have diabetes and expected to rise to 578 million by 2030, with multifactorial causes like infection, genetic predispose and environmental factors.[3,4]

Autoimmune disorders are incurable. Although they are chronic illnesses, many of them are treatable.[5] Immune pathogenesis of autoimmune diseases related to a breakage in the central and peripheral tolerance, persistence T-lymphocytes activation, and imbalance between T-helper1 and T-helper2 inflammatory response with the production of cytokines, leading to the progressive destruction of the β- cells and the gradual loss of insulin.[6]

Connecting peptide (C-peptide) is released into the bloodstream when proinsulin is cleaved to form insulin. It performs important functions in diabetes and metabolic health. However, C-peptide is used to assess endogenous insulin secretion in patients with T1DM and to differentiate the type of DM.[7] Autoantibodies are considered important biomarkers to diagnosis autoimmune disease but are almost observed in late stages of disease so there is urgent need to seek other or additional biomarkers for early diagnosis.[8]

Interleukin-2 (IL-2) a cytokine plays a crucial role in the immune system by promoting growth, proliferation, and differentiation of T cells towered CD4 $^{+}$Th-cell. It also enhances natural killer cell activity and is involved in differentiating T regulatory cells (Tregs) which help maintain immune tolerance and prevent autoimmune reactions, but it arrests progression toward the Th17 cells.[9] IL-2 plays important roles in pathogenesis and is considered as a marker of inflammatory response of T1DM.[10]

MicroRNA (miRNA) is an epigenetic factor involved in regulating several processes, such as immune cell maturation and functions, organ development, cell proliferation, differentiation, death, and signal transmission.[11] MicroRNA-155 is a member of the miRNA's family. It regulates gene expression by binding to the three prime untranslated region (3'UTR) of target mRNA. MicroRNA-155 has a critical role in both innate and adaptive immunity.[12] MicroRNA-155 is abnormally expressed in inflammatory autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, diabetes mellitus type 1and inflammatory bowel disease.[13] Consequently, the current study aimed to investigate the role of miRNA-155 and IL-2 in diagnosis of T1DM in relation to antibodies against glutamic acid decarboxylase 65 (anti-GAD65) and C-peptide.

Subjects and Methods

This case-control study involved 120 participants. Of these, 80 participants were T1DM patients and 40 normal controls. Patients' age ranged from 3 to 17 years and were from both sexes. The patients were collected from the Diabetic Center in Al-Sader medical city in AL- Najaf Al-Ashraf province, Iraq from October 2023 till February 2024. Their diagnoses were made based on clinical and serological parameters.

Sample collection and laboratory assessments

A venous blood sample (3 ml) was collected from each patient and control subject. Of this, an aliquot blood sample (2.5 ml) was placed into a gel tube for serum separation, used for measuring IL-2 level by ELISA kits (BT-Laboratory, China), according to the manufacturer's instruction. C-peptide and Anti-GAD were evaluated by fully automated chemiluminescence immunoassay system (Meglumi, X3 analyzer, China), according to the manufacturer's instruction. A second aliquot blood sample (0.5 ml) was transferred into an Eppendorf tube containing 0.5 ml triazole and immediately stored at -80 °C until used for assessment of miRNA-155 by the reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from whole blood using commercial kits (TransZol™ miRNA, Trans, China), following the manufacturer's protocol. Primers for miR-155 and U6 calibrators were designed by a biotechnology company (Macrogen Inc., Korea). The sequence of the miRNA-155 primers was

F: GTGGCACAAACCAGGAA.
R: GTTGAACATCCCAGTGACCAG.

The expression of U6, as an internal control, was used for the normalization of miRNA expression. The first Step of the RT-qPCR reaction was prepared according to Promega company and the thermocycling conditions are shown in Table 1.

Table 1. One step reverse transcription quantitative polymerase chain reaction (RT-qPCR) programs.

Step	Temperature	Duration	No of Cycles
Reverse transcription	37℃	15 min.	1
RT inactivation/ Hot-start activation	95℃	10 min.	1
Denaturation	95℃	10 sec	
Annealing	58℃	30 sec.	50
Extension and data collection	72℃	30 sec.	

Calculations:

Gene expression or gene fold (Relative quantification) value was calculated according to the method described by Mansor and Alammar.[14]

Relative quantification $_{(RQ)} = 2^{-(\Delta\Delta CT)}$.

At first, we determined the gene fold for each triplicated sample by obtaining the CT (cycle threshold) average value from the real-time PCR equipment. Next, we computed the ΔCT value for each sample in the following manner:

Δ CT = CT (tested miRNA155, miRNA181a) – CT (reference gene U6)

ΔΔ CT = Δ CT (tested sample) – Δ CT (reference gene)

Fold gene expression RQ = $2^{-(\Delta\Delta CT)}$

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20. Data are expressed as means ± standard deviation (SD). Statistical analyses were performed through an independent T-test, one-way ANOVA, the receiver operating characteristic (ROC) curve analysis. $p< 0.05$ was considered significant.

Results

Demographic Distribution of T1DM patients and control subjects

This case-control study involved 120 participants. They were 80 T1DM patients and 40 controls. The female: male sex ratio was 50% for both groups. The age range of patients was between 3-17 years with mean age (11.19± 3.58 years) and the highest age between 9-14 years. The results showed no significant difference between patients and controls, as shown in table 2.

The results showed that 100% of patients had Anti-GAD positive results with higher mean titer of 375.01 U/ml. While all controls had negative results for anti–GAD IgG autoantibody with mean titer of 5.66 U/ml, as shown in Table 3. This work confirmed that 100% of patients had significantly lower C-peptide serum levels with mean titer of 0.64±0.29 ng/ml than controls, who gave 98% negative results (2.91±0.73 ng/ml), ($p< 0.001$), as shown in Table 4.

Table 2. Distribution of study subjects according to gender, age and duration of disease

Variable	Type 1 Diabetes Mellitus patients n (%)	Control group n (%)
Sex		
Male	40 (50)	20 (50)
Female	40 (50)	20 (50)
Age		
Age years (mean)	11.19± 3.58	10.0 ± 2.84
Age years (range)	3-17	3-16
3-8 years	20 (25%)	10 (25%)
9-14 years	40 (50%)	25 (62.5%)
15-17 years	20(25%)	5 (12.5%)
Total	80(100%)	40 (100%)

Table 2. Continued.

Variable	Type 1 Diabetes Mellitus patients n (%)	Control group n (%)
Duration of Disease		
Mean	4.95±3.21	
Range n (%)	Under 5 years 60 (75%) Upper 5 years 20 (25%)	

Table 3. Mean titer and frequency of Anti –GAD IgG in T1DM patients and controls.

Parameters	Anti–GAD IgG			*p* value
	Positive (≥ 10 IU/ml)	Negative (<10 IU/ml)	Mean	
T1DM	100%	0%	375.01±238.43	<0.05
Controls	0%	100%	5.66±1.95	

$p \leq 0.05$ is significant.

Table 4. Frequency and the mean titer of C-peptide in T1DM patients and controls.

Parameters	C-peptide				*p* value
	Lower than normal >101 ng/ml	Normal ng/ml	Higher than normal 404 >ng/ml	Mean	
T1DM	%100	0%	0%	0.64±0.29	<0.05
Controls	0%	98%	2%	2.91±0.73	

$p \leq 0.05$ is significant.

Estimation the level of IL-2 in T1DM patients and controls

The results also showed that the concentration of IL-2 in the controls (960.67 ±188.05 ng/ml) was significantly higher than in the T1DM patients (330.66 ±129.92 ng/ml) ($p < 0.001$) as shown in Figure 1. The ROC curve analysis showed that IL-2 at area under the curve (AUV) value of 1.0, showed 100 % sensitivity, and 100 % specificity, as shown in Table 5 and Figure 2.

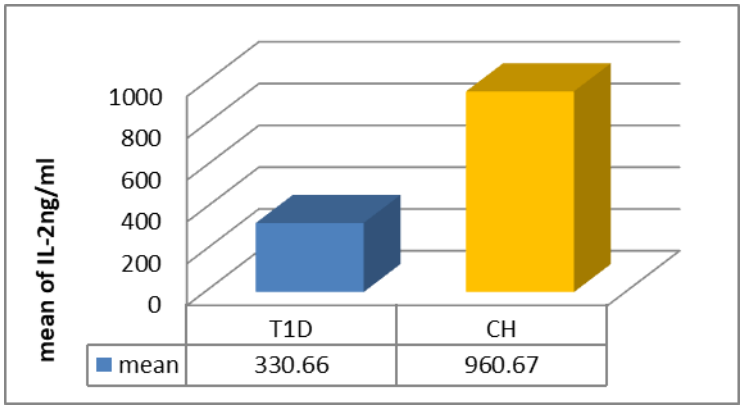

Figure 1. Interleukin 2 (IL-2) Levelin Type 1 Diabetes Mellitus (T1DM) patients and controls.

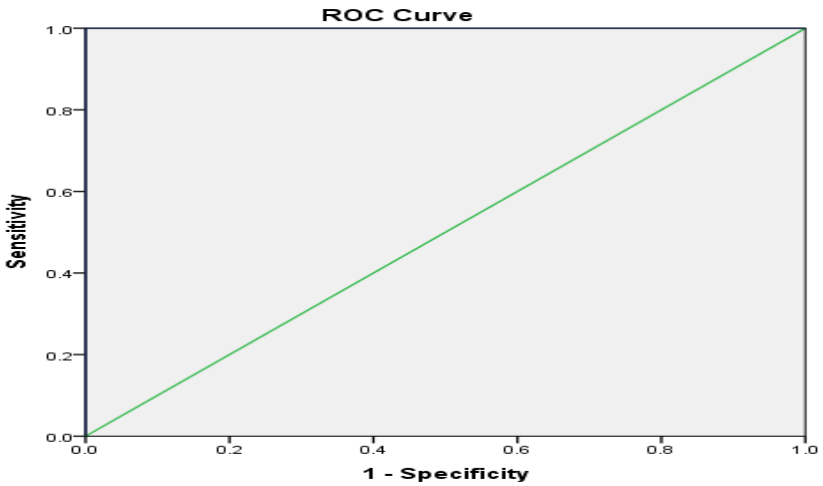

Figure 2. Receiver operating characteristic (ROC) curve analysis of interleukin 2 (IL-2) in Type 1 Diabetes Mellitus (T1DM) patients versus controls.

Table 5. Sensitivity and specificity of interleukin 2 (IL-2) between Type 1 Diabetes Mellitus (T1DM) and controls.

Area under the curve	*p* value	Asymptomatic 95% Confidence interval		Cutoff	Sensitivity	Specificity
		Lower bound	Upper bound			
1.00	0.001	1.000	1000	708.64	100%	100%

*$p \leq 0.05$ is significant.

Estimation of interleukin 2 (*IL-2) serum level in* T1DM *Type 1* (T1DM) *diabetic patients according to the duration of disease.*

The current study showed that IL-2 had significant differences between T1DM patients of more than 5 years and less than 5 years and controls. The IL-2 concentration decreased significantly in T1DM patients of more than 5 years (298.29 ± 87.59 ng/ml), than in less than 5 years (631.70 ± 77.38 ng/ml) and controls (960.67 ± 188.05 ng/ml), as shown in Figure 3.

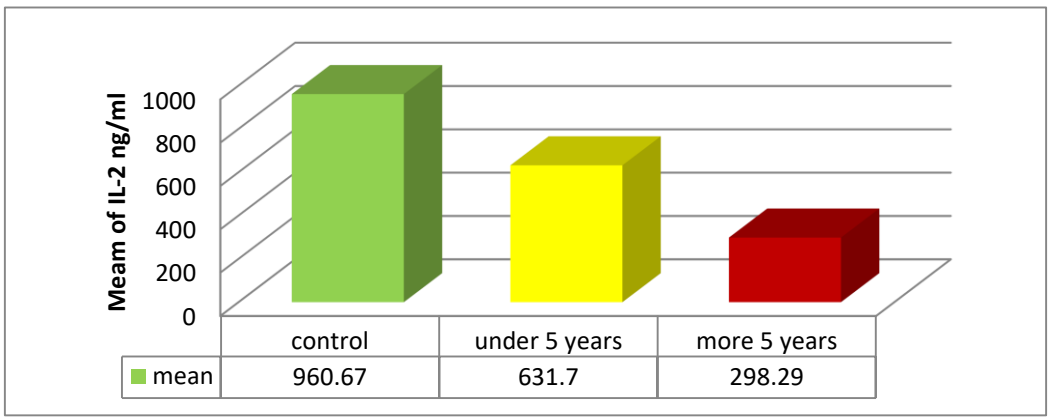

Figure 3. Serum level of interleukin 2 (IL-2) in Type 1 Diabetes Mellitus (T1DM) according to the duration of disease.

Expression of miRNA-155 in Type 1 diabetic patients and controls

The expression of miR-155 gene was significantly higher in the patient group (2.61 ± 1.17) compared to the control group (1.0 ± 0.71), (p< 0.001) as shown in Figure 4 and Figure 5. The appropriate cut-off value of miR-155 was 1.166, which at AUC of 0.91 had 100 % sensitivity, and 80 % specificity (p=0.001), as demonstrated in Table 4 and Figure 7.

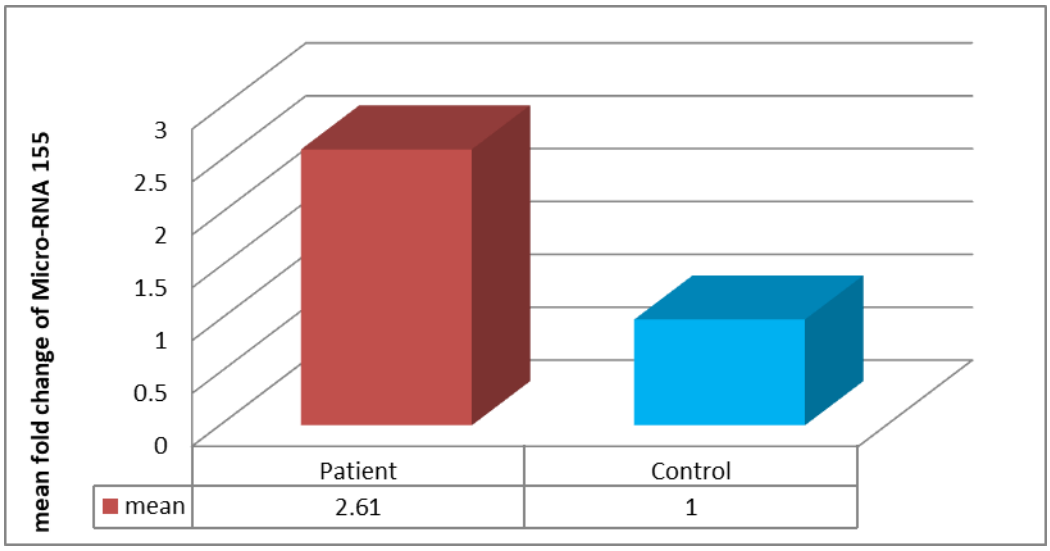

Figure 4. Expression of miRNA-155 in Type1 Diabetes Mellitus (T1DM) patients and controls.

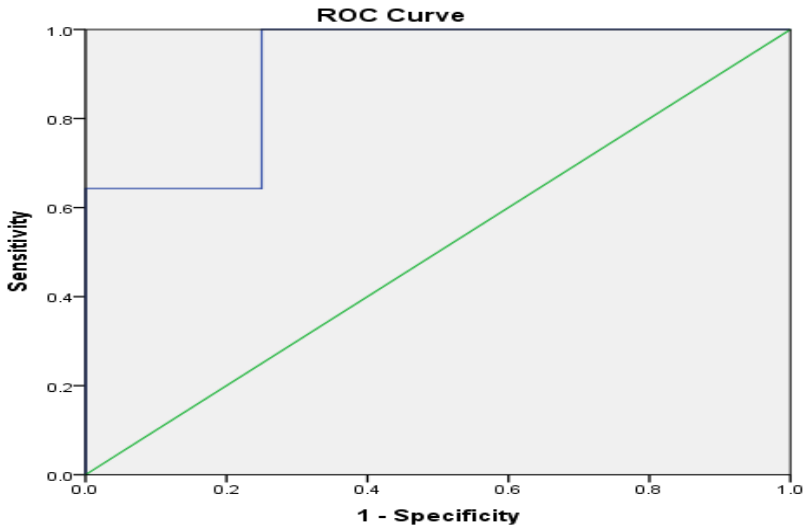

Figure 5. Receiver operating characteristic (ROC) curve analysis of MicroRNA-155 (miR-155) in Type1 Diabetes Mellitus (T1-DM) patients versus controls.

Expression of miRNA-155 in patients with T1DM according to duration of disease

The results showed significant up-regulation of miR-155 gene expression in patients of less than 5 years (1.871 ± 0.873) and more than 5 years (2.862 ± 1.151) compared to controls (1± 0.716), (p<0.05) as demonstrated in Figure 6.

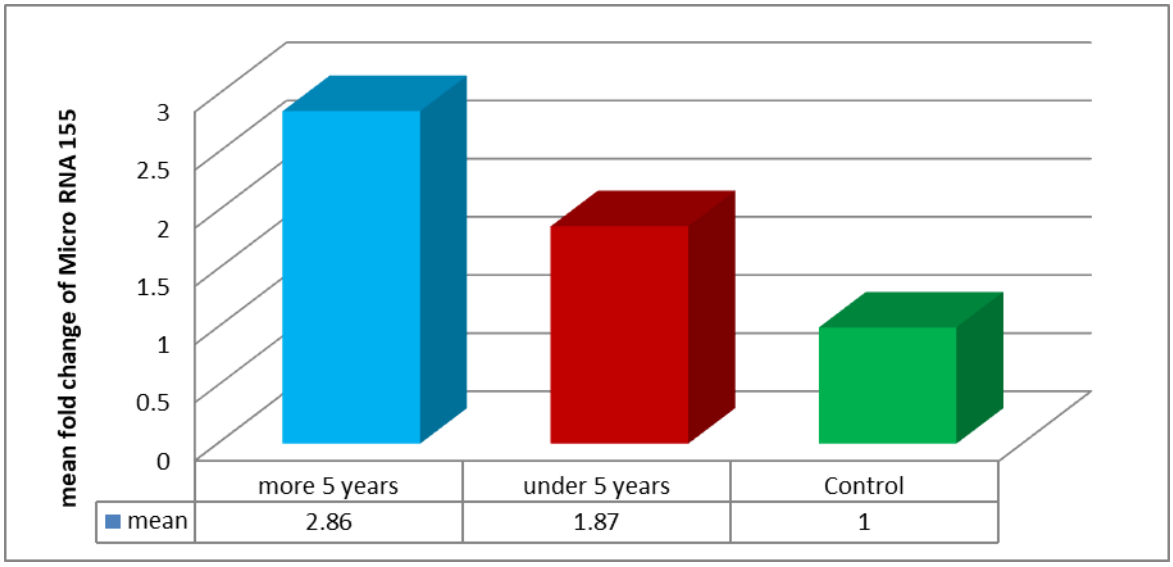

Figure 6. Mean fold change of miR-155 according to the duration of Type 1 Diabetes Mellitus disease.

Table 6. The sensitivity and specificity of miRNA-155 between Type 1 Diabetes Mellitus (T1DM) patients and controls.

Parameter	AUC	*p*-value	Cut off	Sensitivity	Specificity
miRNA-155	0.91	0.001	1.166	100%	80%

p≤0.05 is significant.

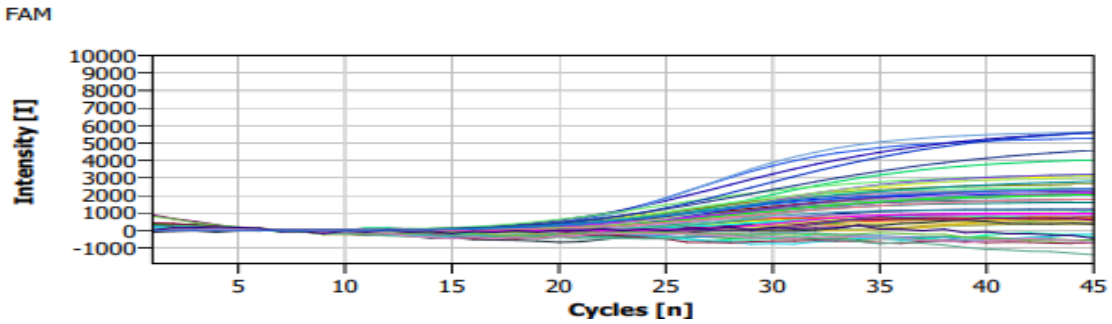

Figure 7. Real time PCR image showing cycle threshold (Ct) values of microRNA -155.

Discussion

The present study aimed to investigate the role of miRNA155 and IL-2 in diagnosis of T1DM in relation to anti-GAD65 and C-peptide. The results of this work indicated a number of philosophical and practical applications. The rising incidence of T1DM is a serious medical condition that affects millions of children and adolescents and requires lifetime care, including insulin therapy, restricted diet, and regular blood glucose assessment which needs further research. The chronic nature of the disease affects people's quality of life as well as healthcare systems. Early intervention and therapy may be possible by discovering biomarkers for early identification and illness progression through research. Additionally, knowledge of immunopathology may result in the creation of immunotherapies that enhance patients' outcomes and quality of life.

This study found that the number of females equals males. The mean age of the T1DM patients was 11.19± 3.58 years and ranged 3-17 years. The observation of this work is consistent with previous findings, which indicated that 64% of the patients aged ≤ 10 years with mean age at diagnosis of 5.22± 2.51 years with a range of 2-11 years.[15] In 2023, similar results were obtained from a local Iraqi study in the Diabetic Center in Al-Sader medical city /AL- Najaf Al-Ashraf province, found that male: female were equal but males exhibited more severe clinical manifestations. They concluded that sex hormones (estrogen and testosterone) can affect the immune function, metabolism, and the risk of disease.[16]

The current results are in line with a prior study, which observed that T1DM disease is often linked to anti-GAD which act as an indicator of autoimmune attack on the pancreatic beta cells and correlated with a more severe form of the disease and a faster progression to insulin dependence islet cells.[17] Also, another study compared between T1DM patients less than and more than 10 years, and observed that the positive anti-GAD antibodies and insulinoma-associated protein 2 autoantibody (IA-2A) were 89.04% and 38% among the studied T1DM patients, respectively, and the mean of anti-GAD was correlated with age and the development of disease. The mean concentration of anti-GAD65 was 69.03±46.14 IU/ml, and the age group of 6-10 years has a mean of 50.21 ±42.07 IU/ml, while patients with age group 1-5 years had the highest mean concentration of 82.04 ±55.48 IU/ml indicating that this autoantibody is correlated with disease progression.[18]

C-peptide plays a crucial role in insulin production and can be used as a direct indicator of B-cell damage caused by autoimmune processes. Findings of this work are in line with a previous study, which found that C-peptide level indicated how much insulin is naturally produced and illustrated that the median interquartile range (IQR) C-peptide concentration was 0.59 nmol/l and C-peptide concentration of ≤0.16 nmol/l showed 92.9% sensitivity, and 1-specificity suggesting a good marker of blood insulin levels and pancreatic cell activity.[19]

The results of the current study are in line with the hypothesis of our study that miRNA155 and IL-2 may have role in diagnosis of T1DM. IL-2 serum level decreased in T1DM patients. This cytokine has an essential role in regulating and activating immune responses by maintaining the function of regulatory T cells (Tregs). This observation is also consistent with the findings of a prior search which concluded a significant decrease in the mean concentration of IL-2 among T1DM patients compared to controls and confirmed that lesions associated with reduced Tregs frequency and induce pro-inflammatory gene expression in T1DM.[20]

Decreased IL-2 concentration is identified as contributors to breakdown of central and peripheral tolerance, which led to development of diabetes by lowered Tregs frequency.[21] Another study found that decreased IL-2 and insulin-like growth factor 1 level lead to development of diabetes disease and when treated with low dose IL-2 lead to clinical remission by preservation of C-peptide level, decreased level of HbA1c, decreased insulin requirement, normal blood glucose, absence or reduced adverse events including vascular leak syndrome.[22] Tregs play a major role in

controlling effector T cells by responding to self-antigens to prevent T1DM autoimmune diseases. It now appears that activating Tregs with low-dose IL-2 could provide immune regulation without immune suppression in T1DM.[23]

This study estimated the impact of epigenetic processes on T1DM such as miR-155 that may be involved in autoimmunity. These outcomes concur with other studies, which showed that the overall miR-155 expression was significantly higher in T1DM patients than in controls.[24] Also, the basal expression of miRNA-155 is elevated in T1DM patients compared to control subjects.[25] miRNA-155 expression has negative correlation with level of C-peptide, which is involved in insulin production and suggest that miR-155 is associated with progressive TIDM autoimmunity.[26] Serum miRNA-155 was found to be considerably overexpressed in T1DM patients with fold change 2.1 compared to controls and confirmed that overexpression of miRNA-155 in CD4 and lamina propria T cells (LPT) decreases IL-2 concentration.[27] MiRNA-155 stands out as an immune-associated miR-155 that is up regulated in response to multiple stimuli and in multiple immune cells such as Toll-like receptor ligands, inflammatory cytokines, and specific antigens in monocytes, macrophages, B- cells, and T cells.[28] A previous study observed that miRNA-155 regulates IL-12 and IL-18 mediated interferon-γ (IFN-γ) production by binding to and down-regulating inositol polyphosphate 5-phosphatase 1 (SHIP1) mRNA and observed a negative correlation between expression of miRNA-155 and IL-2, IFN-γ serum level.[29]

In conclusion, this study illustrates that anti-GAD65 and C-peptide measures with miRNA155 and IL-2 levels, can assist in the diagnosis of T1DM. These results highlight how crucial it is to evaluate immunological and metabolic markers for diagnosis and detection of disease progressive.

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Author Contributions

ZAAT, collected the data and wrote the draft of the manuscript. MFD, proposed the topic of this research and designed the study, and revised draft of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine, University of Kufa (reference no. HK/1070, dated 15/10/2023).

Informed consent

An informed written consent was obtained from each study subject before included in the study.

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