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Fc receptor-like 1–5 molecules are similarly expressed in progressive and indolent clinical subtypes of B-cell chronic lymphocytic leukemia

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Fc receptor-like (FCRL) 1–5 molecules are exclusively expressed in B-cells and have recently been considered as potential targets for immunotherapy of B-cell malignancies. In this study, the expression pattern of FCRL1–5 molecules was investigated in Iranian patients with B-cell chronic lymphocytic leukemia (B-CLL). Our RT-PCR results have demonstrated that all FCRL molecules, except FCRL4, were expressed in the vast majority of the patients with B-CLL. However, comparison of the relative mRNA expression levels of FCRL between B-CLL ($n = 86$) and elderly normal subjects ($n = 10$) revealed significantly lower expression levels of FCRL1 ($p < 0.0001$), FCRL3 ($p = 0.01$) and FCRL4 ($p = 0.002$), but not FCRL2 or FCRL5, in cases with B-CLL. No significant differences were observed between the indolent and progressive subtypes of patients with B-CLL. Comparison between the mutated and unmutated subtypes revealed a significantly higher expression level of FCRL3 ($p = 0.017$) in patients with mutated CLL. Surface and intracytoplasmic expression of FCRL1, 2, 4 and 5 in leukemic cells of 12 patients by flow cytometry revealed similar results to those obtained by RT-PCR with a few exceptions. Thus, while FCRL4 was expressed in only 2 samples at intracytoplasmic level, FCRL1 and 2 were expressed in the majority of samples, both at surface and intracytoplasm. FCRL5 protein was also detected in 10 samples, but surface expression was confirmed in only 2. Analysis of B-cells from 5 normal subjects by flow cytometry revealed higher expression levels of FCRL molecules compared to CLL. Our results indicate differential expression of FCRL molecules in B-CLL and suggest the potential implication of FCRL1 and 2 for immunotherapeutic interventions.

Key words: FCRL; CLL; RT-PCR; flow cytometry

Recently identified Fc receptor-like (FCRL) molecules,[1,2] previously known as Fc receptor homolog (FcRH),[3] immunoglobulin superfamily receptor translocation associated (IRTA),[4] immunoglobulin superfamily-Fc receptor-gp 42 (IFGP),[5] SH2 domain-containing phosphatases anchor protein (SPAP)[6] and anti-IgM activating sequence (BXMAS),[7] are exclusively expressed in B-cell lineage, with the exception of one member of the family (FCRL6), which is mainly expressed in T and NK cells.[8] Owing to the presence of immunoreceptor tyrosine-based activating/inhibitory motifs (ITAM/ITIM) in their cytoplasmic region,[9] some immunomodulatory roles have been attributed to these molecules. FCRL1 may act as activating coreceptor on B cells,[10] whereas FCRL4 can potently inhibit B cell receptor signaling.[11] mRNA expression analysis in normal B cells indicates that FCRL1 and FCRL2 are highly expressed in naïve B cells.[12] In contrast, FCRL3 and FCRL5 are predominantly expressed in centrocytes and postgerminal center (GC) cells.[12] FCRL4 transcripts are exclusively detected in memory B cells.[11,12] FCRL3 molecule has also been localized on marginal zone B-cells as well as B1 cells, both of which are implicated in T-cell-independent antibody response.[13]

Several studies have recently investigated the expression pattern of FCRL molecules in a variety of B-cell malignancies.[4,7,8,14,15] Secretory form of FCRL5, also known as soluble CD307, has been shown to be elevated in the blood of patients with CLL, multiple myeloma (MM) and mantle cell lymphoma (MCL), and its level was correlated with tumor burden.[16]

Limited reports have so far been published regarding the expression profile of FCRL family members in the leukemic cells of B-cell chronic lymphocytic leukemia (B-CLL). Initial studies were conducted employing the microarray methodology.[17] Expression of FCRL molecules at protein level has recently been studied by immunocytochemistry or flow cytometry.[15,16,18] Comparative analysis of FCRL expression at mRNA and protein levels has not been studied yet. Meanwhile, all published data has been derived from studies conducted on Western populations. In the present study, the expression profile of FCRL1–5 molecules was investigated for the first time in 86 Iranian patients with progressive or indolent B-CLL by RT-PCR. Protein expression of these molecules was also studied both at surface and intracytoplasmic levels in 12 patients and 5 normal subjects by flow cytometry.

Material and methods

Patients and controls

Heparinized peripheral blood was collected from eighty-six Iranian patients with CLL attending the Oncology Clinics of Vali-Asr and Firozgar Hospitals, affiliated to Medical Sciences/University of Tehran and Iran University of Medical Sciences, respectively. A consent letter was taken from all patients and the study was approved by the Ethical Committee of Medical Sciences/ University of Tehran. The patients were clinically categorized in indolent ($n = 41$), progressive ($n = 38$) and newly diagnosed ($n = 7$) subtypes. Twenty-one patients were under treatment of 6 months to 3 years before the time of sampling. Disease progression was identified on the basis of either of the following criteria: lymphocyte count doubling time of less than 1 year; progression to a more advanced Rai stage; development of systemic symptoms; development of Richter's syndrome; downward trend of hemoglobin

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TABLE I – LIST OF SPECIFIC PRIMERS USED FOR AMPLIFICATION OF FCRL AND β-ACTIN GENES

Gene name	Primer sequence	Amplicon size (bp)
FCRL1		
F	5′-CTC AAC TTC ACA GTG CCT ACT GGG-3′	793
R	5′-TCC TGC AGA GTC ACT AAC CTT GAG-3′	
FCRL2		
F	5′-CCA GTG TAT GTC AAT GTG GGC TCTG-3′	430
R	5′-CAT TCT TCC CTCAAATCT TTA CAC-3′	
FCRL3		
F	5′-CAG CAC GTG GAT TCG AGT CAC-3′	890
R	5′-CAG ATC TGG GAA TAA ATC GGG TTG-3′	
FCRL4		
F	5′-TCT TCA GAG ATG GCG AGG TCA-3′	686
R	5′-TTT TGG GGT GTA CAT CAA CAT ACAAC-3′	
FCRL5		
F	5′-TGT TGC CCT GTT TCT TCCAAT ACA-3′	768
R	5′-CAG AGT TGG CCG ACC TACGC-3′	
β-actin		
F	5′-CCTTCCTGGGCATGGAGTCCTG-3′	203
R	5′-GGACCAATGATCTTGATCTTC-3′	

F: forward primer; R: reverse primer.

(Hb) concentration or platelet count to below the normal range (Hb $<$ 13.5 g/dl for males and $<$11.5 g/dl for females; platelet count $< 150 \times 10^9$/l) even when not meeting the criteria for stage III or IV disease (Hb $<$ 10 g/dl; platelet count $< 100 \times 10^9$/l). Possession of one of these characteristics was sufficient to qualify as progressive disease.[19] Nucleotide sequence analysis of the immunoglobulin variable region heavy chain (IgVH) of the leukemic cells has allowed classification of the patients into mutated (n = 55) and unmutated (n = 31) groups, based on the presence of more than 2% mutation rate.[20] Major demographic features of all cases with CLL have been shown in supplementary Table 1. Ten elderly normal subjects (mean age 60 years) were also included to determine normal cut-off levels of FCRL mRNA expression. Peripheral blood mononuclear cells (PBMCs) of all subjects were isolated by density-gradient centrifugation using Histopaque (Sigma, St. Louis, MO). RNA extraction and cDNA synthesis were performed as previously described.[21]

Isolation of B- and T-cells from PBMC by magnetic bead separation

B- and T-cells were isolated from peripheral blood of 5 normal subjects using MACS negative selection kits (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, PBMCs were isolated and $CD19^+$ and $CD3^+$ cells were enriched by negative selection method using MACS microbeads and midiMACS columns. The purity of cells were $>$95% as determined by flow cytometry.

Polymerase chain reaction

Amplification of FCRL1–5 and β-actin mRNA was performed using specific primers listed in Table I. Twenty-five-microliter reaction mixture of PCR was prepared using 2.5 μl 10× PCR buffer, 3.5 μl (for β-actin) and 1 μl (for FCRL1 and 4) or 1.5 μl (for FCRL2, 3, and 5) 25 mM $MgCl_2$, 1.5 μl 10 mM dNTPs, 1 μl each primer (10 pmol/μl), 0.2 μl Taq-DNA polymerase (10 U/μl) (CinnaGen, Tehran, Iran) and 1 μl template cDNA. Each amplification reaction underwent initial denaturation at 94°C for 5 min followed by 39 cycles of denaturation at 94°C for 30 sec, annealing at 60°C (for FCRL2 and β-actin) or 62°C (for FCRL1, 3–5) for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplified products were visualized in 1.5% agarose gel containing ethidium bromide and documented with gel documentation system (UVP, CA, USA). Amplicon sizes were 793, 430, 890, 686 and 768 base pairs for FCRL1–5, respectively. Burkitt's lymphoma cell lines were used as positive controls for amplification of FCRL1, 3, and 5 (Ramos), FCRL2 (BL41) and FCRL4 (Raji). The densities of FCRLs and β-actin PCR product bands were determined by Labworks 4.0 software (UVP, CA), and the ratio of the two bands was calculated for each sample, as follows: (density of FCRL band/β-actin density band) × 100.

Indirect immunoflourescence

The staining was performed on PBMCs from 12 patients with CLL and 5 normal controls at both surface and intracytoplasmic levels. After separation of the PBMCs and twice washing with washing buffer (PBS 0.15 M, 0.5% BSA, 0.1% NaN_3), 10^6 cells were incubated with 2.5 μg/ml of biotinylated goat antihuman FCRL1, FCRL2, FCRL4 and FCRL5 antibodies (R&D Systems, Minneapolis, MN) as primary antibodies for 45 min. Biotinylated goat antihuman Ig and biotinylated unimmunized goat IgG (prepared in our laboratory) were also included as positive and negative controls, respectively. For intracytoplasmic staining, the cells were initially fixed with 2% paraformaldehyde in PBS (0.15 M, 0.1% NaN_3) for 10 min and, after twice washing, were incubated with permeabilizing solution (Beckton Dickinson, CA) for 10 min. Afterwards, the cells were washed twice with washing buffer and incubated with appropriate dilution of primary antibody. After incubation, the cells were washed twice with washing buffer and then incubated with RPE-conjugated streptavidin (DAKO, Glostrup, Denmark) as detector, for 45 min. Cells were then washed twice before scanning by flow cytometer (Partec, Nuremberg, Germany). Double staining of B cells from 5 normal subjects and 2 CLL patients was performed using FITC-CD19 monoclonal antibody (clone HD37, Cytomation; Dako Corp, Glostrup, Denmark) and biotinylated anti-FCRL Abs to determine the frequency of FCRL-expressing B cells. To calculate relative expression levels, mean fluorescence intensity (MFI) was determined by subtraction of the MFI of the antigen-specific fluorochrome-conjugated Abs from the MFI of the irrelevant fluorochrome-conjugated isotype-matched negative control. Data analysis was performed using Flomax flow cytometry analysis software (Partec, Nuremberg, Germany). Background staining with the biotinylated unimmunized goat IgG was used to eliminate nonspecific binding of the goat anti-FCRL antibodies.

Statistical analysis

Statistical analyses of the results were performed using Pearson Correlation, Chi-Square, student's t test, and Mann–Whitney U tests as appropriate. Analyses were conducted using the SPSS statistical package (SPSS, Chicago, IL). p-values of less than 0.05 were considered significant.

Results

FCRL1–5 mRNA expression

Qualitative expression of FCRL1–5 mRNA was determined in patients with B-CLL and normal controls by visualization of the corresponding PCR product following electrophoresis. Representative RT-PCR results obtained for a number of patients are illustrated in Figure 1. Our results showed expression of FCRL1–3 and 5 molecules in 91–100% of patients, with no significant differences between patients with B-CLL and normal elderly controls. FCRL4, however, was less frequently expressed in different groups of patients compared to normal subjects ($p < 0.05$) (Table II). Relative expression of FCRL1–5 mRNA levels in patients and normal subjects was determined by calculation of the ratio of FCRL PCR product band density to that of β-actin. Comparison of the expression levels of these molecules in PBMC obtained from CLL and normal elderly subjects demonstrated significant down-regulation of FCRL1 ($p < 0.0001$), FCRL3 ($p = 0.01$) and FCRL4 ($p = 0.002$) in cases with CLL (Table III and Fig. 2*a*). FCRL2 and 5, however, were similarly represented in patients with CLL and normal controls.

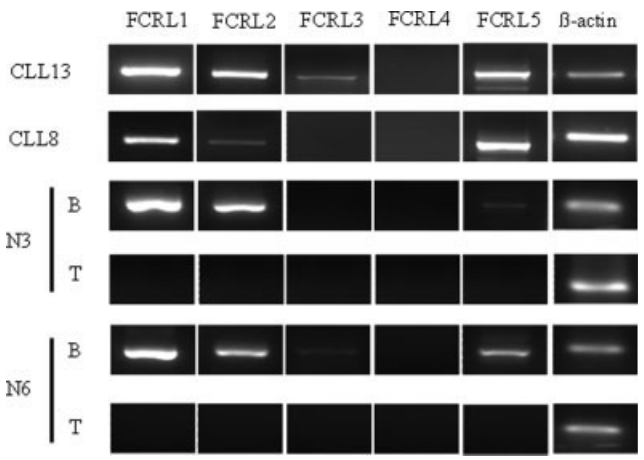

Figure 1 – Representative RT-PCR results of FCRL1–5 genes expression in patients with CLL and normal subjects. B and T refer to purified B- and T-lymphocytes isolated from PBMC by magnetic beads separation.

Relative mRNA expression levels of all FCRL genes were also determined in purified B-cell and T-cells isolated by magnetic beads from PBMC of 5 normal subjects. Representative results are shown in Figure 1. Expression of FCRL molecules was exclusively detectable in B-cells with no or negligible expression in the isolated T-cells population.

No significant differences were observed for any of the FCRL molecules between progressive and indolent patients and also between these 2 groups and patients undergoing treatment (treated patients with CLL) (Table III). A similar expression pattern was also observed in patients with CLL with mutated and unmated VH regions, with the exception of FCRL3, which was expressed at higher levels in mutated patients ($p = 0.017$) (Table III and Fig. 2*b*).

Intracytoplasmic and surface expression of FCRL protein

Flow cytometric analysis of surface and intracytoplasmic expression of FCRL1, FCRL2, FCRL4, and FCRL5 molecules was performed in leukemic cells from 12 patients with CLL (6 progressive, 5 indolent and 1 newly diagnosed patients). Ten PBMC samples were tested by single-staining using biotinylated FCRL polyclonal antibodies, and 2 samples were analyzed by double-staining using FITC-CD19 MAb and biotinylated FCRL polyclonal antibodies. FCRL3-specific antibody was not available for this study. Representative results obtained for 2 patients and 2 normal subjects are illustrated in Figure 3. Overall, the protein expression pattern was similar to that obtained by RT-PCR, with a few exceptions (Table IV). Thus, while FCRL4 was positive in only 2 out of 12 samples at intracytoplasmic level, FCRL1 and FCRL2 were expressed in 11/12 and 9/12 cases, respectively, in most cases at both surface and intracytoplasmic levels. FCRL5 molecule was also detected in 10/12 cases, but surface expression was confirmed in only 2 samples, both of which displayed mutated VH and indolent disease (Table IV). Expression of FCRL protein was studied in B-cells of 5 normal subjects by double-staining of PBMC using FITC-CD19 MAb and biotinylated FCRL antibodies detected by RPE-streptavidin. Representative results are illustrated in Figure 3. FCRL1, 2, 4 and 5 molecules were detected at both surface and intracytoplasm of B-cells from all subjects, with the exception of 1 sample being negative for FCRL4 (Table V). Comparison of mean fluorescence intensity (MFI) obtained for all FCRL molecules, both at surface and intracytoplasm, showed a significantly higher MFI for FCRL1, 2 and

TABLE II – FREQUENCY OF FCRL1–5 EXPRESSION IN IRANIAN PATIENTS WITH B-CLL AND ELDERLY NORMAL SUBJECTS

FCRL	Normal ($n = 10$)	All CLL ($n = 86$)	Progressive CLL ($n = 38$)	Indolent CLL ($n = 41$)	Mutated CLL ($n = 55$)	Unmutated CLL ($n = 31$)	Treated CLL ($n = 21$)	Untreated CLL ($n = 65$)
FCRL1	10 (100)	82 (95)	37 (97.4)	39 (95.1)	53 (96.4)	29 (93.5)	21 (100)	61 (93.8)
FCRL2	6 (60)	81 (94)	37 (97.4)	39 (95.1)	52 (94.5)	29 (93.5)	21 (100)	60 (92.3)
FCRL3	10 (100)	78 (91)	36 (94.7)	36 (87.8)	51 (92.7)	27 (87.1)	19 (90.5)	59 (90.8)
FCRL4	7 (70)	16 (19)	5 (13.2)	11 (26.8)	11 (20)	5 (16.1)	6 (28.6)	10 (15.4)
FCRL5	10 (100)	86 (100)	38 (100)	41 (100)	55 (100)	31 (100)	21 (100)	65 (100)

The results represent number (percent) of positive cases based on visualization of amplified PCR product of the specified FCRL genes.

TABLE III – RELATIVE EXPRESSION OF FCRL1–5 mRNA LEVELS IN IRANIAN PATIENTS WITH B-CLL AND NORMAL SUBJECTS

FCRL	Normal ($n = 10$)	All CLL ($n = 86$)	Progressive CLL ($n = 38$)	Indolent CLL ($n = 41$)	Mutated CLL ($n = 55$)	Unmutated CLL ($n = 31$)	Treated CLL ($n = 21$)	Untreated CLL ($n = 65$)
FCRL1	184 (71)	103 (71)*	103 (56)	109 (57)	109 (56)	93 (54)	108 (63)	102 (53)
FCRL2	42 (51)	67 (51)	73 (57)	66 (44)	68 (50)	64 (51)	71 (56)	62 (47)
FCRL3	116 (51)	72 (51)*	71 (41)	76 (60)	82 (55)	53 (37)*	77 (63)	71 (47)
FCRL4	15 (15)	6 (14)*	4 (12)	9 (17)	6 (15)	5 (13)	8 (14)	6 (15)
FCRL5	122 (47)	117 (40)	120 (38)	118 (41)	121 (40)	109 (40)	127 (55)	113 (33)

The results represent the mean (standard deviation) ratio of FCRL PCR product band density to that of β-actin multiplied by 100.

*Comparison between all patients with CLL with normal subjects revealed significant differences for FCRL1 ($p < 0.0001$), FCRL3 ($p = 0.01$) and FCRL4 ($p = 0.002$). No significant differences were observed for any of the FCRL molecules when progressive, indolent and untreated; treated patients with CLL were compared with each other, with the exception of FCRL3, which was expressed at significantly higher levels in mutated compared to unmutated patients ($p = 0.017$).

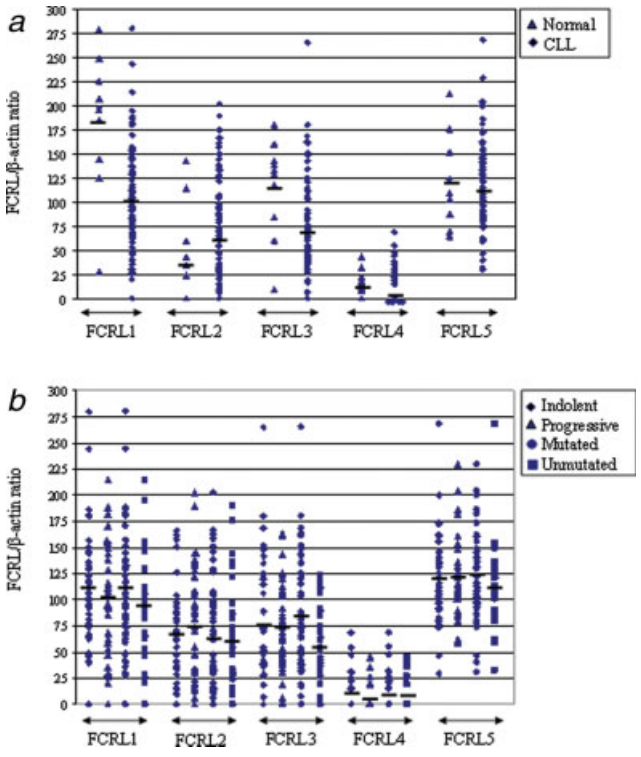

Figure 2 – Distribution of FCRL1–5 mRNA levels in PBMC from different groups of patients with CLL and normal elderly subjects. The results are expressed as the ratio of FCRL to β-actin PCR product band density in (*a*) all patients with CLL and elderly normal subjects and (*b*) different subtypes of patients with CLL. Comparison of the relative expression levels of FCRL1–5 of all subjects with CLL and elderly normal subjects demonstrated significant down-regulation of FCRL1 ($p < 0.0001$), FCRL3 ($p = 0.01$), and FCRL4 ($p = 0.002$) in cases with CLL. FCRL2 and 5 were similarly represented in patients with CLL and normal controls. No significant differences were observed between indolent, progressive, mutated and unmutated groups of patients with the exception of FCRL3, which was expressed at higher level in mutated compared to unmutated patients ($p = 0.017$). PBMC, peripheral blood mononuclear cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

5 as well as immunoglobulin in normal subjects compared to patients with CLL (Table VI).

Discussion

In the present study, we have demonstrated the expression of FCRL1–3 and 5, but not FCRL4 mRNA in the majority of our B-CLL samples. However, comparison of the FCRL mRNA expression level in PBMCs of the patients with that of the normal elderly subjects revealed a significant down-regulation of FCRL1, 3 and 4, but not FCRL2 or 5 in the patients. Consistent with its lack of expression on circulating normal B-cells,[14,22] FCRL4 could not be appreciably detected in leukemic cells from the majority of our patients with B-CLL (Tables II, III).

There are some important methodological issues regarding our semiquantitative RT-PCR assay and the source of cells isolated from patients and normal subjects, which need special consideration. In this study, representation of *FCRL* expression as a ratio to the housekeeping gene β-*actin* was intended to normalize the data and avoid variations due to differences in cDNA concentration or loading different amounts of PCR product on the electrophoresis gel. Further caution was exercised by simultaneous electrophoresis of FCRL and β-actin PCR products for each subject on the same gel. This would minimize variations due to technical shortcomings. The other important issue is the difference of the source of cells collected from patients and controls. Although, PBMCs were collected from peripheral blood of both groups of subjects, the PBMCs isolated from patients with B-CLL were predominantly (60–90%) leukemic B-cells (supplementary Table 1), whereas only 10–20% of the PBMCs isolated from normal subjects are B-cells. Therefore, comparison of FCRL mRNA expression in these 2 groups of subjects may not be appropriate. However, since constitutive expression of FCRL1–5 molecules is restricted to B-cells and other blood cells are totally negative[3,4,10,13,15,22] with the exception of FCRL3, which is also expressed in NK and T-cells,[10,13,15] it appears that the significant down-regulation of FCRL1, 3 and 4 observed in our study would have been more magnified and signified if pure B-cells could have been isolated from the normal subjects and tested. Employment of PBMCs from normal subjects reduces the PCR product band densities of FCRL molecules to that of β-actin for each sample tested. Thus, much higher significant differences are expected if pure normal B-cells are employed and compared for FCRL mRNA expression with leukemic CLL B-cells. This is clearly indicated in our results obtained in isolated B- and T-cells from a limited number of normal subjects showing higher expression of FCRL mRNA levels in B-cells compared to T-cells, which are essentially negative (Fig. 1).

Initial studies using cDNA clones present on the lymphochip microarray demonstrated up-regulation of FCRL1–4 in B-CLL, as well as diffuse large B-cell lymphoma and follicular lymphoma.[17] At protein level, surface membrane expression of FCRL1–5 family members was examined in leukemic CLL B-cells by flow cytometry using monoclonal antibodies.[15] The expression pattern was reported to be similar to that of the normal mature peripheral B-cells, with the exception of FCRL3, which was significantly up-regulated in patients with B-CLL. Of the B-CLL cases tested, leukemic cells from 15/15, 15/20, 14/20, 1/20 and 14/15 of the subjects displayed FCRL1–5, respectively.[15] Membrane expression of FCRL1 and FCRL5[16,18] and FCRL1–5 molecules has also been investigated separately in 2 other recent studies by flow cytometry. The results presented in these 2 papers showed that 12/14 and 23/29 of the CLL samples tested were positive for FCRL1 and FCRL5, respectively.

Overall, our flow cytometry results are similar to those obtained from previous studies (mentioned above). Of the 12 randomly selected cases with B-CLL tested, 11/12, 9/12, 2/12 and 10/12 were found to express FCRL1, FCRL2, FCRL4 and FCRL5 molecules, respectively (Table IV). We could not study FCRL3 molecule at protein level because of unavailability of the antibody. Despite the overall similarity observed in our study and previous reports regarding the frequency of FCRL protein expression, FCRL5 was detected only in the cytoplasm, but not on surface membrane of the leukemic cells in the majority of our patients. Surface staining was observed in only 2/12 samples studied. Lack of surface expression of FCRL5 in our patients does not seem to be associated to ethnicity of the patients, because B-cells from all 5 normal subjects studied by flow cytometry displayed the membrane-bound FCRL5 molecule (Table V). Rather it may reflect the weak reactivity of the antibody in flow cytometry technique as well as the lower expression level of this molecule at the surface of the leukemic cells, which renders them undetectable by the weak polyclonal antibody employed in this study. Significantly higher MFI of FCRL5 in normal B-cells confirms our proposition. Accumulation of FCRL5 in the cytoplasm would naturally result in quantitatively higher expression levels compared to the membrane-bound form of this molecule. The anti-FCRL antibodies employed in our study are all polyclonal antibodies with potential cross-reactivity to other members of the FCRL family. However, as indicated by the supplier (R&D Co.), apart from the anti-FCRL1 Ab, which shows minor (25%) cross-reactivity with recombinant human FCRL2 molecule, none of the other Abs

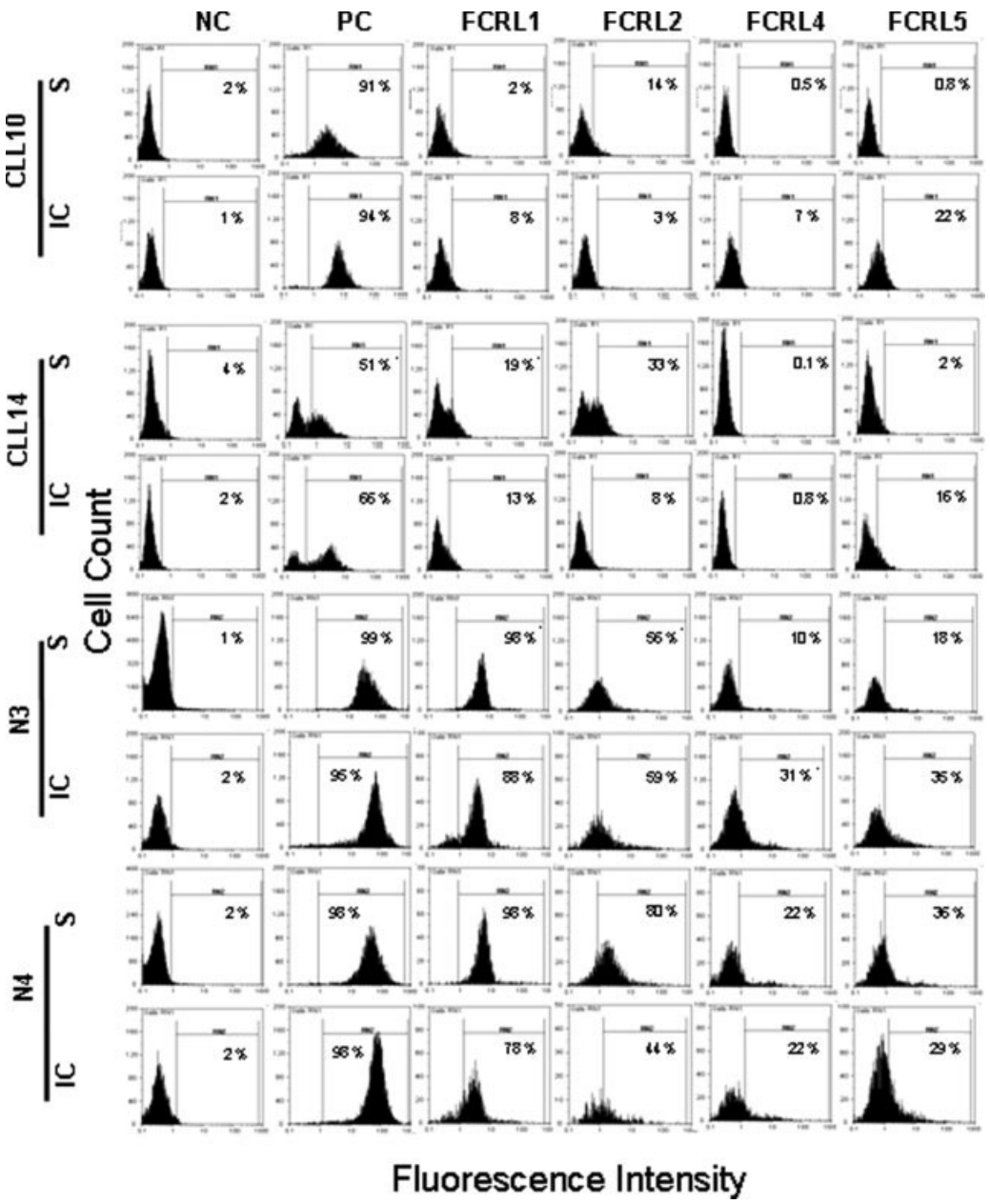

Figure 3 – Representative flow cytometry results of FCRL protein expression in a number of patients with CLL and normal subjects. Values presented in each figure represent percent of positive cells. Biotinylated goat IgG and biotinylated goat antihuman immunoglobulin were used as negative (NC) and positive controls (PC), respectively. S, surface expression; IC, intracytoplasmic expression.

cross-react with human FCRL proteins. The cross-reactivity of the other Abs has been stated to be less than 2–5%, which is essentially negligible.

In previous flow cytometry studies, 1/15 and 6/29 of the cases with B-CLL tested were found to be negative for membrane-bound FCRL5.[15,16] Intracytoplasmic staining was not reported in these articles. This discrepancy could be attributed to the specificity of the antibodies employed in these studies or transcription of different forms of FCRL5 in different groups of patients with different ethnic backgrounds. FCRL5 has been proposed to have 2 membrane-bound and 1 secretory forms.[16] High levels of soluble FCRL5 (also known as CD307) were detected in serum of a large number of patients with B-CLL.[16] It would be interesting to quantitate soluble FCRL5 level in serum of our patients to find out any correlation with the membrane-bound or intracytoplasmic expression pattern detected by flow cytometry. Quantitation of soluble FCRL5 molecule might also be valuable to differentiate between indolent and progressive B-CLL clinical subtypes, because the level of soluble FCRL5 was associated to WBC count and disease burden in patients with CLL.[16]

Chronic lymphocytic leukemia follows an extremely variable clinical course with overall survival times ranging from months to decades.[23] Immunoglobulin variable region heavy chain (IgVH) mutational status separates CLL into 2 different forms of the disease, mutated and unmutated forms, which are closely associated to the indolent and progressive clinical courses, respectively.[20,24] In an attempt to large scale genotyping of CLL using microarray technology, however, it has been shown that CLL displays a com-

TABLE IV – SURFACE AND INTRACYTOPLASMIC EXPRESSION OF FCRL1, 2, 4, AND 5 MOLECULES IN FRESH LEUKEMIC CLL CELLS BY FLOW CYTOMETRY

Patients	Disease progression	VH mutation status	NC		PC		FCRL1			FCRL2			FCRL4			FCRL5		
			S	IC	S	IC	S	IC	mRNA	S	IC	mRNA	S	IC	mRNA	S	IC	mRNA
CLL8	P	M	−	−	++++	++++	−	+	+	−	−	+	−	−	−	−	−	+
CLL10	I	U	−	−	++++	++++	+	NT	+	NT	−	−	−	−	−	−	+	+
CLL13	I	M	−	−	++	++++	+	+++	+	+++	+	+	−	−	−	++	++	+
CLL14	P	M	−	−	+++	++++	+	+	+	++	NT	+	−	−	+	−	+	+
CLL15	I	M	−	−	++	++++	++	+++	+	++++	+++	+	−	NT	−	++	++++	+
CLL20	I	M	−	−	++	++++	−	−	+	−	−	+	−	−	−	−	−	+
CLL23*	ND	U	−	−	++++	++++	++++	++++	+	+++	++	+	−	+	−	−	+	+
CLL33	P	M	−	−	+++	++++	+++	++++	+	+++	++	+	−	−	−	−	+++	+
CLL62	P	U	−	−	++	+++	−	+	+	−	+	+	−	+	+	−	+	+
CLL63*	P	U	−	−	++++	++++	+++	+++	+	++	NT	+	−	−	−	−	+	+
CLL64	P	M	−	−	++++	++++	++	+	+	++	NT	+	−	−	−	−	+	+
CLL66	I	M	−	−	++++	++++	+++	++++	+	+++	+++	+	−	−	+	−	+++	+

The flow cytometry results are presented as follows: 0–10% = −; 10–20% = +; 20–40% = ++; 40–60% = +++; >60% = ++++.

I, indolent; P, progressive; U, unmutated; M, mutated; S, surface; IC, intracytoplasmic; NC, negative control (biotinylated unimmunized goat IgG); PC, positive control (biotinylated goat antihuman immunoglobulin); NT, not tested; ND, newly diagnosed.

*CLL23 and CLL63 samples were analyzed by double staining of PBMC cells using FITC-CD19 MAb and biotinylated RPE-labelled anti-FCRL antibodies; all other samples were single stained with the FCRL-specific antibodies.

TABLE V – SURFACE AND INTRACYTOPLASMIC EXPRESSION OF FCRL1, 2, 4, AND 5 MOLECULES IN $CD19^+$ B CELLS OF NORMAL SUBJECTS BY FLOW CYTOMETRY

Normal subjects	NC		PC		FCRL1		FCRL2		FCRL4		FCRL5	
	S	IC	S	IC	S	IC	S	IC	S	IC	S	IC
N1	–	–	++++	++++	++++	++++	++++	+++	+	+	+++	++
N2	–	–	++++	++++	++++	++++	++++	++++	+	++	++	NT
N3	–	–	++++	++++	++++	++++	+++	+++	–	++	+	++
N4	–	–	++++	++++	++++	++++	++++	+++	++	++	++	++
N5	–	–	++++	++++	++++	++++	++++	+++	++	++	++	+++

Double staining was performed on PBMC using FITC-CD19 MAb and biotinylated RPE-labelled anti-FCRL antibodies.

The flow cytometry results are presented as follows: 0–10% = –; 10–20% = +; 20–40% = ++; 40–60% = +++; >60% = ++++.

S, surface; IC, intracytoplasmic; NC, negative control (biotinylated unimmunized goat IgG); PC, positive control (biotinylated goat antihuman immunoglobulin); NT, not tested.

TABLE VI – COMPARISON OF MEAN FLUORESCENCE INTENSITY (MFI) OF SURFACE AND INTRACYTOPLASMIC FCRL AND IMMUNOGLOBULIN EXPRESSION BETWEEN PATIENTS WITH CLL AND NORMAL CONTROLS

Molecule	CLL (n = 12)	Normal (n = 5)	p value
FCRL1			
S	0.65 (0.54)	7.5 (0.7)	0.001
IC	0.73 (0.6)	4.0 (0.5)	0.001
FCRL2			
S	0.77 (0.6)	4.7 (2.0)	0.002
IC	0.75 (1.2)	5.9 (1.6)	0.002
FCRL4			
S	–	1.8 (0.4)	–
IC	0.93 (0.9)	2.5 (1.4)	0.190
FCRL5			
S	0.22 (0.3)	2.3 (0.69)	0.095
IC	0.64 (0.6)	2.1 (0.9)	0.008
Ig			
S	2.6 (2.4)	70.2 (7.7)	<0.0001
IC	6.3 (4.3)	69.2 (12)	<0.0001

The results represent mean (SD) fluorescence intensity calculated by subtraction of the MFI of the FCRL or human immunoglobulin-specific fluorochrome-conjugated antibodies from the MFI of the irrelevant fluorochrome-conjugated isotype-matched negative control antibodies.

S, surface; IC, intracytoplasmic; Ig, immunoglobulin.

mon characteristic gene expression profile that is largely independent of their IgVH genotype.[25,26] Our results appear to follow a similar pattern with no significant differences for any of the FCRL molecules between indolent and progressive patients, though FCRL3 mRNA levels were expressed at higher levels in mutated compared to unmutated patients (p = 0.017) (Tables II, III). More studies need to be performed to assess and extend our findings.

Since a number of our patients, mostly from the progressive group, have already been treated before the time of sampling, these patients were classified in a separate group and compared with indolent, progressive and newly diagnosed patients for the frequency of FCRL mRNA expression. We found no significant difference between these groups of patients, suggesting lack of effect of previous treatment on FCRL expression in leukemic CLL B-cells. This is also inferred from the high percentage of lymphocytosis, mostly with leukemic origin, observed in treated patients despite a substantial decrease in WBC count in a number of these patients (supplementary Table 1).

No RT-PCR results have so far been published regarding FCRL expression in progressive and indolent patients with B-CLL to be able to compare them with our results. Initial microarray studies have identified FCRL2 and 3 to be associated to the indolent disease.[17] Comparison of flow cytometry results in a limited number of patients has recently demonstrated a significantly higher mean fluorescence intensity only for FCRL2 molecule in progressive patients compared to the indolent group.[15] We have not been able to perform statistical analysis on our flow cytometry results due to the small sample size of the progressive and indolent samples checked by flow cytometry, though the frequency and intensity of expression of all FCRL molecules tested were overall similar in these 2 groups of patients (Table IV).

Comparing CLL genetic profile with those of purified normal B cell populations indicated that the common CLL profile is more related to memory B cells than to those derived from naïve B cells, $CD5^+$ B cells and germinal center centroblasts or centrocytes.[25] Expression of CD27 in leukemic B-cells of the majority of patients with CLL suggests a memory phenotype for these cells.[27] This proposition, however, does not seem to fit with the lack of expression of FCRL4 molecule in the majority of our patients with CLL and also

those reported by other investigators.[15] FCRL4 is considered as a B-cell memory marker.[11] The B-CLL cells, therefore, may originate from the recently identified FCRL4 negative memory pool.[22]

Altogether, our results confirm and extend previous studies indicating differential expression of FCRL molecules in B-CLL. The profile of expression of FCRL molecules was similar between progressive and indolent patients. A similar expression pattern was also observed in mutated and unmutated groups of patients, with the exception of FCRL3, which was expressed at higher levels in mutated patients. Contrary to previous findings, expression of FCRL5 protein was confined to the cytoplasm of the leukemic cells in the majority of the patients. This observation may argue against selection of this molecule for targeted immunotherapy of B-CLL, which has previously been proposed by some investigators.[15,16] FCRL1 and to a lesser extent FCRL2, which are widely expressed in B-CLL cells, both as membrane-bound and intracytoplasmic forms, seem to be more suitable targets for such therapeutic interventions, as already suggested by others.[15,18]

Note added in proof: During resubmission of the revised version of this manuscript, we noticed the article of Li et al. published online in the most recent issue of Blood describing the expression profile of FCRL1–5 molecules at protein level in IgVH mutated and unmutated forms of B-CLL.[28] Contrary to our findings of significantly higher expression level of only FCRL3 in mutated samples at mRNA level, they reported increased expression of FCRL1–3 and 5, more significantly FCRL2, in the mutated group of patients compared to unmutated samples. They also reported a similar expression level of all FCRL molecules in leukemic B-cells of patients with CLL and polyclonal B-cells of normal subjects, which contradicts our results. These discrepancies could be due to ethnic differences between the subjects studied in these 2 studies. Alternatively, such differences could partly be due to methodological aspects, *i.e.*, determination of FCRL expression at mRNA level in our study as compared to protein analysis performed by Li et al. More comparative studies need to be performed to clarify this issue.

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