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Evaluation of miR-210 expression in common variable immunodeficiency: patients with unsolved genetic defect

Fateme Babaha[a,b], Reza Yazdani[b], Sepideh Shahkarami[b,c,d], Zahra Hamidi Esfahani[a,b], Hassan Abolhahassani[b,e], Maryam Sadr[f], Ahmad Zavaran Hosseini[a]*, Asghar Aghamohammadi[b]*

[a]*Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran. Iran*
[b]*Research Center for Immunodeficiencies, Children's Medical Center, Tehran University of Medical Sciences, Tehran, Iran*
[c]*Department of Pediatrics, Dr. von Hauner Children's Hospital, University Hospital, Ludwig-Maximilians-Universität München (LMU), Munich, Germany*
[d]*Medical Genetics Network (Megene), Universal Scientific Education and Research Network (USERN), Tehran, Iran*
[e]*Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska Institute at Karolinska University Hospital Huddinge, Stockholm, Sweden*
[f]*Molecular Immunology Research Center, Tehran University of Medical Sciences, Tehran, Iran*

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Abstract
Background: Common variable immunodeficiency (CVID) is one of the most prevalent forms of primary immunodeficiency diseases (PID). CVID is characterized by failure in the final differentiation of B lymphocytes and impaired antibody production but the pathogenesis is not known in the majority of patients. We postulated that the expression pattern of miRNAs in unsolved CVID patients might be the underlying epigenetic cause of the disease. Therefore, we aimed to assess the expression of hsa-miR-210-5p and FOXP3 transcription factor in CVID cases in comparison with healthy individuals.
Methods: Eleven CVID cases with no genetic defects (all PID known genes excluded) and 10 sex and age-matched healthy individuals were enrolled in the study. T lymphocytes were purified from PBMC, and expression levels of miR-210-5p and FOXP3 mRNA were evaluated by real-time PCR.
Results: We demonstrated that miR-210 expression in patients was significantly higher than the control group (P=0.03). FOXP3 expression was slightly lower in patients compared with healthy controls (P=0.86). There was a negative correlation between miR and gene expression (r: −0.11, P=0.73). Among various clinical complications, autoimmunity showed a considerable

*Corresponding authors: Ahmad Zavaran Hosseini, PhD. Ahmad Zavaran Hosseini, Department of immunology Tarbiat Modarres University, P.O. Box. 14155-4838, Tehran, Islamic Republic of Iran. *Email address*: Zavarana@modares.ac.ir;
Asghar Aghamohammadi, MD, PhD. Children's Medical Center Hospital, 62 Qarib St., Keshavarz Blvd., Tehran 14194, Iran.
Email address: aghamohammadi@sina.tums.ac.ir

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rate in high-miR patients (P=0.12, 42.8%), while autoimmunity was not observed in normal miR-210 patients.
Conclusions: Our results suggest a role for miR-210 in the pathogenesis of autoimmunity in CVID patients. Further studies would better elucidate epigenetic roles in CVID patients with no genetic defects.

Introduction

Common variable immunodeficiency (CVID) is a primary immunodeficiency (PID) characterized by defects in B cell differentiation and antibody production. Affected patients manifest various clinical manifestations such as recurrent respiratory tract infections, autoimmunity, interstitial lung disease, lymphoproliferation, enteropathy, malignancies, and allergic disease.[1] Increased susceptibility to infections, marked reduction of serum IgG, IgA, and/or IgM levels along with poor specific antibody production against protein and polysaccharide antigens are the major diagnostic parameters of CVID.[2] Immunoglobulin (Ig) replacement and antimicrobial agents are commonly highly effective at preventing and treating infections in CVID patients.[3] Several monogenic disorders including the lipopolysaccharides responsive beige-like anchor (*LRBA*) and cytotoxic T-lymphocyte- associated protein 4 (CTLA4) which affect the function of regulatory T cells (Treg) can clinically present as CVID.[1] However, these affect less than 40% of CVID patients in non-consanguineous cohorts and approximately 60% of CVID patients in consanguineous cohorts. These mutations could be involved in the differentiation of B cells and T cells, as impaired B and T cell subsets (including Treg) have been reported so far.[1,4,5] Although genetic mutations have clarified some aspects of the pathogenesis of CVID, there are some cases with no known PID or candidate genetic mutations.

Alteration of epigenetic profile could be considered as a pathogenic cause for the manifestation in patients with unsolved CVID.[6] Epigenetic regulate gene expression without altering the DNA sequence. DNA methylation, histone-chromatin modification, transcription factor, and non-coding RNAs expression are the epigenetic mechanisms influencing the development and functions of rapidly evolving immune cells.[6] MicroRNAs are a subgroup of short non-coding RNAs acting as small immune regulators of B and T cells. The importance of miRNAs action in the immune system was first highlighted in studies that reported that Argonaute 2 breakage in hematopoietic stem cells impairs B cell differentiation.[7] Several studies revealed the role of miRNAs in the regulation, development, and differentiation of lymphocytes. In this regard, the aberrant expression of miRNAs has been reported to be related to immune system malfunction in various immunologic complications.[8-10] As an example, miR-210-5p directly inhibits FOXP3 expression, which is known as the essential transcription factor in the development and function of regulatory T (Treg) lymphocytes, and its upregulation is associated with pathological conditions.[11,12] Since immune cells are constantly under severe morphologic and functional changes to exert proper immune response, the immune system is always susceptible to drastic epigenetic changes.[13]

Epigenetic studies on PID patients are scarce, especially in CVID. Few studies investigated the alteration of DNA methylation in CVID patients.[6,11,12] In the present study, we aimed to evaluate the expression pattern of miR-210-5p that can regulate Treg cells of CVID patients without the genetic mutation.

Material and methods

Study population/sampling and preparations

We evaluate all registered available CVID patients[14] who referred to the PID clinic of the Children's Medical Center affiliated to Tehran University of Medical Sciences, Tehran, Iran. The ethics committee of Tarbiat Modares University approved the study (ID number: IR.TMU.REC.1396.731); participants were informed about the project and written consent was obtained from them and/or their parents. The patients were clinically diagnosed as CVID according to the European Society for Immune deficiencies disease (ESID) criteria.[2] In addition to ESID criteria, we excluded patients who have demonstrated genetic defects after whole-exome sequencing (WES) in known PID genes[15] or candidate PID genes.[16] The procedure of WES has been described in our previous study.[4] The demographic and clinical data of the patients were extracted from the Iranian National Registry of PID patients. Moreover, age- and sex-matched individuals with no records of immune-based diseases were chosen as a healthy control group (HC).

Peripheral mononuclear cell isolation and T cell purification

Blood samples (5 mL) were obtained from CVID patients 4 weeks after routine monthly intravenous immunoglobulin (IVIG) treatment and were collected in 6 mL K2 ethylenediaminetetraacetic acid (EDTA) tubes. Peripheral blood mononuclear cells (PBMCs) of patients and controls were isolated by Ficoll-PaqueTM plus density gradient (Biosera, France) and centrifugation at 600 g for 30 min. After washing twice, the number of cells was determined using trypan blue staining. T lymphocytes were negatively purified using a human pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer guidelines. Firstly, 107 cells were magnetically labeled with 10 µL

of a cocktail of biotin-conjugated antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a (GlycophorinA). After 5 min of incubation at 4◦C, 20 µL of anti-biotin microbeads were added to the labeled cells. A further 10 min of incubation prepared the cells for the following step. MACS LS column was retained with 3 mL of the buffer, then cell suspension was loaded into the column. Flow-through contained untouched enriched T lymphocytes. The purity of the T cell population was assessed above 95% by flow cytometry (Figure S1). Lymphocyte subsets of the patients were evaluated based on our previous study.[17]

RNA extraction

Total RNA was extracted using TRIzol™ Reagent (Invitrogen™, USA) and at room temperature (RT). Freshly purified T cells were homogenized with 1 mL of Trizol reagent and were incubated for 5 min at RT for complete dissociation of nucleoproteins complex.

0.2 mL chloroform was added to separate the homogenate into the upper clear layer of RNA, interphase (containing DNA and protein) and lower red-phenol layer. The aqueous phase was transferred to a new tube and 0.6 mL isopropanol was added to precipitate RNA. After centrifugation, a white gel-like pellet of RNA formed at the bottom of the tube. The supernatant was discarded, and the RNA pellet was washed with 1 mL of 75% ethanol to remove impurities. After centrifugation at 7500 g for 5 min, the supernatant was discarded and the pellet was air-dried for 10 min to prevent phenol contamination. The pellet was re-suspended with 20 µL of nuclease-free water and was incubated in a water bath set at 58◦C for 15 min.

Quantitative PCR for miR-210 and FOXP3 expression

According to the bioinformatics software programs Targetscan and miRwalk, miR-210 has potential target sites in the 3′ untranslated region (UTR) of *FOXP3* gene (Figure S2). Stem-loop double-stranded cDNAs for hsa-miR-210-5p were synthesized through a two-step procedure using a BON-Stem miR cDNA synthesis kit (Bon Yakhte, Iran). All steps were performed on ice. First, 500 ng (5 µL) of extracted RNA and 1 µL of Bon-RT stem-loop adaptor (10 µM) were mixed in a 0.2 mL tube, then 6 µL of diethyl pyrocarbonate (DEPC) water was added reaching the volume to 12 µL. After 5 min of incubation at 75°C, tubes were placed on ice and 4 µL of 5X buffer, 2 µL of deoxynucleotide (dNTP) mix (10 mM), and 1 µL of reverse transcriptase (RT) enzyme (5 U/µL) were added to the reaction. Reverse transcription was then performed using a thermocycler (Applied Biosystem, USA) with the following cycling conditions: one cycle of 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min. The expression level of miR-210 was measured by quantitative real-time PCR, using RealQ Plus 2x Master Mix Green High ROX (Amplicon, Denmark). Twenty microliter reactions containing 10 µL of mastermix, 7 µL of DEPC water, 1 µL of miR-210 specific reverse and forward primers (Bon Yakhte) plus 2 µL of cDNA were run in duplicate at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Small nucleolar RNA (SNORD, Bon Yakhte) was used as an endogenous control. The relative expression levels of miRNA in patients and control groups were calculated using the comparative cycle threshold (Ct) method and fold changes were calculated by the expression $2^{-\Delta\Delta Ct}$.

For FOXP3 quantitative PCR analysis, cDNA was synthesized using the FIREScript RT cDNA synthesis kit (Solis Biodyne, Latvia). The procedure was conducted on ice and in two steps according to the manufacturer's protocol. Briefly, 500 ng of template RNA was mixed with 1 µL of Oligo (dT) primer (100 µM) and random primers (100 µM). The reaction volume was reached to 16 µL with nuclease-free water and incubated at 65°C for 5 min and was then placed on ice for the following step. 2 µL of 10x RT reaction buffer with dichloro-diphenyl- trichloroethane (DDT), 1 µL of FIREScript RT plus 0.5 µL of dNTP and RNase inhibitor were added to the tubes and were reverse-transcribed according to the following program: one cycle of 25°C for 10 min and 50°C for 60 min, followed by a final step of 85°C for 5 min for enzyme inactivation. Real-time PCR was performed using FOXP3 specific reverse and forward primers (Pishgam, Iran) and the same master mix was used. Reactions were prepared at the total volume of 20 µL and were run in duplicate at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) was used as an internal control. The relative expression levels of FOXP3 in patients and control groups were calculated using the comparative Ct method and fold changes were calculated by the expression $2^{-\Delta\Delta Ct}$. Primer sequences used in RT-PCR are presented in Table S1.

Statistical analysis

Statistical analysis of qRT-PCR data was performed using Graphpad Prism version 8.2. (La Jolla, CA, USA). Further analyses were carried out using SPSS version 22 (SPSS Inc., Chicago, IL, USA). Frequencies were reported with mean ± standard deviation and median (interquartile range, IQR), as appropriate. In order to assess the normality of the variables, Kolmogorov–Smirnoff tests were used, then the following parametric and non-parametric tests were carried out based on this assumption. Independent sample T-test and Mann–Whitney tests were used in order to compare numerical variables of parametric and non-parametric distribution, respectively. For 2 × 2 comparison of categorical variables, chi-square and Fisher's exact tests were used. Pearson's and Spearman correlation coefficients were used to assess the correlation between parametric and non-parametric values, respectively. In each test, a *P*-value of 0.05 or less was considered significant.

Results

Patients

Eleven unsolved CVID patients (three females [27.27%]) with a median (IQR) age at diagnosis of 13 (10–27) years were included. Patients were followed for a median (IQR) of 11 (7–15) years. Seven (63.6%) patients were from

Table 1 Demographics and immunological data of CVID patients.

Parameters	Total (N=11)
Sex (M/F), N (%)	8/3 (72.7/27.3)
Consanguinity; N (%)	7 (63.6)
Age at the study time, years	
Mean (SD)	30.09 (11.7)
Median (IQR)	25 (21-42)
Age of onset, years	
Mean (SD)	8.8 (10.5)
Median (IQR)	4 (1-11)
Age at diagnosis, years	
Mean (SD)	18 (8.3)
Median (IQR)	13 (10-27)
Delay of diagnosis, years	
Mean (SD)	9.09 (8.3)
Median (IQR)	8 (2-11)
Course of disease, years	
Mean (SD)	20.64 (13.14)
Median (IQR)	15 (13-31)
Follow-up, years	
Mean (SD)	11.27 (5.51)
Median (IQR)	11 (7-15)
IgG*, mg/dL	
Mean (SD)	333 (237)
Median (IQR)	250 (197-519)
IgM*, mg/dL	
Mean (SD)	34.64 (26.63)
Median (IQR)	29 (10-51)
IgA*, mg/dL	
Mean (SD)	19.44 (32.5)
Median (IQR)	4 (1.5-35)
IgE*, IU/mL	
Mean (SD)	2.14 (3.4)
Median (IQR)	1 (1-1)
Lymphocyte*, cell/mL	
Mean (SD)	2556.36 (1740.96)
Median (IQR)	2142 (1235-2750)
CD4+ lymphocyte*, cell/mL	
Mean (SD)	27.68 (6.09)
Median (IQR)	27 (24-34)
CD8+ lymphocyte*, cell/mL	
Mean (SD)	36.5 (8.23)
Median (IQR)	37 (29-46)
Regulatory T cells*, cell/mL	
Mean (SD)	1.2 (1.1)
Median (IQR)	0.7 (0.43-2.2)

CVID: common variable immune deficiency; M: male; F: female, N: count; AOO: age of onset; AOD: age at diagnosis.
*Evaluated at the time of diagnosis.

consanguineous parents. Table 1 depicts the demographic and immunological data of the studied patients. The most prevalent clinical manifestations observed in our cases were recurrent infections (81.9%) and most patients experienced otitis and sinusitis, recurrent diarrhea (82.7%), and lymphoproliferation (63.6%). Only three patients (27.3%) had a history of autoimmunity and none showed evidence of malignancy (Table 2).

Table 2 Clinical manifestations of CVID patients.

Parameters	Total (N=11)
First presentation with respiratory infection, N (%)	6 (54.5)
First presentation with non-respiratory infection, N (%)	7 (63.6)
Recurrent infection, N (%)	9 (81.8)
Otitis, N (%)	8 (72.7)
Sinusitis, N (%)	8 (72.7)
Pneumonia, N (%)	6 (54.5)
Pulmonary infection, N (%)	6 (54.5)
Allergy, N (%)	3 (27.3)
Autoimmunity, N (%)	3 (27.3)
Organomegaly, N (%)	7 (63.6)
Arthritis, N (%)	3 (27.3)
Recurrent diarrhea, N (%)	8 (72.7)

Increased miR-210 expression in CVID T cells

To investigate miR-210 expression in T cells, we compared the expression of miR-210 between CVID patients and 10 healthy age-sex matched controls by real-time PCR. Our results showed that miR-210 expression had a significant increase (with a fold change of 5.6) in CVID patients compared with healthy controls (P=0.03, Figure 1). This means that miR-210-5p relative expression increased compared with the control group. In addition, we compared each patient's expression to the average of miR-210 expression in patients and observed that five individuals (P4, P6, P7, P8, P9) had miR-210 expression above the average of patients (Figure S3).

Decreased FOXP3 expression in CVID patients

Aligned with the reduction of Treg cells in eight studied patients, analyses of qRT-PCR showed that FOXP3 mRNA in CVID patients had lower expression compared with healthy controls. However, as shown in Figure 1, it was a slight decrease (mean fold change: 0.87) and the difference was not significant between controls and patients (P=0.75). Evaluating the relative expression in patients individually, showed that seven patients (P1, P2, P6, P7, P8, P10, P11) had lower expression than the average of FOXP3 expression

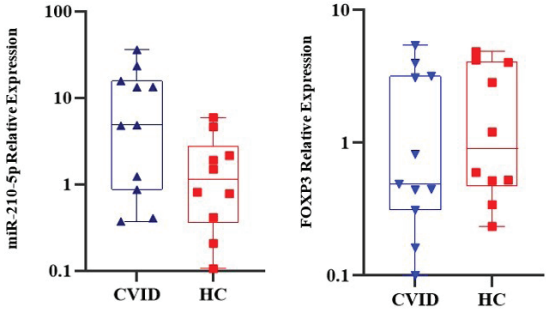

Figure 1 miR-210 and Foxp3 mRNA relative expression in study groups. HC: healthy control.

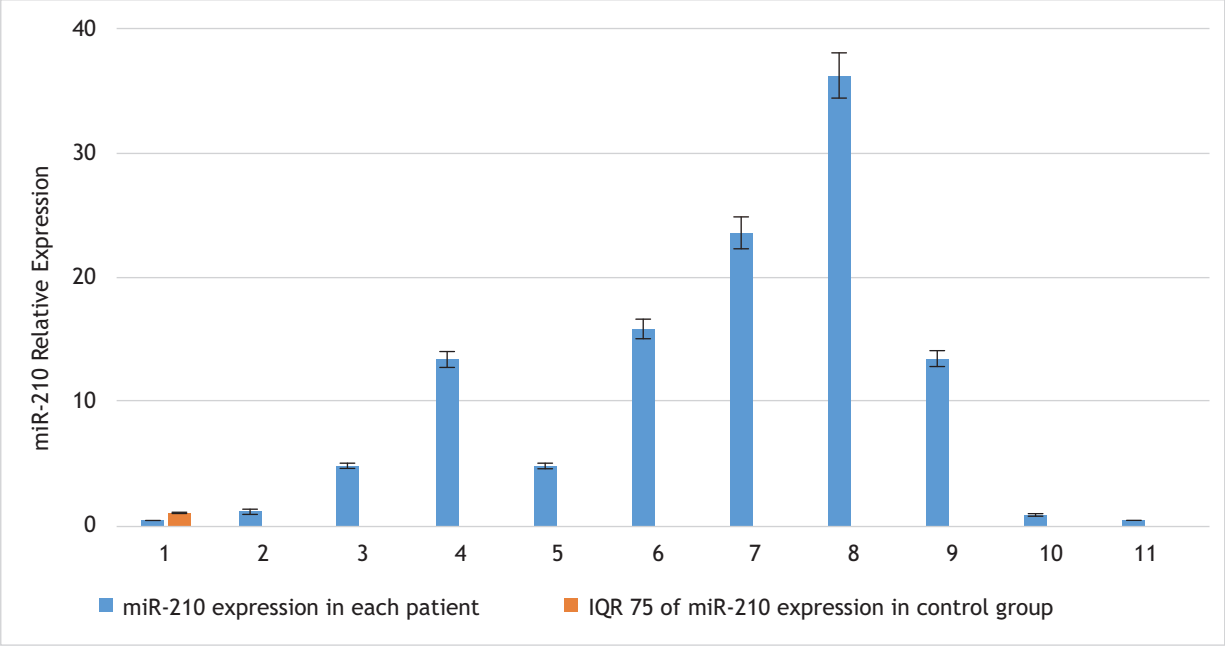

Figure 2 miR-210 relative expression in each sample compared with IQR of control.

in patients (Figure S4). There was a negative correlation between miR-210-5p and gene expression (r: -0.11, P=0.73).

miR-210 Upregulation correlates with autoimmune manifestations in patients

In order to assess whether there is a correlation between clinical manifestations of patients and miR-210 expression, we first compared the miR-210 expression of each patient with the median of miR expression in healthy controls. We calculated that the median (IQR) of miR-210 expression in the control group was 1.1 (0.36-2.7). We observed that the miR-210 expression of seven patients was higher than the 75 percentile (more than 2.7) and the remaining four were within the normal range; between 0.36 and 2.7 (Figure 2). Accordingly, we categorized our patients into two groups of higher miR-210 expression (P3, P4, P5, P6, P7, P8, P9) and normal miR-210 expression. Three patients (P7, P8, P9) with autoimmunity were in the high-miR group (P=0.12, 42.8%), while autoimmunity was not observed in normal miR-210 patients. Pulmonary infection was also significant (P=0.02), however, this complication was more frequent among patients who were in the normal range. The data are provided in the supplementary Table S2.

Decreased expression of CD4+ T cells and Treg upon miR-210 upregulation

Using the data of T cell subsets, we evaluated the correlation of T cell and miR-210 expression. Chi-square test showed that the frequency of patients with low CD4+ T cells and Treg was more within the high-miR expression group, while patients with increased CD8+ T cells were more frequent upon miR-210 upregulation (Table 3).

Table 3 Chi-square T cell subset quality and miR-210 expression.

T-cell subsets	7 patients (high)	4 patients (normal)	*P*-value
CD8+ T cell	Normal: 2 Increase: 5 Decrease: -	Normal: 1 Increase: 3 Decrease: -	0.89
Activated CD8+ T cell	Normal: 0 Increase: 7 Decrease:	Normal: 1 Increase: 3 Decrease:	0.16
Cytotoxic CD8+ T cell	Normal: 0 Increase: 7 Decrease:	Normal: 1 Increase: 3 Decrease:	0.16
CD4+ T cell	Normal: 0 Increase: Decrease: 7	Normal: 1 Increase: Decrease: 3	0.16
Naïve CD4+ T cell	Normal: 2 Increase: Decrease: 5	Normal: 0 Increase: Decrease: 4	0.23
Central memory CD4+ T cell	Normal: 2 Increase: 1 Decrease: 4	Normal: 0 Increase: 3 Decrease: 1	0.11
Regulatory T cells	Normal: 2 Increase: 0 Decrease: 5	Normal:1 Increase: 0 Decrease: 3	0.89

Discussion

Epigenetic alteration is one of the possible causes affecting CVID patients. In the present study, we aimed to study a miRNA expression, as an epigenetic factor, in a selected group of unsolved CVID cases. To the best of our

knowledge, this is the first study to investigate a miRNA profile epigenetic mechanism in CVID cases.

CVID patients manifest a broad range of clinical manifestations including infectious and non-infectious disorders.[18] In the present study, the most frequent clinical complications were recurrent respiratory tract infections as well as gastrointestinal complications such as recurrent diarrhea and organomegaly. Other less frequent manifestations were autoimmunity and allergy. Several studies on CVID cases have reported infections as the most prevalent clinical phenotype with mostly respiratory and gastrointestinal tracts being involved.[19,20] Other frequent non-infectious complications in CVID patients are immune dysregulation and malignancy. According to Chapel et al.,[21] autoimmunity is observed in almost 42% of CVID cases. Another study estimated that 2.5% of CVID patients have early onset of malignancy and 8.5% show late-onset for malignancy.[22] We suggest that the lower rate of autoimmunity, allergy, and malignancy in our cases might be related to our inclusion criteria. Generally, patients who are a monogenic form of CVID present with more severe clinical complications. On the other hand, our patients who had no known genetic mutations reveal more moderate types of clinical symptoms with the infectious only presentation.

Our results showed that miR-210 expression was significantly increased in CVID patients. According to previous studies, miR-210 is an inhibitory miRNA that is normally downregulated or is expressed at low levels in healthy individuals.[23] According to several reports, miR-210 is over-expressed in hypoxic environments and is regarded as a major hypoxy-miR.[24] It is reported that miR-210 is upregulated in different types of immune-related disorders such as breast,[25] head and neck,[26] pancreatic[27] and renal cancers,[28] psoriasis vulgaris,[29] and occupational allergic disease.[30] The upregulation of miR-210 is associated with the severity of these pathological conditions. Furthermore, over-expression of miR-210 directly promotes angiogenesis in patients suffering from breast or hepatocellular cancer by increasing vascular endothelial growth factor (VEGF)[31] and fibroblast growth factor receptor like 1 (FGFRL1) expression.[32] It has been reported that inhibition of miR-210 expression led to remission or alleviation of these disorders. There is no study investigating miR-210 in PID or CVID patients to compare our results with others. A significant increase of miR-210 expression in CVID patients may suggest a relation between miR-210 upregulation with the disease and immune dysfunction/dysregulation in this entity of immune deficiency, as immune dysregulation has been previously demonstrated in CVID patients.[33] Given that immune dysregulation, a mechanism that is not entirely elucidated in CVID patients with no genetic defects, evaluating cytokines responsible for the inflammations in this group of patients and its correlation with miR-210 upregulation is suggested for further studies.

Bioinformatic software analyses define innumerable potential target genes for miR-210 such as FOXP3, STAT6 and GATA3, and CTLA-4. In this study, FOXP3 was chosen as one of the experimentally validated target genes in CD4+ T cells for miR-210.[34] Our results showed that FOXP3 expression was slightly decreased in unsolved CVID cases. Studies evaluating Treg function in CVID patients with and without autoimmune manifestation indicate a decrease in Foxp3 expression and Treg population. Genre et al.[35] reported for the first time that in CVID patients with autoimmune manifestations, Treg population and FOXP3 expression was significantly decreased; however, in those without autoimmunity, FOXP3 expression in patients was somewhat like healthy individuals. We observed a slightly negative correlation between miR-210 and FOXP3 expression. Several studies have reported a significant negative correlation between miR-210 and FOXP3 expression. In one study, Lung et al. suggested a potential inhibitory influence for miR-210 on FOXP3 expression during sensitization caused by toluene diisocyanate (TDI). Another study in 2012 by Fayyad-Kazan et al.[34] reported the negative connection between miR-210 and Foxp3. They proved that miR-210 directly inhibits Foxp3 expression by attaching to 3'UTR of the region of the *FOXP3* gene. The inconsistency among our results and previous reports might be related to two issues: (1) our specific and small study population; and (2) the inclusion of only isolated CD4+ T cells. It is suggested that this study be investigated in the future with a larger number of CVID patients with no genetic defects. Moreover, we evaluated miR-210 in isolated CD+4 T cells, and this limitation should be removed in future studies when this evaluation is performed on isolated FOXP3+ T cells.

We observed a correlation between autoimmunity and miR-210 upregulation. Three of the patients with autoimmunity had higher miR-210 expression than normal controls. Of note, FOXP3 expression in two of the three had lower expression than the normal range. Zhao et al.[36] reported that miR-210 overexpression induces immune dysfunction via the reduction of FOXP3 expression in psoriasis vulgaris patients. They observed that miR-210 was significantly increased in CD4+ T cells of psoriasis vulgaris individuals and inhibiting miR-210 reversed the condition and increased FOXP3 expression improved condition. Other studies also reported that miR-210 upregulation correlates with autoimmunities such as type 1 diabetes,[37] systemic lupus erythematosus, rheumatoid arthritis,[38] and Grave's disease.[39] Based on our findings, it is suggested that miR-210 upregulation increases the possibility of autoimmunity phenotype in CVID patients.

In the present study, we observed that patients in whom miR-210 was upregulated, CD4+ T cell and Treg were decreased while the frequency of CD8+ T cell was increased. Previous studies have reported that CD4+ T cells in CVID patients are decreased while CD8+ T cell increases.[40] On the other hand, Zhao et al.[36] demonstrated that miR-210 had a significant inhibitory effect on the CD4+ T cell population. Therefore, miR-210 may also be a responsible factor in decreasing CD4+ T cells in CVID cases.

Conclusions

Epigenetic studies in CVID are in their first stages and only a few studies are investigating the role of epigenetic in CVID patients. Evaluating miRNA expression in CVID cases has not been reported before and this study is the first initiative focusing on the role of miRNA and immune dysregulation in such patients. Further studies regarding the correlation of miRNA expression with B and T cell development and differentiation could help to clarify the

pathogenesis of different CVID phenotype in patients with no genetic defects. It is expected that more studies in this regard promise new approaches for the diagnosis and treatment of CVID.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Supplementary Data

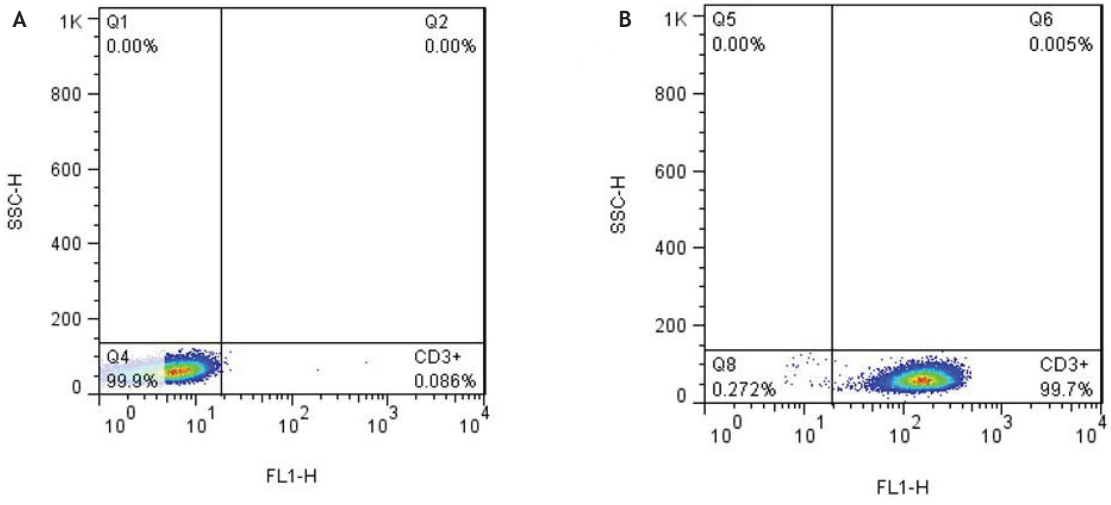

Figure S1 Flow cytometry for isolated T lymphocytes. (A) Isotype; (B) T cells.

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile
Position 32-38 of FOXP3 3' UTR hsa-miR-210-5p	5' ...AGGAGGAUGGACGAACAGGGGCC... 3' GUCACACGCCACCCGUCCCCGA	7mer-m8	-0.28	94
Position 530-537 of FOXP3 3' UTR hsa-miR-210-5p	5' ...CAGCCUCAGGGCCCACAGGGGCA... 3' GUCACACGCCACCCGUCCCCGA	8mer	-0.26	92

Figure S2 Bioinformatics software program for miR-210-5p.

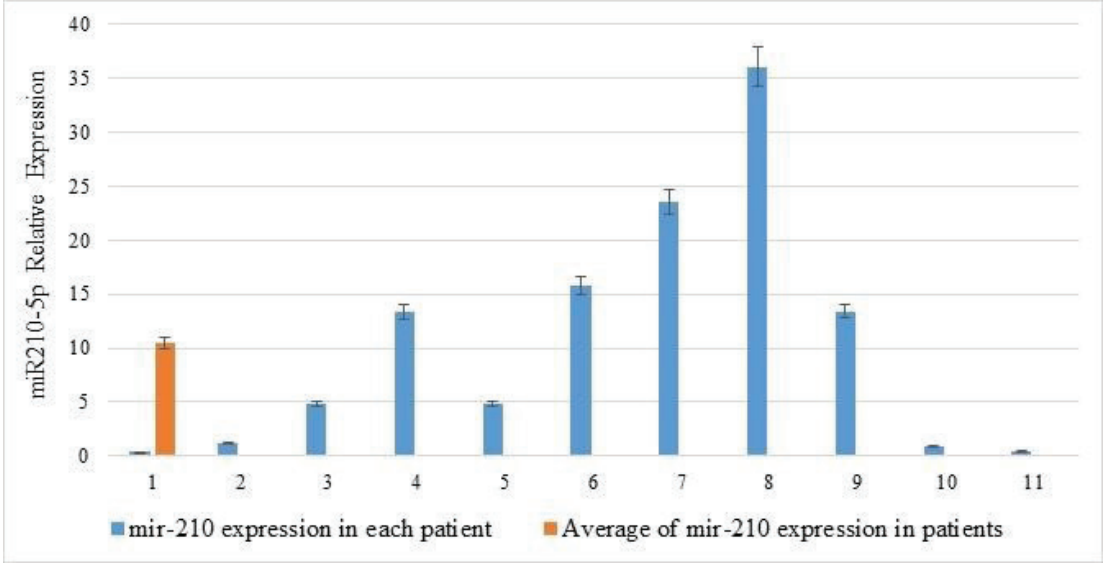

Figure S3 Relative expression of miR-210 in each patient.

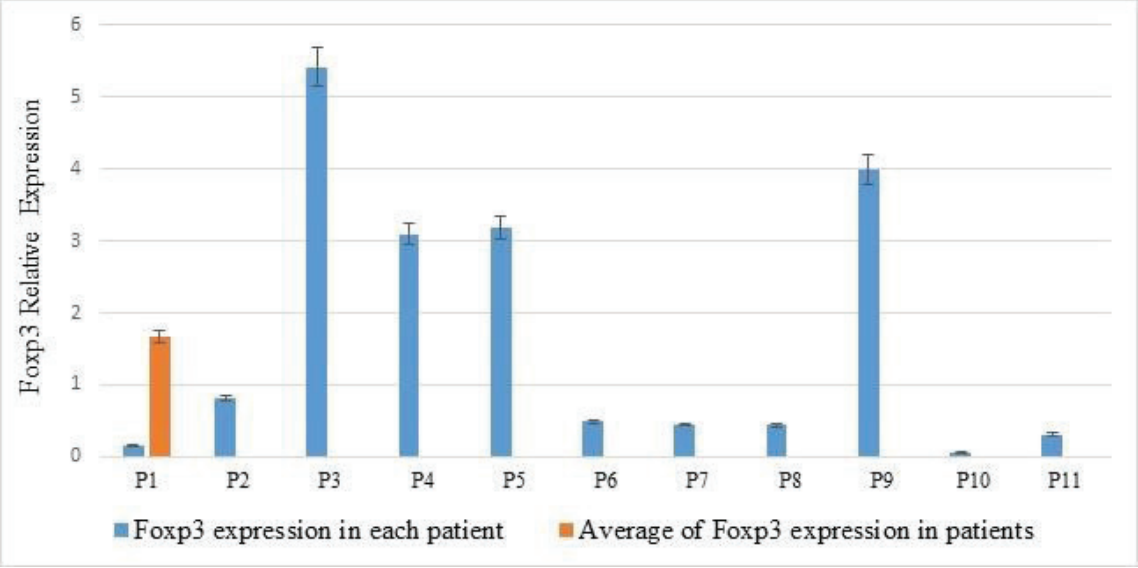

Figure S4 Relative expression of Foxp3 mRNA in each patient.

Table S1 Primers used for quantitative real-time PCR.

Gene	Direction	Sequence
FOXP3	Forward	TTCCACAACATGCGACCCC
	Reverse	CATGCGTGTGAACCAGTGG
HPRT	Forward	CCTGGCGTCGTGATTAGTGAT
	Reverse	AGACGTTCAGTCCTGTCCATAA

Table S2 Crosstab of clinical manifestation and patients' miR-210 expression.

Clinical manifestation	4 patients (in range)	7 patients (high)	*P* value
First presentation with respiratory infection; N (%)	2 (50%)	4 (57.14%)	0.81
First presentation with non- respiratory infection; N (%)	3 (75%)	4 (57.14%)	0.55
Upper respiratory infection; N (%)	3 (75%)	6 (85.71%)	0.31
Lower respiratory infection; N (%)	3 (75%)	6 (85.71%)	0.65
Recurrent infection; N (%)	4 (100%)	5 (71.42%)	0.23
Otitis; N (%)	3 (75%)	5 (71.42%)	0.89
Sinusitis; N (%)	3 (75%)	5 (71.42%)	0.89
Pneumonia; N (%)	2 (50%)	4 (57.14%)	0.302
Pulmonary infection; N (%)	4 (100%)	2 (28.57%)	0.02*
Allergy; N (%)	1 (25%)	2 (28.57%)	0.89
Autoimmunity; N (%)	0	3 (42.85%)	0.12
Organomegaly; N (%)	4 (100%)	3 (42.85%)	0.058
Arthritis; N (%)	1 (25%)	2 (28.57%)	0.89
Lymphadenopathy; N (%)	1 (25%)	1 (14.28%)	0.65
Recurrent diarrhea; N (%)	3 (75%)	5 (71.42%)	0.89

**P*-value <0.05 statistically significant.