Original Article

The Relationship of Circulatory miRNA-21 Expression with IL-15 Level in Celiac Disease Patients in Karbala Province

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Abstract

Background: Celiac disease (CeD) (celiac sprue) is a condition of immune-mediated disorder characterized by inflammation of the small intestine as a result of gluten protein ingestion in susceptible patients (HLA-DQ2/DQ8 positivity). MicroRNAs (miRNAs) are a type of ribonucleic acids presented as small non-coding single-chain ribonucleic acids, which hinder different types of messenger RNAs (mRNAs) translation, hence silencing their target genes. Upregulation of miRNA expression and its role in immune system is contributed to a wide range of diseases. **Objectives:** Evaluating the level of miRNA-21 and its relation to the proinflammatory cytokine (IL-15) in CeD patients, and determine their role as biomarkers for disease diagnosis. **Materials and Methods:** A total of 100 patients of both genders and various ages diagnosed positively with CeD who attended the OesophagoGastroDuodenoscopy unit. The patients included both males and females of different ages. Their biopsy results were confirmed with serological testing. In addition, this study included 100 participants who were in good health as control group. **Results:** CeD is more common in females (60%) than in males (40%). ELISA was used to measure the concentration of IL-15 in CeD patients which was significantly higher in about (10.100 ng/mL), while the control group recorded (0.365 ng/mL) ($P \leq 0.001$). The expression of miRNA-21 was very highly statistically significant up regulated in CeD patients compared to control ($P \leq 0.001$). Receiver operating characteristic curve (ROC) measurement as follows: cutoff value was 8.763, area under the curve 0.922, sensitivity 0.922%, specificity 0.955%, and (CI 95% 0.453–0.814). **Conclusion:** IL-15 levels are higher in CeD patients which can be considered as a hallmark for CeD diagnosis. miRNA-21 expression was higher in CeD patients, the ROC curve was done for miRNA-21 in serum for early diagnosis of the disease because it shows good sensitivity and specificity. Finally, these results revealed that IL-15 and miRNA-21 can be a promising biomarker for CeD diagnosis.

Keywords: Autoimmune disease, celiac disease, IL-15, miRNA-21

Introduction

The celiac disease (CeD) can be defined as a chronic autoimmune condition occurs as a result of protein termed gluten, characterized by enteropathy of small intestine in persons with genetic susceptibility (HLA-DQ2/DQ8). CeD is diagnosed based on certain tests (serologic) which include IgG-DGP, IgA-TG2 or IgA-EmA, confirmation of these tests accomplished with histological changes such as enteropathy that consists of increased intraepithelial lymphocytes (IELs) present in the biopsies of duodenum, crypt hyperplasia, and villous atrophy.[1]

CeD is a T-cell-mediated intestinal disease, causes chronic irritation of the upper small intestine because of intolerance against gluten protein. It is more common in genetically susceptible people. Western nations consume large amounts of gluten protein on a daily average (10–20 g per person).[2] Gluten is the most significant environmental component in CeD. Gluten peptides pass through the epithelial layer and undergo deamination by the enzyme tissue transglutaminase 2. There are specific molecules on antigen-presenting

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cells named as (HLA-DQ2/8), in which deaminated gluten peptides attached with, resulting in $CD4^+$ T lymphocytes gluten-specific activation as a consequence the proinflammatory mediators were secreted, including tumor necrosis factor, TFN γ, IL-2, IL-15, and IL-21 that are specific to gluten. The immunological reaction therefore led to the inflammation and damage to the gut tissue.[3] Environmental factors in addition genes at non-HLA loci must be implicated for disease development, even if the existence of DQ2/DQ8 genotypes is necessary but insufficient on its own. About 65% of the genetic vulnerability in the progress of CeD comes from non-HLA genes, while susceptibility of DQ2/DQ8 accounting for 35%.[4] Gluten-specific $CD4^+$ T lymphocytes response against gluten consumption appear with the production of IL-2, IL-15, as well as the inflammatory factors that cause gastrointestinal signs such as nausea and vomiting.[5] In healthy subjects, IL-15 is expressed only on villous enterocytes and almost absent in the lamina propria. In contrast, in inflammatory conditions, it is over-expressed by enterocytes and lamina propria mononuclear cells.[1]

Interleukin-15 is a proinflammatory cytokine present on surface of all cell types, that is, over-expressed under stress or inflammation.[6] It is also part of the family of four α helix bundle of cytokines, known as interleukin 21, interleukin 9, interleukin 7, interleukin 4, and interleukin 2.[7] IL-15 produced by different spectrum of cells, for example, intestinal epithelial cells (non-hematopoietic cells); their argument was enhanced in the gut milieu under infection or tissue stress.[8]

IL-15 over-secretion occurs nearly in all CeD patients as IL-15 can interfere with the activation of IELs as enhances intestinal mucosal permeability. IL-15-activated IELs in celiac patients act more like NK cells than conventional antigen-specific T cells; under the influence of further activation the IELs induce direct injury to the mucosal cells.[9]

MicroRNAs (miRNAs) currently encompass one of the most progressive concepts in the field of novel diagnostics and follow-up biomarkers. They are non-coding ribonucleic acids (RNAs) of 19–24 nucleotides playing significant effect in post-transcriptional gene regulation. They can be detected in various body fluids and organ biopsies.[10] miRNAs constitute a type of prospective biomarker because of their function in the diagnosis and treatment of various digestive illnesses, such as CeD, therefore helping in better understanding the pathogenetic pathways of CeD.[11]

In current study, ELISA performed to assess IL-15level in CeD patients as well as qRT-PCR technique which also used to assess the different levels of expression of miRNA-21 in CeD patients and controls beside to receiver operating characteristic curve specificity and sensitivity to investigate its capability of miRNA-21 as biomarker.

Materials and Methods

Subjects

The clinical samples were gathered from individuals attending the Karbala Center for Gastroenterology and hepatology, 250 patients enrolled in this study, 100 healthy as well as 100 control and 50 clinical samples were excluded, in Imam AL Hussein medical city in Karbala Province. All the participants met the criteria to be included in this study (patients 3–45 years old, who attended gastrointestinal tract unit and were diagnosed with CeD by specialists). Diagnosis was done based on the clinical features, serological markers, and histopathological pictures of CeD in duodenal mucosa all together according to the current international guidelines.[12] While any age bellow 3 years or if there any suspicion in the family history for CeD, if the person was on GFD or if there was any autoimmune disease other than CeD were excluded criteria. The patient subject and controls were matched as possible as could by age and gender.

Ethical approval

The study agrees with the ethics of the Training and Developing Center in Karbala province, in which hospitals samples were taken and all participants provided the needed approvals for the study be preceded at date January 8, 2023, number (004).

ELISA assay for determination of IL-15

The ELISA assay was performed according to the manufacturer's instructions. Hunan IL-15 (Interleukin-15) ELISA kit was supplied by Elabscience Biotechnology/ USA, catalog No: E-EL-H0222. Figure 1 clarifies the typical standard curve of human IL-15 concentration.

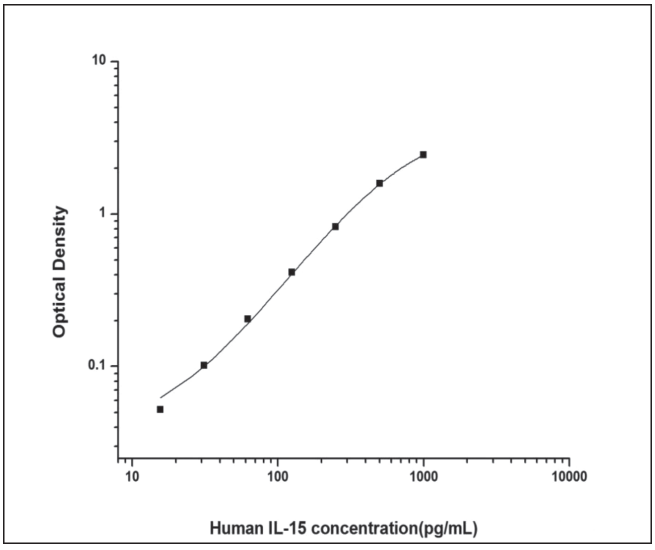

Figure 1: Typical standard curve of human IL-15 concentration

Ribonucleic acids purification

Gene expression analysis carried out using the reverse transcription polymerase chain reaction (RT-PCR) technique, it was necessary to isolate the RNA from samples. The validity and precision of gene expression assessed by RT-PCR and were totally determined by the quality, purity, and integrity of the starting genetic material.[13,14] RNA was isolated according to the manufacture's instruction of TRIzol™ Reagent. Thermo Scientific, USA, catalog No: 5346994. Determination of cDNA using Quantus Fluoromete, Promega, USA (cDNA concentration was 3.5–8 ng/μL).

Selection of reference genes

The criteria for determination of optimal reference gene were stability and expression across all samples,[15] the expression level almost high and further demonstrated convergence in expression levels across all samples. Hence, the best and longest target gene presented in the serum samples was chosen as endogenous control gene or housekeeping gene.

Real time polymerase chain reaction program

The PCR program used in this study is a two-step amplification response (reverse transcription quantitative polymerase chain reaction [RT-qPCR]) to assess miRNA-21 and U43 reference gene expression. All reagents were prepared as recommended by manufacture instruction of Macrogen, Korea, as shown in Table 1. The first step RT-qPCR reaction is shown in Table 2. The second step of RT-PCR is shown in Table 3, while as shown in Table 4 illustrated the PCR cycling program.

Calculating gene expression (gene fold)

Standard dilutions that caused errors while constructing the standard curve can be eliminated. Furthermore, the count of the relative gene between two treatment groups can be more interesting than the exact deoxyribonucleic acid/RNA molecular counts.[16] Thus, the most used strategy is relative quantification. Using the PfaffI equation gene expression values were calculated.

$$RQ = 2^{-(\Delta\Delta CT)} \quad RQ \text{ is relative quantification}$$

Gene fold was computed:

$$\Delta CT = CT \text{ (gene of interest)} - CT \text{ (reference gene) } CT \text{ (cycle threshold)}$$

ΔCT is the difference in CT values between the gene of interest and the reference gene in a given sample. This is necessary to normalize the gene of interest to a gene unaffected by the experiment.

Calculating ΔΔCT:

$$\Delta\Delta CT = \Delta CT_{(\text{treated sample})} - \Delta CT_{(\text{untreated sample (control)})}$$

After calculating ΔΔ CT of all samples, gene expression (fold change) calculated as follows:

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

Statistical analysis

The data of the study participants, including both CeD sufferers and the healthy group, were input, organized, and tested by (SPSS) version 25 software for Windows. The ANOVA F test is employed to compare the average levels of certain parameters. A significance *P* value of 0.05 or less can be considered statistically significant. Data and

Table 1: Primer preparation

Primer	Nuclease free water (μL) vol.	Concentration (pmol/μL)
miR-21-5p-RT	300	100
miR-21-5p-F1	300	100
RNU43_RT	300	100
RNU43_F	300	100
Universal reverse	300	100

Table 2: First step reverse transcription polymerase chain reaction

1st reaction	Volume per sample (μL)
RT primer	1
Ribonucleic acid	4
Volume (total)	5

Table 3: Gotaq 2-step reverse transcription quantitative polymerase chain reaction master mix

Component	Volume per sample
Goscript reverse transcriptase	0.25
dNTPs	0.5
Rnasin	0.25
$MgCl_2$	1
Goscript 5× reaction buffer	2
Nuclease free water	3.5
Volume (total)	7.5
Aliquot/single rxn	Second reaction mix (7.5 μL)/ tube then add template (5 μL)

Table 4: Realtime polymerase chain reaction program

Steps	Degree (°C)	Time	Cycle
Original denaturation	95	5 min	1
Denaturation	95	20 s	40
Annealing	55 (or) 62	20 s	
Elongiation	72	00:20 s	

findings were presented in tables and/or figures using the Microsoft Word program 2010 for Windows.

Results

This study included a total of 250 individuals, of which 100 clinical samples were positively diagnosed as CeD which included both males (four children and 36 adult males) and females (40 children and 20 adult females), age range of 3–45 years and a mean age of 2.75 years. In addition, 100 samples in the control group, while the 50 clinical samples were excluded because they do not match the study criteria, during the period from February to August 2023 in Imam Al-Hussain Teaching Hospital in Karbala Governorate. The current study indicated that CeD in females at a significantly higher ratio of CeD than males, with females representing 60% of the total CeD ratio while in male occur with 40%.

IL-15 serum concentration in CeD patients and control subjects

According to results of this study in accessing IL-15 concentration in the serum of CeD patients which indicated the excess secretion of IL-15, in which the patients group show higher level rather than in healthy controls, who did not show any elevation in cytokine concentration at *P* value ≤ 0.001. As shown, Table 5 represents the mean concentration of IL-15 in patients group as 10.100 ng/L, while in the control group was 0.365 ng/L.

The high percentage of IL-15 secretion appears in the age group of 33–42 years with a mean range of 65.25 ng/mL followed by the age group of 23–32 years. As shown, Table 6 illustrates the details of IL-15 secretion.

Concentration of miRNA-21 5p among CeD patients and controls.

miRNA-21 5p expression was significantly upregulated among CeD patients (fold change = 13.565), compared to control (fold change = 4.544), at *P* = <0.001, as shown in Table 7.

The ROS curve explain in details miRNA-21 test characters in which cutoff value was 8.763, area under the curve 0.922, sensitivity 0.922%, specificity 0.955%, and (CI 95% 0.453–0.814), as shown in Table 8 and Figure 2.

Association of microRNA-21 with IL-15 in CeD

Pearson correlation used in the study to determine the relationship between miRNA-21 in serum and IL-15 serum levels to assess their clinical application. This correlation indicates that there was a positive association between miRNA-21 and IL-15 in CeD patients, as shown in Figure 3.

Table 5: Mean concentration of IL-15 in patients and control group

IL-15	Mean ± SEM	*P* value
Patients	10.100 ± 0.411	<0.001
Control	0.365 ± 0.026	

Table 6: IL-15 secretion according to age group

Variable	Age	No	Mean rank	Chi-square	*Df*	*P* value
IL-15	3–12	44	41.05	13.319	4	0.01*
	13–22	5	36.60			
	23–32	20	63.95			
	33–42	12	65.25			
	43–52	19	52.58			
	Total	100				

*Significant result

Table 7: Mean expression of microRNA in patients and control

miR-21	Mean ± SEM	*P* value
Patients	13.565 ± 0.736	<0.001
Control	4.544 ± 0.580	

Table 8: The sensitivity and specificity of the test

Variable	Cutoff	AUC	Sen%	Spec%	Std. error	CI 95%	*P* value
MiRNA	8.763	0.922	0.955%	0.824%	0.042	0.453–0.814	0.000***

95% CI: confidence interval, AUC: area under the curve; values are significant at *P* ≤ 0.00

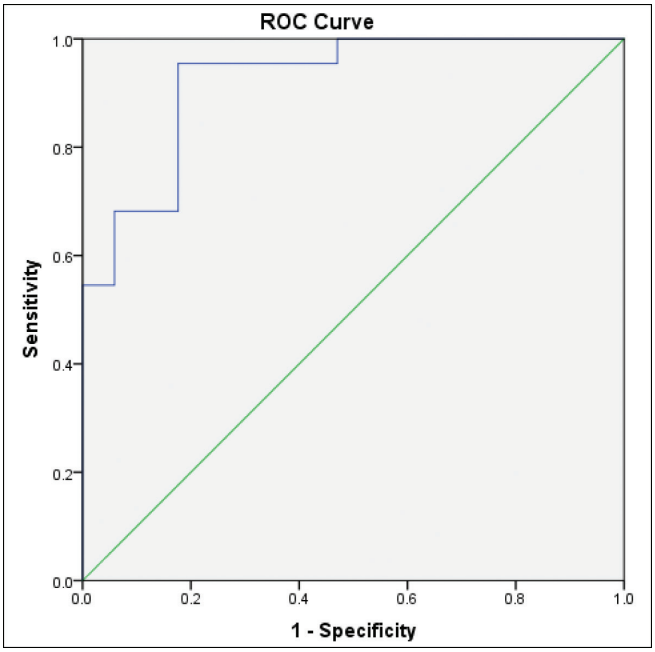

Figure 2: Receiver operating characteristic curve for microRNA-21-5p in celiac disease

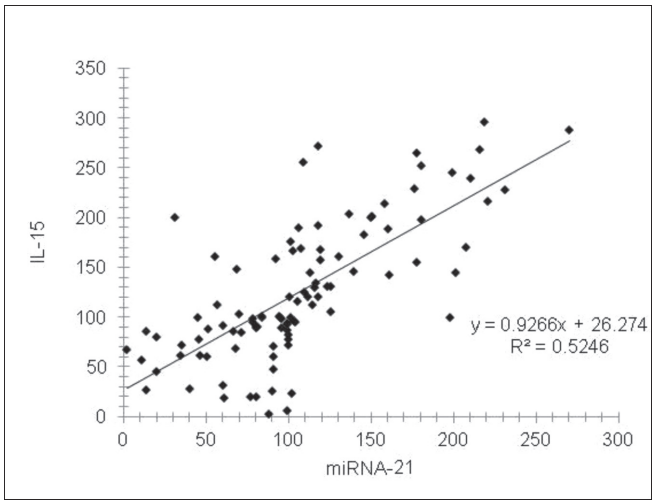

Figure 3: The correlation of microRNA-21 with IL-15

Discussion

miRNAs non-coding small single-chain RNAs, inhibit various mRNAs translation, hence silencing their own target genes. Because of their significance in the diagnosis and treatment of different absorption disorders, such as CeD, miRNAs may comprise a type of potential biomarker; hence, they could help better understand the disease's infectious pathways.[11]

As demonstrated by Pfaffl[17] who revealed that the proinflammatory cytokine (IL-15) is upregulated in CeD, which is triggered by gluten peptides. This causes the activation of Th17 and Th1 responses, disruption of intestinal immune homeostasis, the promotion of the NK-like phenotype in $CD8^+$ T lymphocytes, and different of other inflammatory consequences, whereas the critical function of miRNAs, which may be attributed to a type of short non-coding RNAs that engaged to complementary area of the target genes' 3′UTRs, resulting in translational inhibition or target gene destruction.[18]

The study conducted by Jabri and Sollid[19] indicated that IL-15, which is a hallmark for CeD, is upregulated in both the epithelial barrier and the lamina propria. Together, these cytokines orchestrate a cascade that leads to increased stress in intestinal epithelial cells, migration of CD^{8+} cells to the epithelial layer, and activation of B cells. On arrival in the epithelial layer, the CD^{8+} T cells (designated CD^{8+} IELs) are effectively "licensed to kill" epithelial cells, thereby damaging the villous structure of the small intestine (villous atrophy).

Other studies demonstrated that enterocytes and lamina propria mononuclear cells overexpress IL-15 in CeD,[20] and this was confirmed by another study, which considered IL-15 a hallmark and a basic blood marker of CeD.[21] Other research indicated the reasons for the previous studies on the connections between autoimmunity and miRNA function, which focused on the critical functions of miRNAs in immune cell formation and immune response regulation. There has been conjecture on the potential significance of miRNA-21-5p as a target in the avoidance and management of specific autoimmune disorders.[22]

There are numerous efforts employed to inhibit or reduce the effect of IL-15 signaling pathway in CeD patients because of the importance of IL-15 in GFD (non-responding) form of CeD (refractory CeD).[23]

While in the study of Park *et al*.[24] found that in rheumatoid arthritis, the synovial fibroblasts and macrophages release IL-15, which acts on NK, T cells and neutrophils. However, others discovered that in CeD patients these mechanisms and the production of desired miRNAs are reduced, implying the fact according miRNAs could be implicated in the pathophysiology of intestinal barrier failure and specific clinical characteristics.[25]

Due to their role in the diagnosis and treatment of several digestive diseases, including CeD, miRNAs could represent a class of promising biomarkers; thus, they could help us better understand the pathogenetic mechanisms of the disease.[11,26-29]

Conclusion

It is clear from the study that CeD is chronic, inflammatory, and autoimmune intestinal disease result from gluten consumption in genetically susceptible patients, the only effective treatment for CeD is a GFD, miRNA-21 and IL-15 are possible biomarkers to positively confirm the diagnosis of CeD is. In which upregulation of the small regulatory RNA (miR-21) has

an important role in CeD development which directly affect IL-15 secretion in serum patients with CeD. The MARSH grade not taken into consideration as this research has several limitations.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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