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Evaluation of MicroRNA-125b-5p and Transcription Factors *BLIMP1* and *IRF4* Expression in Unsolved Common Variable Immunodeficiency Patients

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ABSTRACT

Common variable immunodeficiency (CVID) is the most prevalent form of symptomatic primary humoral immunodeficiencies characterized by failure in the final differentiation of B lymphocytes. The majority of CVID cases have no identified genetic defect, and epigenetic alteration could be involved in the pathogenesis of CVID**.** Hence, we aimed to evaluate the expression of hsa-miR-125b-5p -and, B lymphocyte-induced maturation protein-1(*BLIMP-1*) and interferon regulatory protein-4 (*IRF-4*) in a group of CVID patients with no definitive genetic diagnosis in comparison with healthy individuals.

Ten CVID patients (all known genes excluded) and 10 age and sex-matched healthy controls participated in the study. B lymphocytes were isolated and expression of miR-125b-5p, *IRF4,* and *BLIMP1* were evaluated by real-time polymerase chain reaction (RT-PCR). Moreover, B cell subsets were analyzed by flow cytometry.

The results showed that the relative expression of miR-125b-5p in CVID patients was increased while it was decreased for the *BLIMP1* and IRF4 transcription factors compared with the healthy controls. Although a reduction was observed in switched and non-switched memory B cells among all high-miR patients, these subsets were decreased in patients with normal miR

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expression (71.0% and 85.0%, respectively).

Our results suggest that overexpression of miR-125b-5p affects the terminal differentiation of B cells in a selected group of CVID patients by downregulating the *BLIMP-1* gene and more intensively for the *IRF-4* gene expressions.

Keywords: Common variable immunodeficiency; Epigenesis; MicroRNAs; Primary immunodeficiency diseases

INTRODUCTION

Common variable immunodeficiency (CVID) is the most prevalent symptomatic form of primary immunodeficiency deficiencies (PIDs), known by remarkably decreased serum antibody concentrations, impaired specific antibody response to protein and polysaccharide antigens.[1] CVID patients manifest heterogeneous clinical manifestations including recurrent bacterial infections, gastrointestinal complications, autoimmunity, inflammatory diseases, allergies, lymphoproliferation, and malignancies.[2] Although some specific monogenetic defects have been identified in some CVID-like patients, the underlying reasons for other CVID patients remain largely unknown. Only 30-50% of CVID patients have specific mutations.[3-5] A group of identified genes suggested intrinsic B cell defects including *CD19, CD20, CD81, CD21,* transmembrane activator, and calcium modulator and cyclophilin ligand interactor *(TACI),* B cell-activating factor receptor *(BAFFR),* inducible T-cell costimulator *(ICOS),* SEC61 translocon subunit alpha 1 *(SEC61A1)* and interferon regulatory factor 2 binding protein 2 *(IRF2BP2).*[6,7] The other patients might be influenced by other alternative theories, mainly polygenetic state or epigenetic abnormalities.[5]

Epigenetics mechanisms alter gene expression without changing germ-line DNA and are consisted of DNA methylation, histone and chromatin modification, and non-coding RNAs notably miRNAs.[8] MicroRNAs (miRNAs) are small single-stranded non-coding RNA molecules about 19–23 nucleotides in length, that regulate gene expression at the post-transcriptional level by targeting mRNA for degradation or translational repression. These molecules are also involved in a variety of biological processes and immune cell functions like B-cell development and differentiation.[9,10] In the majority of CVID patients, B cell counts have been reported within the normal range, therefore the main defect possibly exists in the terminal differentiation and long-term survival of B cells.[11] Dysregulation of miRNAs can have an impact on transcription factors and consequently B cell differentiation.[12] Several studies reported that hsa-miR-125b-5p directly regulates the expression of B lymphocyte-induced maturation protein-1 (*BLIMP-1*) and interferon regulatory protein-4 (*IRF-4*) which are known as essential transcription factors for plasma cell differentiation.[13-15]

Only a few studies investigated epigenetic alterations in PID patients, especially in CVID.[5,16,17] In this study, we aimed to evaluate the expression of miR-125b-5p that regulates B cell differentiation through *IRF-4* and *BLIMP-1* in a group of CVID cases with an unsolved genetic defect.

PATIENTS AND METHODS

Study Population

We included 10 CVID patients who were referred to the PID clinic of Children's medical center affiliated with Tehran University of Medical Sciences, Tehran, Iran. The study was approved by the ethics committee of Tarbiat Modares University (IR.TMU.REC.1396.730) and written consent was received from the participant and/or their parents. These patients were diagnosed based on the European Society for Immunodeficiencies (ESID) criteria including a marked reduction level of at least two serum immunoglobulins (IgG and IgA) with or without low IgM levels by two standard deviations (SDs) from normal mean values for the patient's age, the elimination of defined causes of hypogammaglobinemia, confirmed diagnosis after the 4th year of life, poor antibody responses to vaccines and/or absent isohemagglutinins, and no evidence of profound T-cell deficiency, defined as 2 out of the following (y=year of life): 1) CD4 numbers/microliter: 2-6y<300, 6-12y <250, >12y<200, 2) % naive CD4: 2-6y<25%, 6-16y<20%, >16y<10%, and 3) T cell proliferation absent. In addition to ESID criteria, included patients demonstrated no genetic defects after whole-exome sequencing (WES)

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in known PID genes[18] or candidate PID genes.[19] The procedure of WES and its analysis were described in our previous study.[6] Also, age and sex-matched healthy individuals without any history of immune disorders were included as healthy controls (HCs).

Bioinformatics Studies

By reviewing previous studies and due to the importance of miR-125b-5p and its potential target genes on terminal B cell development, we selected this miRNA for this study.[13-15] The score of miR-125b-5p for binding to its predicted genes was checked in Targets can (http://www.targetscan.org/vert_71/)[20] and miRDB (http://www.mirdb.org/),[21] Both algorithms confirmed that miR-125b-5p has potential target sites in 3′ untranslated region (UTR) of *BLIMP-1* and *IRF-4* genes (Figure S1). MiRNA sequence was obtained from miRBase database (http://www.mirbase.org/).[22]

Cell Isolation and Purification of $CD19^+$ B cells

Peripheral blood samples (10 ml) were collected four weeks after intravenous immunoglobulin (IVIG) treatment in K2 ethylenediaminetetraacetic acid (EDTA) tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll-paque™ density gradient centrifugation (Lymphodex innotrain, Germany). The viability of separated PBMCs was more than 98%, as evaluated using the Trypan blue viability test. B cell populations were purified from PBMCs by negative selection; using a human pan B-cell isolation kit (Miltenyi Biotec, Germany), based on standard manufacturer's instruction. The purity of sorted B lymphocytes was>95% based on flow cytometry assessment (Anti-CD19 allophycocyanin, eBoscience, USA, Figure S2).

RNA Extraction and Reversed Transcription Reactions

Total RNA was extracted from $1x10^6$ purified $CD19^+$ B cells with TRIzol™ reagents (Invitrogen™, USA) according to the manufacturer's instructions. The RNA purity and concentration were evaluated by NanoDrop OneMicrovolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). The absorbance ratio of isolated RNA at A260/280 nm was between 1.8 and 2.0, showing that it was pure and could be used for further analysis. 500 ng of total RNA was reversed-transcribed into complementary deoxyribonucleic acid (cDNA) for miR-125b-5p using BON-Stem miR cDNA Synthesis kit (Bon Yakhte, Iran) based on manufacturer's protocol. Also, 500 ng of total RNA was converted to cDNA using FIREScript RT cDNA Synthesis Kit (Solis BioDyne, Estonia) according to the manufacturer's protocol for quantitative analysis of *BLIMP-1* and *IRF4* genes.

Real-time Polymerase Chain Reaction Analysis

The expression levels of *BLIMP-1* and *IRF-4* genes and miR-125b-5p were measured by quantitative real-time PCR; using SYBR Green PCR master mix Ampliqon (RealQ Plus 2x Master Mix Green High ROX, Denmark). The amplification conditions were 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min in StepOne Real-Time PCR System (Applied Biosystems). The relative expression of mRNAs and microRNA were calculated by the $2^{-\Delta\Delta Ct}$ method and were normalized with the expression of Hypoxanthine-guanine phosphoribosyltransferase (*HPRT)* and Small nucleolar RNA 47 (*SNORD47*), respectively. The gene-specific reverse and forward primers were designed; using Primer-BLAST (NCBI) and their quality was checked in UCSC (https://www.genome.ucsc.edu/cgi-bin/hgPcr). The primer pairs of the related genes are indicated in (Table S1). All the tests were carried out in duplicate.

B Cell Subset Assay

B cells where were stained for 20 min at 4°C with a mixture of Anti-CD19 allophycocyanin (APC), Anti-CD27 fluorescein isothiocyanate (FITC), Anti-CD38 (FITC), Anti-CD21 phycoerythrin (PE), Anti-IgD (PE), Anti-IgM peridinin chlorophyll protein (PerCp)-eFluor710 (all from eBoscience). Isotype controls were purchased from eBioscience to detect unspecific staining. B cells were divided into two panels of B1 and B2. Naive B-cells ($CD19^+CD27^-IgM^+IgD^+$), marginal zone-like B-cells ($CD19^+CD27^+IgM^{++}IgD^+$), switched memory B-cells ($CD19^+CD27^+IgM^-IgD^-$), and IgM-only memory B-cells ($CD19^+CD27^+IgM^{++}IgD^-$) were in Panel B1. Transitional B cells ($CD19^+CD21^{int}CD38^{++}IgM^{++}$), $CD21^{low}$ expressing B-cell ($CD19^+CD21^{low}CD38^{low}IgM^+$) and Plasmablast ($CD19^{low}CD21^{int}CD38^{+++}IgM^{-(+)}$) were in Panel B2. Data were analyzed by the FACS caliber instrument and FlowJo 10 software (eBoscience).

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics, version 22(IBM Corp, USA). Using

Kolmogorov-Smirnov and Shapiro-Wilk tests, we estimated whether data were normally distributed. Correlations between variables were investigated by Spearman correlation coefficients. The values were described as the frequency (numbers and percentage) and median (interquartile range, IQR). A nonparametric test Mann-Whitney *U* Test was applied to compare quantitative data. Figures were drawn by using GraphPad Prism software version 8.0.2 (GraphPad Software, Inc., USA).

RESULTS

Demographic Data and Clinical Phenotypes of Patients

Ten unsolved CVID patients (7 males and 3 females; median age [IQR] 28.5 [21.5-42]; age range) and ten healthy cases (7 males and 3 females; median age [IQR] 30 [24.5-38. 5]; age range) at the time of the study were included. The median age at onset of exhibiting symptom was 6.25 (range: 2-10) years, the median age of diagnosis was 17 (range; 10-24) years, the median age of diagnosis delay was 9 (range; 2-15) and the median age of follow-up was 12 (range; 8-16). 60% of patients were from consanguineous parents while the rate of consanguineous marriage was 0% among HC. The demographic and immunologic data of the patients are summarized in (Table 1). The most prevalent clinical manifestation among patients was recurrent infections of 90% mostly in the form of the upper 80% or lower 90% respiratory tracts. Other non-infectious complications were less frequent and were manifested as lymphadenopathy 20% and autoimmunity 20% (Table 2).

Measurement of *MiR-125b-5p*, *IRF4*, and *BLIMP1* Expression

We assessed miR-125b-5p expression between CVID patients and healthy donors. Our findings showed that the expression of miR-125b-5p was higher (median: 4.2; range:1.71-17.72) in patients compared with healthy donors (median: 1.2; range: 0.15-7.24), although this difference was not significant (p<0.09, Figure 1). We next investigated whether upregulation of miR-125b-5p could also affect expression levels of its target genes, *BLIMP-1* and *IRF-4*. We found that the expression of *IRF-4* in CVID patients was significantly lower (median: 0.24; range: 0.07-0.42) compared with controls (median: 0.84: range: 0.41-3.84) (p<0.008; Figure 1). In addition, *BLIMP-1* expression in patients (median: 0.42; range: 0.11-1.07) was slightly less than healthy donors (median: 1.26; range: 0.5-3.15, p<0.07; Figure 1). Also, the relative expression of miR-125b-5p, *IRF-4,* and *BLIMP-1* for each patient is demonstrated in (Figure S3). However, there was no significant correlation between the expression level of *IRF-4*, *BLIMP-1,* and miR-125b-5p. Low expression of IRF4 results in induction of activation-induced cytidine deaminase (AID) expression which is responsible for somatic hypermutation (SHM) and class switching recombination (CSR) in late stages of B cell differentiation, while optimal expression of IRF-4 favors BLIPM-1 expression and subsequent plasma cell formation (Figure 3).

Comparison of Clinical Manifestations and B-cell Subsets between CVID Patients with MiR Overexpression vs. Normal Expression

We evaluated the relation of miR over-expression on clinical manifestations of patients and their B cell subsets. First, based on the median of miR-125b expression in the control group (1.2 [0.15-7.2]) unsolved CVID patients were categorized into high-miR and normal-miR expression groups according to the 75th percentile of HCs. In three patients (P2, P5, and P6) the expression of miR-125b was higher than the 75th percentile (more than 7.2) and they were considered as high-miR patients (n=3, Figure 2). The remaining patients had normal miR expression; between 0.15 and 7.2. Chi-square test for clinical manifestations and miRNA-overexpression showed that all high-miR patients 100% had recurrent infections, however, it was not significant (p=0.49). More specifically, two (P5 and P6) had otitis 66% and two (P5 and P6) had sinusitis 66%. Other severe manifestations like autoimmunity, lymphadenopathy and clubbing were not seen in any of high-miR patients. These data are summarized in (Table 3). According to the results for B cell subsets and miR-125b-5p overexpression, we observed that all high-miR patients had decreased switched and non-switched memory B cells 100%, while these subsets were decreased only in 71.0% and 85.0% in patients with normal miR expression, respectively (Table 4). Also, naïve B cells and transitional B-cell were increased in all high-miR patients 100%, while these subsets had an increase in 71.0% and 85.0% of patients with normal miR expression, respectively (Table 4). In addition, the correlation between demographics data and the expression level of miR-125b-5p was not significant. (Table S2).

Table 1. Demographics and immunological data of CVID patients at the time of diagnosis

Variable	Total (N=10)
Sex (M/F); N	7/3
Consanguinity; N	6/4
Age of onset, years Median (IQR)	6.5 (1.75-10.25)
Age of diagnosis, years Median (IQR)	17 (10-24)
Delay of diagnosis, years Median (IQR)	9 (1.75-14.75)
Course of disease, years Median (IQR)	19.5 (14.5-34)
Follow-up, years Median (IQR)	12 (8-15.75)
Serum IgG*, mg/dl Median (IQR)	361 (152.5-629.5)
Serum IgM*, mg/dl Median (IQR)	34 (22.75-53.25)
Serum IgA*, mg/dl Median (IQR)	3.5 (0.5-47.5)
White blood cells (mm^3); median (IQR)	7475 (6450-9950)
Lymphocytes (cells/µl); median (IQR)	2192.5 (1632-3310)
CD19+ B cells (%); median (IQR)	11.5 (6.82-17.23)
CD3+ Tcells (%); median (IQR)	76 (67-78.5)
CD4+ Tcells (%); median (IQR)	29 (27-31.5)
CD8+ Tcells (%); median (IQR)	41 (32.5-49.5)

CVID: Common Variable Immune deficiency, M: Male, F: Female N: Count * Evaluated at the time of diagnosis

Table 2. Clinical manifestations of CVID patients

Parameters	Total (N=10)
URI; N(%)	8 (80)
LRI; N (%)	9 (90)
Recurrent infection; N(%)	9 (90)
Otitis; N (%)	7 (70)
Sinusitis; N (%)	7 (70)
Pneumonia; N (%)	6 (60)
Allergy; N (%)	4 (40)
Autoimmunity; N (%)	2 (20)
Lymphoproliferation; N (%)	5 (50)
Arthritis; N (%)	3 (30)
LAP; N (%)	2 (20)
Recurrent diarrhea; N (%)	8 (80)
Malignancy; N (%)	0
Poor growth; N (%)	2 (20)
Clubbing; N (%)	4 (40)

CVID: Common Variable Immune deficiency, URI: Upper Respiratory tract Infection, LRI: Lower Respiratory tract Infection, LAP: Lymphadenopathy

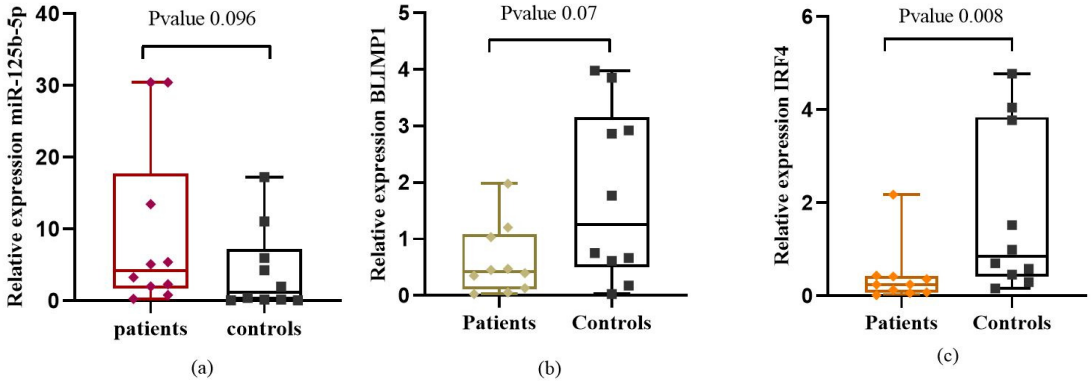

Figure 1. MiR-125b-5p is overexpressed in B-cell of common variable immunodeficiency (CVID) patients compared with healthy donors (a) (p=0.096) while the expression level of BLIMP1 (b) and IRF4 (c) were downregulated in B-cell of CVID patients compared with controls. A nonparametric test Mann-Whitney U Test was applied to compare quantitative data between 10 patients and 10 healthy controls. The median is represented by a horizontal line, the interquartile range by box, and the 10th and 90th percentiles by whiskers

Table 3. Comparison of immune-related clinical manifestations between CVID patients with MiR overexpression vs. normal

Clinical manifestation	3 patients(high)	7 patients(normal)	*p*
URI; number (%)	2(66.67%)	6(85.71%)	0.49
LRI; number (%)	3(100%)	6(85.71%)	0.49
Recurrent infection; number (%)	3(100%)	6(85.71%)	0.49
Otitis; number (%)	2(66.67%)	5(71.43%)	0.88
Sinusitis; number (%)	2(66.67%)	5(71.43%)	0.88
Pneumonia; number (%)	1(33.33%)	5(71.43%)	0.26
Allergy; number (%)	1(33.33%)	3(42.86%)	0.778
Autoimmunity; number (%)	0	2(28.57%)	0.301
Arthritis; number (%)	1(33.33%)	2(28.57%)	0.88
LAP; number (%)	0	2(28.57%)	0.301
Recurrent diarrhea; number (%)	2(66.67%)	6(85.71%)	0.49
Poor growth; number (%)	1(33.33%)	1(14.28%)	0.49
Clubbing; number (%)	0	4(57.14%)	0.091

CVID: Common Variable Immune deficiency, URI: Upper Respiratory tract Infection, LRI: Lower Respiratory tract Infection, LAP: Lymphadenopathy. p-value <0.05 is statistically significant.

Table 4. Chi-square of B cell subsets between CVID patients with miR overexpression vs. normal expression

B-cell subsets-Quality	3 patients(high)	7 patients (normal)	*p*
$CD19^{+}$ B-cell	Normal: 2 Decreased: 1 Increased: -	Normal: 5 Decreased: 2 Increased: -	0.88
Naïve B-cell	Normal: - Decreased: - Increased: 3	Normal: 2 Decreased: - Increased: 5	0.301
Marginal zone B-cell	Normal: 1 Decreased: 2 Increased: -	Normal: 0 Decreased: 5 Increased: 2	0.202
Switch memory B-cell	Normal: 0 Decreased: 3 Increased: -	Normal: 1 Decreased: 5 Increased: 1	0.585
IgM^{+} memory B-cell	Normal: 3 Decreased: - Increased: -	Normal: 4 Decreased: 2 Increased: 1	0.399
Non-Switch memory B-cell	Normal: 0 Decreased: 3 Increased: -	Normal: 1 Decreased: 6 Increased: -	0.49
Transitional B-cell	Normal: 0 Decreased: - Increased: 3	Normal: 1 Decreased: - Increased: 6	0.49
$CD21^{low}$ B cells	Normal: 1 Decreased: - Increased: 2	Normal: 0 Decreased: - Increased: 7	0.107
Plasmablast	Normal: 1 Decreased: 2 Increased: -	Normal: 2 Decreased: 5 Increased: -	0.88

CVID: Common Variable Immune deficiency, $p<0.05$ is statistically significant

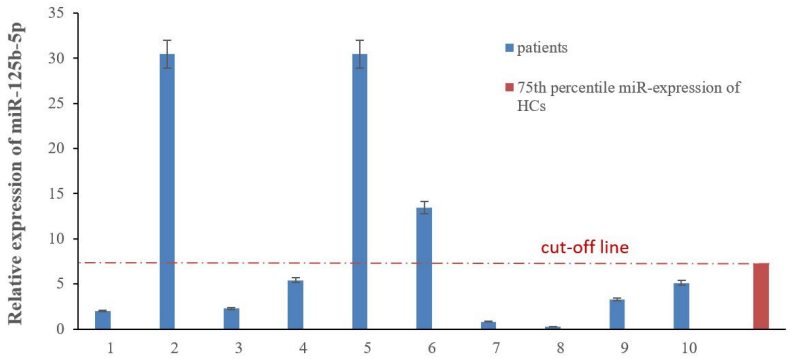

Figure 2. miR-125b-5p relative expression in each patient compared with interquartile range (IQR) of control. Cut-off line: Patients were categorized into high-miR and normal-miR expression groups according to the 75th percentile of HCs.

DISCUSSION

In recent years, epigenetic abnormalities have been noticed as features that play an important role in the dysregulation of B-cell subsets in CVID individuals.[16,17] This study is the first report of alteration in expression levels of microRNAs in unsolved CVID with excluded known PID gens and PID candidate genes. Here we specifically evaluated miR-125b-5p expression which has a major impact on the terminal differentiation of B cells in CVID patients. We demonstrated that the expression level of miR-125b-5p in B lymphocytes of CVID patients with no definitive genetic diagnosis was increased compared with healthy donors. In contrast, the expression levels of *IRF-4* and *BLIMP-1* were decreased.

As reported in previous studies, the majority of CVID cases experience respiratory infections in the upper and lower respiratory tract.[1] Non-infectious complication like autoimmunity and lymphoproliferative disorder are also frequent among these patients.[23] In this study, the frequencies of clinical manifestations in CVID patients included upper and lower respiratory tract infections 80-90% mostly in forms of sinusitis 70%, otitis 70%, and pneumonia 60%. Other severe complications like autoimmunity 20%, lymphadenopathy 20% were less frequent among our unsolved patients compared to total registered CVID patients.[24] Since our patients were excluded for genetic defects and monogenic patients present more severe clinical manifestations. Therefore, the reason that our patients showed milder complications might be due to our inclusion criteria.[25-27] Moreover, we also observed high-miR patients with terminal B cell defects were presented exclusively with infectious-only phenotype, suggesting the remaining unsolved patients with non-infectious complications may have extrinsic B cell defects.

We previously reported that plasma cells and memory B cells were decreased in the majority of our registered CVID patients.[11] Nevertheless, these unsolved patients had usually normal circulating B cell counts, indicating that there is an impairment in the terminal stages of B-cell differentiation.[6] In the present study, we observed that all high-miR patients had decreased switched and non-switched memory B cells and naïve B cells and transitional B-cell compared to patients with normal miR expression. These findings highlight defective terminal stages of B-cell differentiation in unsolved CVID patients. In this sense, Ahn et al have reported that despite normal B cell count in CVID patients, immunoglobulin levels were decreased and suggested that this is related to an impairment in final B cell differentiation.[28] Also, Blance et al, evaluated B cell subsets in 61 CVID cases and their results indicated a lack of plasmablast and reduced memory B cells in these patients.[29] Defects in B-cell differentiation could be explained by different hypothesizes including increased apoptosis of B-cells or defect in B-cell activation and demethylation of specific CpG sites at genes which might contribute to the transition from naïve to memory B-cells in CVID patients.[17,30,31] But due to the regulatory/inhibitory effect of miR125b-5p on *IRF-4* and *BLIMP-1* expression, which are essential transcription factors for plasmablast differentiation, suggesting that the increase in miR125-b expression, at least in a selected group of patients, downregulates these factors and subsequently affects final B cell differentiation. Since there was not a significant correlation between overexpression of miR125-b and these factors, further investigations in a larger patient population are needed to confirm this hypothesis.

The differentiation of B lymphocytes in the germinal center occurs in sequential events of isotype switching, affinity maturation, and plasmablast formation.[32] IRF-4 is known as a key transcription factor in the late stages of B-cells development. Depending on the amount of IRF-4 concentration, B cell undergoes two different developmental pathways. The low or intermediate concentration of *IRF-4* induces *AID* expression which leads to CSR and somatic hypermutation (SHM). A higher concentration of *IRF4* drives plasmablast formation by inducing *BLIMP-1* expression[33] (see the result). Supporting these data, Indrevaer et al,[32] have observed defects in B lymphocyte function of CVID individuals followed by high expression of *IRF-4* which leads to abnormal expression of AID in these patients. BLIMP-1 is regarded to be a key factor of plasma cell formation and it is required for the differentiation of activated B-cell into plasma cells (see the result). Though Taubenhelm et al, have indicated that despite normal B cell count and *BLIMP-1* expression in lymph nodes of 3 CVID cases, the serums of these patients lacked immunoglobulins. Since B cell differentiation was normal until plasma blast formation, they suggested that the defect might be related to factors downstream

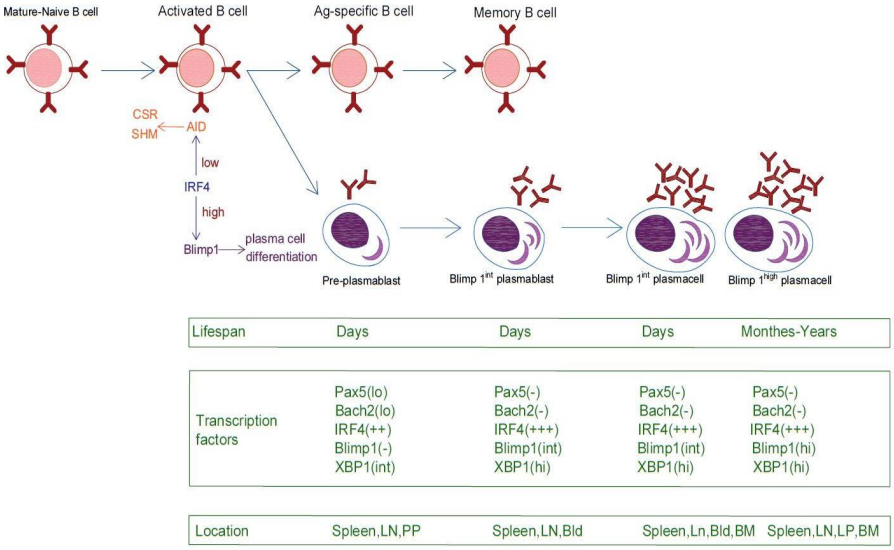

Figure 3. Low to moderate expression of IRF4 leads to activate AID expression which is responsible for SHM and CSR in late stages of B cell differentiation, while optimal expression of IRF-4 favors BLIPM-1 expression and subsequent plasma cell formation. LN: Lymph Node, Bld: Blood, PP: Peyer's patches, BM: Bone Marrow

of BLIMP-1 as an alternative mechanism for the CVID pathogenesis.[34] As explained above, there is a positive correlation between the expression of IRF-4 and BLIMP-1. In line with the notion, our results showed a noticeable decrease in *IRF-4* and subsequent *BLIMP-1* expression along with overexpression of miR-125b-5p. However, Afshar Ghasemlou et al and Farrokhi, et al have reported that the expression of IRF4 and BLIMP-1 were increased in CVID cases respectively.[35,36] One of the most important reasons for this discrepancy can be our inclusion criteria. In this study, we included patients who demonstrated no genetic defects after whole-exome sequencing. it could be assumed that in such patients' epigenetic mechanisms have an important role in the differentiation of B cells into antibody-secreting plasma cells. Another possibility could be that Afshar Ghasemlou et al and Farrokhi, et al evaluate IRF-4 and BLIMP-1 expression in stimulated B lymphocytes; while we assess the genes in unstimulated B-cells.[35,36] As Farrokhi, et al has reported we are not able to compare expression levels of BLIMP-1 and IRF-4 in both stimulated and unstimulated conditions, and more studies are required to elucidate this discrepancy in this pathway.

Few studies are investigating epigenetic factors in CVID pathogenesis and clinical manifestations. Rodriguez-Cortez et al have shown that alteration in DNA methylation patterns in a discordant CVID twin is involved in the presentation of clinical manifestations.[16] There is not any report that evaluated miRNA profile in CVID individuals, however, Karmer et al have suggested the role of miRNAs in developing CVID-like manifestations in mouse models with deletion of miR-142-3p.[37] Similarly, in this study, we observed that all patients especially the high-miR group developed infectious only clinical phenotypes related to CVID. Therefore, we suggest that the up-regulation of miR-125b might be the underlying reason for these complications. Unfortunately, there is no similar study in this field that we can compare our results with.

To the best of our knowledge, this is the first study to investigate the expression of miR-125b-5p in CVID patients. Based on the effect of this microRNA on the expression level of *IRF-4* and *BLIMP-1* transcription factors, we proposed that the overexpression of miR-125b-5p might be responsible for reduced levels of *IRF-4* and *BLIMP-1* expressions which results in an impairment in terminal differentiation of B cells in a

selected group of CVID patients. However further studies are required to elucidate the long-term correlation of miR-125b-5p overexpression and clinical outcomes of CVID patients.

In this study, we tried to focus on the role of miRNA expression and immune dysregulation in CVID patients which has not been reported in previous studies. We observed reduced *IRF-4* and *BLIMP-1* expressions followed by overexpression of miR-125b-5p. Also all patients especially the high-miR group developed infectious-only clinical phenotype. Although these findings highlight the importance of epigenetic control and specially altered microRNA expressions on terminal B lymphocyte differentiation defects, further researches are required to clarify the correlation of microRNA expression with B cell development and to identify underlying mechanisms in the pathogenesis of CVID cases with no genetic defects.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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