



Prognostic significance of Fc receptor-like 1 in patients with chronic lymphocytic leukemia, hairy cell leukemia, and various B-cell non-Hodgkin's lymphoma

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ARTICLE INFO

Keywords:

Fc receptor-like 1
Hairy cell leukemia
Chronic lymphocytic leukemia
B-cell non-Hodgkin's lymphoma

ABSTRACT

Fc receptor-like 1 (FCRL1) positively regulates B-cell responses and may involve in the pathogenesis of B-cell malignancies. This study examined the expression pattern of FCRL1 in B-cell non-Hodgkin's lymphoma patients using real-time PCR and flow cytometry. The results revealed higher levels of FCRL1 expression in diffuse large B-cell lymphoma, hairy cell leukemia, and Burkitt lymphoma patients compared with control groups. There was a significant reduction in the levels of FCRL1 expression in chronic lymphocytic leukemia and mantle cell lymphoma patients compared with healthy individuals. These findings suggest FCRL1 as an excellent marker for the prognosis or immunotherapy of B-cell malignancies.

1. Introduction

B-cell non-Hodgkin's lymphoma (B-NHL) represents a wide spectrum of malignancies with different clinical outcomes [1]. To date, various studies have been conducted to understand the biology and causes of these B-cell malignancies and their results have improved the prognosis and treatment of patients suffering from these diseases. Previous studies have also demonstrated that the majority of patients with distinct subtypes of B-NHL respond well to current therapeutic strategies. However, there is still no suitable therapy for aggressive types of B-cell leukemia and lymphomas [2,3]. More research is needed to find new diagnostic biomarkers and appropriate therapies for B-NHL disorders. Molecular analysis and gene profiling studies have recently demonstrated the involvement of Fc receptor-like (FCRL) molecules in several disorders such as B-cell-derived hematological malignancies [4–9].

FCRL molecules are an interesting group of lymphocyte receptors that have been suggested as useful targets for the classification or immunotherapy of different B-cell-related disorders [4–14]. They are also known as Fc receptor homologs (FcRH), IgSF receptor translocation-associated genes (IRTA), Src homology 2 domain-containing phosphatase anchor proteins (SPAP), B-cell cross-linked by anti-immunoglobulin M-activating sequences (BXMAS), and immunoglobulin-FcR-gp42-related genes (IFGP) [12,13,15–20]. However, the unified

nomenclature FCRL was selected to describe these molecules by the Mouse Genomic Nomenclature Committee and the Human Genome Organization Gene Nomenclature Committee [21]. Moreover, the Ninth HLDA Workshop assigned CD307a-e to characterize FCRL1-5 molecules [22]. The FCRL genes are located in close proximity on human chromosome 1q21–23, and they encode two intracellular receptors (FCRL A and B) and six transmembrane glycoproteins type I (FCRL1-6) consisting of three to nine immunoglobulin (Ig)-like domains in the extracellular region and immunoreceptor tyrosine-based activation (ITAM)/inhibition (ITIM) motifs in their cytoplasmic domain [11,20,23–25]. FCRL1-5 molecules are preferentially expressed in B-lineage cells, although expression of FCRL3 is also recognized in natural killer (NK) cells and different subsets of T lymphocytes, including CD8⁺, CD4⁺, and regulatory T cells [26–29]. In contrast to FCRL1-5 molecules, the human FCRL6 gene is located in the proximity of high-affinity IgE receptor genes and extensively expressed by mature NK cells (CD16⁺, CD56dim), NKT cells, cytotoxic CD4⁺ T cells, cytotoxic CD8⁺ γδ T cells, and effector and memory subtypes of CD8⁺ T cells [19,30–33]. Differential expression of FCRL1-5 molecules by lymphocyte cells and the presence of ITAM/ITIM motifs in their cytoplasmic domains suggest some immunoregulatory roles to these molecules, which affect the development and regulation of human B cell responses [12,20,23,24]. Recent studies have evaluated the expression profile of FCRL molecules in various B-cell malignancies to determine whether

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<https://doi.org/10.1016/j.lrr.2019.100181>

Received 15 December 2018; Received in revised form 6 June 2019; Accepted 9 August 2019

Available online 14 August 2019

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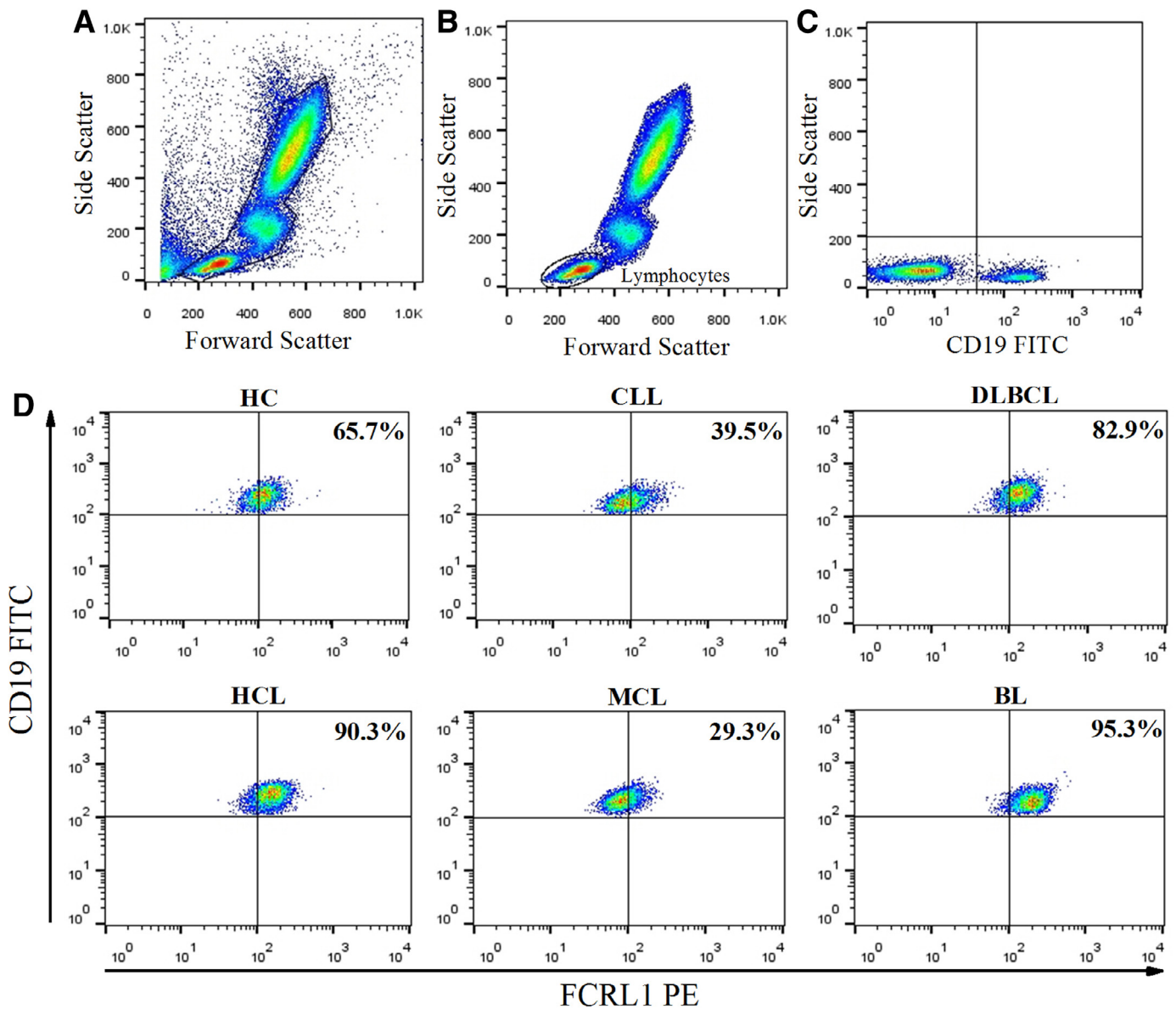


Fig. 1. Gating strategy and flow cytometric dot-plots data of FCRL1 expression in the blood cells. (A) The live cells were first gated based on the forward scatter/side scatter (FSC/SSC) signals. (B) Then, total lymphocytes were selected. (C) The lymphocyte gate was further analyzed for CD19, and (D) Fc receptor-like 1 (FCRL1) expression (%) in CD19⁺ B cells of healthy controls (HC) and patients with chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), hairy cell leukemia (HCL), mantle cell lymphoma (MCL), and Burkitt lymphoma (BL).

Table 1
FCRL1 expression in patients with various B-cells non-Hodgkin's lymphoma and healthy individuals: summary of the results.

Groups	No. of samples		Expression levels of FCRL1 molecule		
	Total	Male/ Female	FCRL1 positive (%)	Protein (MFI) Mean ± SD	mRNA (fold) Mean ± SD
Normal	27	17/10		127.37 ± 7.6	2.82 ± 0.71
CLL	13	7/6	2(15.3)	97.17 ± 26.7	1.6 ± 1.07
DLBCL	26	15/11	21(80.7)	163.21 ± 38	6 ± 3.18
HCL	10	9/1	9(90)	172.174 ± 29.09	6.9 ± 2.6
MCL	6	6/0	0(0)	88.05 ± 22.9	1.08 ± 0.3
BL	3	3/0	3(100)	186.5 ± 6.5	8.5 ± 0.6

these molecules have the potential to be used as good markers for the prognosis or immunotherapeutic interventions of cancers. Findings showed aberrant expression of some members of the FCRL family molecules in several types of B-cell-related leukemia and lymphomas, such

as FCRL1-5 molecules in acute lymphoblastic leukemia (ALL) [34] and chronic lymphocytic leukemia (CLL) patients [7,8,35], FCRL4 in diffuse large B-cell lymphoma (DLBCL) subjects [36], and FCRL5 in the patients with multiple myeloma (MM), CLL, mantle cell lymphoma (MCL) and hairy cell leukemia (HCL) [4,7,35,37,38].

Among the FCRL molecules, FCRL1 has two ITAM-like components in its intracellular tail and preferentially expressed on B lineage cells [7,14]. Previous studies have indicated the potential of FCRL1 molecule to amplify B-cell activation and showed a positive correlation between overexpression of this molecule and up-regulation of various activation co-receptors, including CD80, CD86, and CD69 in human B cells [39,40]. Abnormal expression of this molecule was also reported in different Burkitt lymphoma (BL) and DLBCL cell lines [8,12] as well as some diseases, including CLL, MCL, HCL, MM, and follicular lymphoma (FL) [7,8,35]. These data suggest an essential role for FCRL1 in the pathogenesis or development of B-cell malignancies. However, these results were derived from studies performed on Western populations.

The aim of the current study was to investigate the extent of FCRL1

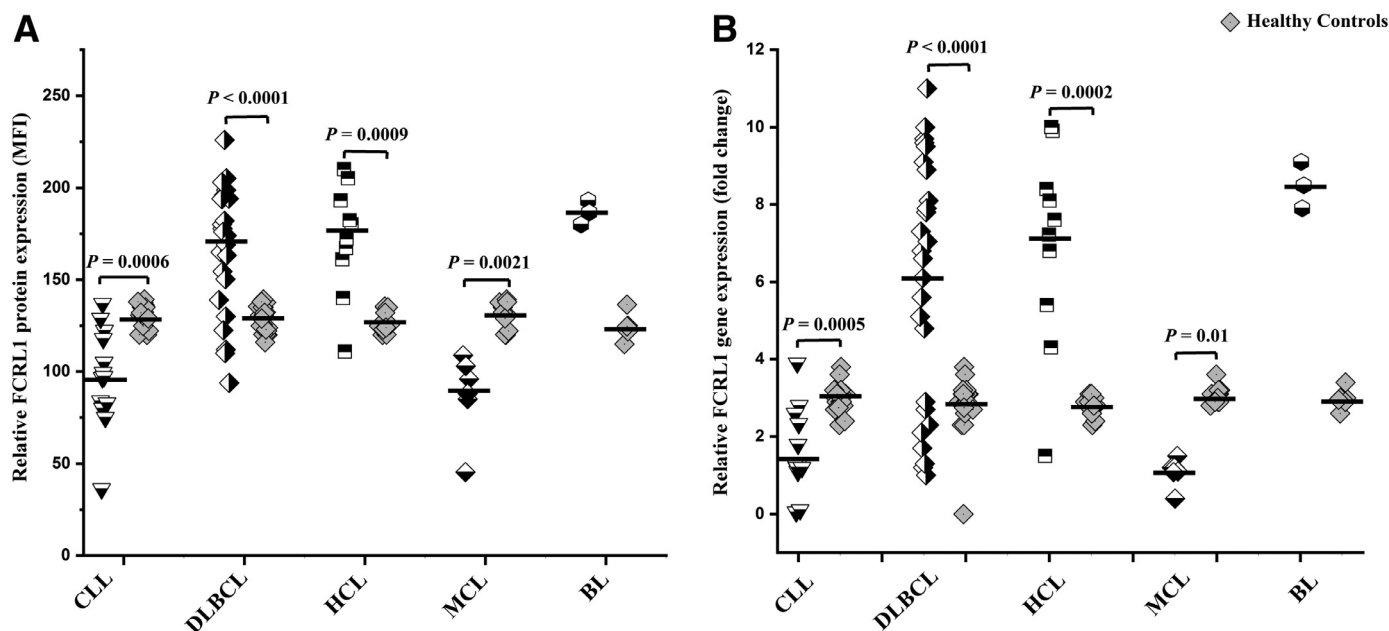


Fig. 2. Expression levels of Fc receptor-like 1 (FCRL1) protein in peripheral blood cells from B-NHL patients compared with healthy controls assessed by flow cytometry and data are presented as mean fluorescent intensity (MFI) in protein levels. (B) Expression levels of FCRL1 mRNA in peripheral blood mononuclear cells (PBMCs) from patient groups compared with healthy controls measured by quantitative real-time PCR and the results are reported as median fold changes in mRNA levels. Data show mean \pm standard deviation (SD), and horizontal bars represent mean values. CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; HCL, hairy cell leukemia; MCL, mantle cell lymphoma; BL, Burkitt lymphoma.

protein and mRNA expression in Iranian patients with DLBCL, CLL, MCL, HCL, and BL compared with healthy individuals to determine whether FCRL1 is a suitable target for prognostic or antibody-based therapies for these B-cell malignancies in Iranian patients.

2. Patients and methods

2.1. Patients

Fifty-eight Iranian patients with distinct subtypes of B-NHL were included in the study. Diagnosis of B-NHL was confirmed based on clinical examinations, diagnostic criteria, and tumor-specific immunophenotyping [41,42]. Included samples consisted of 26 patients with DLBCL (38–79 years old; with a mean age of 65.6 ± 12.8 years; 57.6% male), 13 patients with CLL (54–79 years old; with a mean age of 70.46 ± 7.9 years; 53.8% male), 10 patients with HCL (28–71 years old; with a mean age of 56.2 ± 13.05 years; 90% male), 6 patients with MCL (51–77 years old; with a mean age of 71.3 ± 10.1 years; 100% male), and 3 patients with BL (20–32 years old; with a mean age of 25.6 ± 6.02 years; 100% male). They had not received any treatments at the time of the blood sampling.

To determine the normal cut-off value of FCRL1 expression, twenty-seven age and sex-matched healthy individuals (20–79 years old; with a mean age of 55.5 ± 19.2 years; 62% male) without any history of cancer were also included in this study. All procedures were performed in accordance with the declarations of Helsinki and approved by the Ethics Committee of Isfahan University of Medical Sciences (reference number: 394608). Informed consent was taken from all individuals before taking a blood sample.

2.2. Flow cytometry

Anti-coagulated peripheral blood samples were taken from the participants. About 100 μ l of whole blood from each sample of the patients with DLBCL, MCL, CLL, HCL, BL, and the healthy individuals was stained with a mixture of fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (clone: HIB19) (BD Biosciences, San Jose, CA, USA),

Phycoerythrin (PE)-conjugated anti-FCRL1 (clone: REA440) (Miltenyi Biotec, Auburn, CA, USA), and other tumor-specific antibodies as previously described [41,42]. Then, the red blood cells (RBCs) were removed by BD Pharm Lyse™ lysing buffer $\times 10$ according to the kit instructions (BD Biosciences). Afterwards, the cells were washed twice with phosphate-buffered saline (PBS), and the single cells were suspended in 500 μ l PBS/1% bovine serum albumin (BSA). 1×10^5 cells were gained for each sample and debris excluded based on the side scatter (SSC) vs. forward scatter (FSC) signals, representing granularity and size of the cells, respectively. All dead cells with damaged membranes were also excluded by DNA-binding dye propidium iodide (PI). Lymphocyte cells were selected and CD19⁺ B cells isolated from the lymphocyte population. Finally, the mean fluorescence intensity (MFI) of the FCRL1 expression was determined on CD19⁺ B lymphocytes using FACSCalibur flow cytometry (Becton Dickinson Biosciences, San Jose, CA, USA) and then analyzed by FlowJo software (v10.1, FlowJo, Ashland, OR, USA). The matched isotype control antibodies and Burkitt lymphoma cell line Ramos were used as negative and positive controls, respectively. The Fluorescence Minus One (FMO) controls were also used for the proper gating of cell population and determination of fluorescence spread.

2.3. Isolation of peripheral blood mononuclear cells

Ten milliliters of peripheral blood containing ethylenediaminetetraacetic acid (EDTA) was collected from both patients and healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated from each peripheral blood sample using Ficoll density-gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). After centrifugation, the PBMCs were collected and washed three times in PBS. The percentage of cell viability was determined using the Trypan blue dye exclusion method, and cells were stored at -80°C for further analysis.

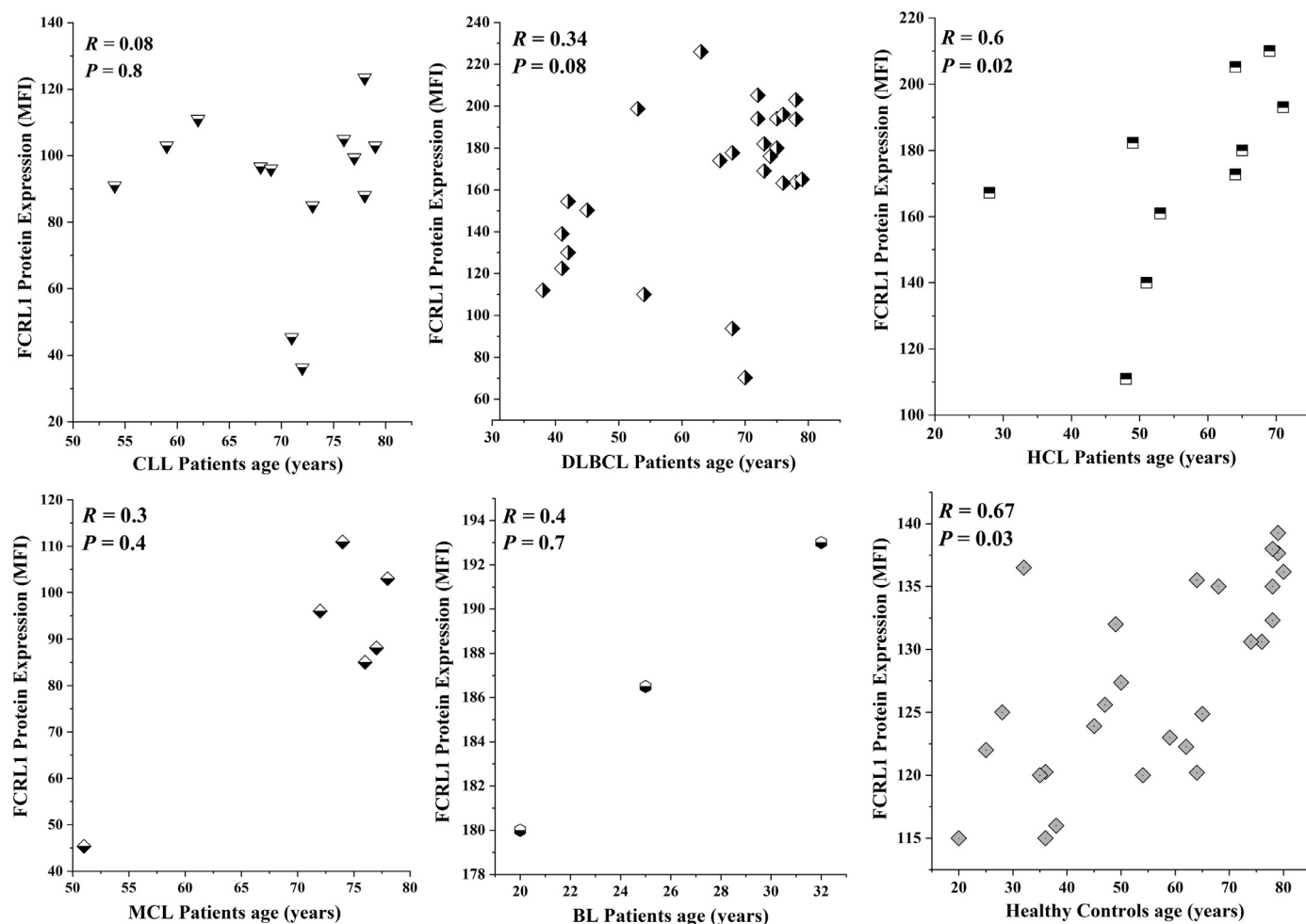


Fig. 3. Correlation between Fc receptor-like 1 (FCRL1) protein expression and age in B-NHL patients and healthy individuals. There was a positive but not significant correlation between FCRL1 protein expression and age in diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and Burkitt lymphoma (BL) patients. FCRL1 protein expression showed a significant positive correlation with age in hairy cell leukemia (HCL) patients and healthy controls. *R*, correlation coefficient.

2.4. RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction

The total RNA was extracted from about 2×10^6 PBMCs using the RNax plus solution (Cinagen Company, Tehran, Iran) according to the manufacturer's guidelines. Afterwards, the quality and concentration of the extracted RNAs were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) in the 260/280 nm absorbance ratio. Synthesis of the first strand of complementary DNA (cDNA) was conducted using the one-step SYBR PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Shiga, Japan) according to the following program: 37 °C for 15 min, 85 °C for 5 s, and 20 °C for 5 min. The synthesized cDNA was stored at -20 °C for further real-time polymerase chain reaction (PCR) analysis.

Quantitative real-time PCR was performed using SYBR Green PCR master mix (Takara Bio Inc.) on the cDNA samples by a Rotor-gene 6000 instrument (Qiagen, Hilden, Germany) to evaluate the relative expression levels of FCRL1 mRNA in the patient groups and healthy individuals. The human β -actin gene was included as the internal control to normalize the results.

Amplification of FCRL1 and β -actin genes were performed using specific primers including *FCRL1*: forward, 5'-CAGAGTTCAGATGCCAGTT-3', and reverse, 5'-TCACATCAGCAGGGAC-3'; β -actin: forward, 5'-GGACTTCGAGCAAGAGATGG-3', and reverse, 5'-AGCACTGTGTTGCGGTACAG-3'. A final volume of 20 μ l reaction mixture including 2 μ l

template cDNA, 10 μ l SYBR Green PCR master-mix, and appropriate amounts of each primer, dH₂O, and ROX reference Dye II was prepared for each sample.

A mixture reaction including 2 μ l of distilled water instead of template cDNA was also prepared for each set of primers as a no-template negative control (NTC). The real-time PCR program was optimized with denaturation at 95 °C for 5 s, followed by 40 cycles as annealing at 60 °C for 30 s and extension at 70 °C for 30 s. To obtain specific amplification, the melting curves of each reaction were checked, and each reaction was repeated three times. The Relative Expression Software Tool 2009 (REST 2009) and $2^{-\Delta\Delta Ct}$ formula were utilized to measure the relative fold changes of FCRL1 mRNA expression in patients and healthy individuals [43,44].

2.5. Statistical analysis

Data were analyzed using SPSS version 17 (SPSS Inc, Chicago, IL) and GraphPad Prism software (version 6.04, GraphPad Software, CA). Differences in the FCRL1 expression levels between the two groups with normal or non-normal distribution were determined using Student's *t*-test and Mann-Whitney *U* test, respectively. The Spearman Correlation test was also used to examine the probable association between FCRL1 expression levels and both gender and age of the patient groups and healthy individuals. Data are shown as mean \pm standard deviation (SD). A *p*-value < 0.05 was considered as statistically significant.

3. Results

3.1. Expression level of FCRL1 protein

The expression level of FCRL1 protein in the blood cells of patients diagnosed with various B-NHL and healthy individuals was examined using the flow cytometry assay. Based on the initial analysis of the MFI values, the FCRL1 protein expression was detectable in all patients and control samples. The flow cytometry results revealed a wide spectrum of FCRL1 positive cells in each group with a variety of MFI values ranging from low levels to high levels, without a distinct cut-off value. Gating strategy for flow cytometry analysis of FCRL1 protein expression in the blood cells is illustrated in Fig. 1A–C. The extent of the expression of FCRL1 protein in the CD19⁺ B cells of a healthy individual and a number of patients diagnosed with CLL, DLBCL, HCL, MCL, and BL are shown in Fig. 1D.

The results of flow cytometry analysis showed that 15.3% of patients diagnosed with CLL (2/13), 80.7% of DLBCL patients (21/26), 90% of HCL patients (9/10), 0% of MCL patients (0/6), and 100% of patients with BL (3/3) expressed higher levels of FCRL1 protein when compared with age and sex-matched healthy controls (Table 1). There was a significant increase in the expression levels of FCRL1 protein in DLBCL ($P < 0.0001$) and HCL ($P = 0.0009$) patients compared with healthy subjects. This study also revealed a significant reduction in the expression levels of FCRL1 protein in CLL ($P = 0.0006$) and MCL ($P = 0.0021$) patients compared with the healthy subjects. In addition, the results showed higher levels of FCRL1 protein expression in BL patients compared with healthy individuals, although it was not significant due to the small number of BL patients in this study (Fig. 2A).

3.2. Expression level of FCRL1 mRNA

The relative expression of FCRL1 mRNA was investigated in PBMCs derived from the patients and controls using the quantitative real-time PCR method. Data showed expression of FCRL1 mRNA in the majority of patients and healthy individuals. There were higher levels of FCRL1 mRNA expression in DLBCL ($P < 0.0001$), HCL ($P = 0.0002$), and BL patients compared with healthy individuals. However, compared with healthy controls, a significant reduction in FCRL1 mRNA expression was observed in both CLL ($P = 0.0005$) and MCL ($P = 0.01$) patients. Representative data for all samples are shown in Fig. 2B.

3.3. FCRL1 expression levels in relation to age and gender

The association between expression levels of FCRL1 protein and age in patients and healthy individuals were also investigated. The results showed a positive but not significant correlation between FCRL1 expression levels and age in CLL, DLBCL, MCL, and BL patients. However, a significant positive association was observed between FCRL1 expression levels and age in both HCL patients ($P = 0.02$) and healthy individuals ($P = 0.03$) (Fig. 3). The results also showed no significant differences in FCRL1 expression levels between males and females in any of the evaluated groups (data not shown).

4. Discussion

FCRL1 is a member of the FCRL family molecules that has positive signaling potential and is encoded by genes on human chromosomal bands 1q21-23, where they are associated with chromosomal abnormalities in various B-NHL and multiple myeloma [25,33,45–47]. The structural features and signaling potential of the FCRL1 suggest that this human B cell receptor plays a crucial role in the pathogenesis or development of B-NHL [16,48]. The investigation of the FCRL1 expression pattern is a significant first step toward understanding the functional roles of this molecule in B-NHL disorders and its possible implications in the clinic as a suitable target for prognosis or treatment

of FCRL1-positive B-cell malignancies. This study investigated the extent of FCRL1 protein and FCRL1 mRNA expression in the blood cells of Iranian patients with various B-cell leukemia and lymphomas compared with healthy individuals. The data revealed the extensive expression of FCRL1 in the majority of the patients. This study showed that FCRL1 expression levels were higher in blood cells derived from patients with DLBCL, HCL, and BL compared with healthy individuals. These findings are consistent with the results of a previous study which showed the wide expression of FCRL1 protein on leukemic cells from patients with HCL and different DLBCL and BL cell lines using flow cytometry assay [8,12]. Nevertheless, little data has been reported on the expression pattern of FCRL1 in DLBCL and BL patients.

This study also revealed a significant reduction in the extent of the expression of FCRL1 protein and FCRL1 mRNA in both MCL and CLL patients compared with healthy subjects. These findings were in contrast to other relevant studies, in which the investigators reported higher levels of protein and mRNA expression for the FCRL1 molecule in MCL and CLL patients compared with the healthy controls [6–8,35,49]. This observed discrepancy could be due to a technical shortcoming or probable differences in the sensitivity of the applied methods. However, further studies are needed to evaluate this event. In addition, these differences in the expression pattern of FCRL1 in various B cell malignancies may be related to the different roles of this molecule in these diseases. Additional investigations are required to explore the biology and functional roles of FCRL1 in various B-NHL, which might be helpful for the immunotherapy of these malignancies.

The small number of samples examined could be considered as a limitation of the present study. In addition, blood cells and PBMCs were used to evaluate the extent of FCRL1 protein and mRNA expression in various B-NHL patients and healthy controls, respectively. The results would have been more reliable if purified B cells had been used for the assessment of FCRL1 expression patterns.

In summary, the current results revealed the differential expression of FCRL1 molecule in various B-NHL patients compared with healthy individuals and suggest this molecule as a suitable marker for the prognosis or treatment of FCRL1-positive B-cell-related leukemia and lymphomas such as DLBCL, HCL, and BL.

Funding

This work was supported by Isfahan University of Medical Sciences (grant no. 394608).

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

Authors would like to thank all individuals who participated in this study.

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