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PRAME is a membrane and cytoplasmic protein aberrantly expressed in chronic lymphocytic leukemia and mantle cell lymphoma

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Abstract

The preferentially expressed antigen in melanoma (*PRAME*) gene is aberrantly expressed in chronic lymphoproliferative disorders (CLD). We produced and characterized an anti-PRAME monoclonal antibody (MoAb), which was then applied in a quantitative flow cytometric (QFC) method to evaluate PRAME expression in leukemic cells from the peripheral blood (PB) of 47 patients with chronic lymphocytic leukemia and seven with mantle cell lymphoma as well as in the PB mononuclear cells (PBMCs) and B lymphocytes from 15 healthy subjects. Approximately 90% of CLD, but none of the normal samples, presented more than 20% of PRAME+ lymphocytes. Moreover, the intensity of PRAME expression was significantly higher in CLD cells compared to normal B lymphocytes and PBMCs. By immunofluorescence microscopy and by permeabilized flow cytometry we demonstrated that PRAME is a membrane antigen and a cytoplasmic protein aberrantly expressed in malignant CLD. Our results suggest that the analysis of PRAME protein may contribute for the distinction between normal and leukemic cells in CLD, and that PRAME may be a potential target for therapy.

Keywords: PRAME; CLL; Mantle cell lymphoma; Quantitative flow cytometry

1. Introduction

PRAME gene is located on chromosome 22 (22q11.22) and encodes for a 509 amino acids protein of elusive function [1]. Small amounts of PRAME transcripts were detected in trophoblasts and testis, normal adrenal, ovarian and endometrial cells. In contrast, PRAME expression has been demonstrated at high levels in several types of cancers, such as in acute myeloid and lymphoid leukemias [2] and multiple myeloma [3]. We have recently reported that PRAME is also aberrantly expressed in lymphoproliferative diseases and its transcripts were detected in 26 out of 58 patients with chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL). Particularly, MCL cells expressed high levels of PRAME mRNA [4].

A clinically relevant feature concerning PRAME protein is that it contains an immunogenic nonapeptide able to elicit a cytotoxic response when presented by HLA-A24, thus, suggesting that PRAME could be a potential target

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for immunotherapy [1]. Recently, a study of the physiologic role of PRAME demonstrates that its transient overexpression induces a caspase-independent cell death in culture cells lines (CHO-K1 and HeLa). Cells stably transfected with PRAME also exhibit a decreased proliferation rate [5]. Another study demonstrates that overexpression of PRAME also confers growth or survival advantages by antagonizing RAR signaling [6], and is causally involved in tumorigenic process.

Nevertheless, the analysis of PRAME protein expression has been hampered by the lack of a specific antibody. Here we describe the production of a monoclonal antibody (MoAb) against PRAME and demonstrate that in the majority of CLL and MCL cases PRAME is aberrantly expressed. Moreover, the antigenic density, as determined by quantitative flow cytometry (QFC), was significantly higher in CLD cells compared to normal peripheral blood B lymphocytes and mononuclear cells (PBMCs).

2. Material and methods

2.1. Patients and controls

Peripheral blood (PB) samples from 54 patients with CLD and from 30 healthy blood donors were analyzed. The diagnosis of CLD was based on clinical, hematological, immunophenotypical and molecular findings and the cases were classified as CLL ($n = 47$) or MCL ($n = 7$). According to the staging system proposed by Binet, 33 CLL cases were at stage A, two at stage B, 11 at stage C and one case transformed into CLL/prolymphocytic leukemia. PB samples were collected on K_3EDTA tubes after informed consent. The study was approved by the Institutional Ethics Committee. The number of lymphoid cells in the PB ranged from 3900 to 67,400 cells/mm^3 (mean: 30,149 cells/mm^3) and 14,600–226,000 cells/mm^3 (mean: 88,000 cells/mm^3).

2.2. Monoclonal antibody production

A 562 bp fragment of the PRAME cDNA that includes the region that encodes for the immunogenic nonapeptide was amplified using specific primers for this region [1], sequenced to confirm specificity for *PRAME* gene, cloned in pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and subcloned into a pET24a expression vector. The 196-amino acid peptide was expressed in BL21 (DE3) *E. coli* cells as His-tagged protein, with 22.9 kDa, and purified by Ni-NTA sepharose chromatography. Hybridomas secreting anti-PRAME MoAbs were generated from splenocytes of immunized BALB/c mice and SP2/0 myeloma cell lines. Detection of positive secretory clone for MoAb anti-PRAME was identified by ELISA using PRAME recombinant protein. Determination of anti-PRAME isotype was performed with the Serotec's Mouse Monoclonal Isotype Kit (Serotec, Raleigh, NC, USA).

2.3. Determination of anti-PRAME MoAb specificity

In order to determine the specificity of the MoAb anti-PRAME, we performed a Western blot using an average of 10^7 cells to obtain the cell lysates of normal CD19+, PRAME+ CLL, PRAME+ MCL (positivity was previously detected by flow cytometry (FC) analysis and RT-PCR for PRAME RNA expression) and NB4 cells (a human promyelocytic cell line that does not express PRAME mRNA nor protein by FC analysis). CD19+ cells were sorted using an immunomagnetic method with anti-CD19 microbeads (BD Biosciences, San Jose, CA, USA). To evaluate cell sorting efficiency, we stained the sorted cells with anti-CD20 fluorescein-conjugated (FITC) MoAb, and FC analysis revealed that all samples had more than 95% of purity. Cells were homogenized in buffer (40 mM Hepes pH 7.4, 2 mM EDTA, 2 mM DTT, 1 mM benzamidine and 1 mM Pefabloc-SC). Homogenates were immediately mixed with SDS sample buffer and heated at 80 °C for 3 min and then loaded on a 16% polyacrylamide SDS gel. After electrophoresis, proteins were transferred to Hybond ECL nitrocellulose (Amersham Biosciences, Little Chalfont, UK) at 30 V for 12 h. The PRAME MoAb and the peroxidase-conjugated secondary antibody goat anti-mouse IgG were used at a 1:20 and 1:2500 dilutions, respectively. To ensure equal protein loading and integrity, we also stained all membranes with an anti-γ-tubulin MoAb which produces a 48 kDa band. The reaction was developed using the ECL kit.

2.4. Subcellular localization of PRAME by immunofluorescence microscopy

PRAME+ CLL cells were plated on polylysine-coated glass coverslips for 15 min. Cells were then briefly rinsed in PBS and fixed for 10 min in a solution of freshly prepared 2% paraformaldehyde in PBS. A solution of 0.3% Triton X-100 was used to permeabilize cells for 10 min. For immunofluorescent staining, cells were blocked using 2% BSA in the same solution for 10 min. Cells were stained for PRAME protein by incubation using MoAb against PRAME at a 1:20 dilution for 1 h at room temperature followed by incubation with an Alexa 488-labeled secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were stained for CD19 by incubation with a 1:50 dilution of anti-CD19 phycoeritrin (PE)-conjugated MoAb for 1 h at room temperature, and then stained with DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) at a 1:5000 dilution in blocking buffer (1% BSA in PBS) for 1 h at 37 °C in the dark. Cells were then washed four times in PBS for 5 min, mounted on glass slides using the Prolong Antifade kit from Molecular Probes and visualized on a Zeiss Axiovert fluorescence microscope. Images were captured and digitalized with Metamorph Software.

2.5. Flow cytometry analysis of PRAME

PBMCs were separated in Ficoll-Hypaque, resuspended in media at a concentration of approximately 5×10^5 cells/ml and incubated with saturating amounts (100 μl) of anti-PRAME MoAb for 30 min. After washing in PBS, the samples were incubated with saturating amounts of goat anti-mouse (GAM, 3 μl) polyclonal antibody previously conjugated with FITC (BD Biosciences, San Jose, CA, USA) for 30 min. Negative controls were incubated with mouse immunoglobulin of irrelevant specificity (normal mouse IgG1, catalog number sc-2866, Santa Cruz Biotechnology, Santa Cruz, CA, USA). In 15 samples of normal PBMCs, as well as in 26 out of 47 CLL and all MCL samples, after being labeled for PRAME, the suspensions were incubated with PE conjugated anti-CD19 MoAb for 15 min, and washed twice in PBS. For analysis, B lymphocytes were gated based on CD19 staining. Alternatively, mononuclear cells were gated based on forward (FSC) and sideward (SSC) light scatter.

In order to elucidate in which PB cell subpopulations PRAME was expressed, normal PBMCs, CLL and MCL samples were stained for PRAME as above and then incubated with the following panel of PE conjugated MoAbs: CD19, CD3, CD16 + CD56, CD33 + CD11b (BD Biosciences, San Jose, CA, USA). In all experiments 10,000 events were acquired and analyses were performed using the using the Cell Quest software (Fig. 1). The cut off values for PRAME positivity were established based on the analysis of isotypic control-stained samples.

For quantitative analysis of PRAME expression in 26 CLL cases, 7 MCL samples and 15 healthy blood donors, 3 μl of GAM antibody were incubated with DAKO QIFIKIT microbeads and the number of PRAME sites per cell was expressed as specific antibody binding capacity (SABC). In order to evaluate the sensitivity of the QFC method, we established a cut-off value for PRAME SABC based on the highest value detected in normal lymphocytes. All suspensions of MCL cells were labeled with anti-PRAME and serially diluted in normal PBMCs. The highest dilution in which PRAME+ cells were distinguishable from normal PB based on PRAME SABC value was determined.

Twenty-one CLL cases and another 15 healthy blood donors were also analyzed for the expression of PRAME in the cell cytoplasm. Approximately 5×10^5 cells/ml were incubated with BD FACS lysing solution for 10 min, washed with PBS and then incubated with 500 μl of FACS Permeabilization Solution 2 (BD Biosciences, San Jose, CA, USA) and washed with PBS. After the membrane permeabilization, cells were incubated with PRAME monoclonal antibody and CD19, as described above. We evaluated the normalized mean fluoresce intensity in permeabilized and non-permeabilized CLL and normal cells using an electronic gate in CD19+ cells (Fig. 4B).

2.6. Real-time RT-PCR quantification for PRAME mRNA

The cDNA from PBMCs was synthesized from 2 μg of RNA by extension with random hexamer primers and 200 U of reverse transcriptase. The PCR amplification was performed in 40 cycles, using TaqMan PCR master mix in a SDS 5700 platform. The TaqMan primers and probe for PRAME were synthesized using assay-by-design according to GeneBank sequence (accession No. NM_006115) as follows: forward primer pra2F-GAGGCCGCCTGGATCAG, reverse primer pra2R-CGGCAGTTAGTTATTGAGAGGGTTT and probe pra2M2 FAM-TCACGTGCCTGAGCAA-MGBQ. The GAPDH housekeeping gene was used to normalize PRAME expression, designed according the pre-developed assay reagent (PDAR, Applied Biosystems, Foster City, CA, USA), and results are presented here as a relative quantification on the basis of ΔCt. A serial dilution experiment was performed to compare PCR efficiency of PRAME and GAPDH [7,8].

3. Results

3.1. MoAb specificity

The isotype of anti-PRAME monoclonal antibody was demonstrated to be IgG1. In order to confirm its specificity of anti-PRAME isotype IgG1, we performed a Western blot analysis using protein extracts of sorted normal CD19+, CLL and MCL cells. The leukemic cell line NB4 was used as a negative control. The Western blot results revealed the presence of a single 33 kDa band, the expected molecular weight for the PRAME protein, in MCL and CLL, but not in NB4 extracts. A weaker band of the same size could be identified in normal CD19+ cells (Fig. 2).

3.2. In situ immmunofluorescence for PRAME

In order to elucidate whether PRAME protein was expressed in cell membrane, we co-stained cells for PRAME and CD19. CLL cells, previously identified as PRAME+ by flow cytometry analysis, were analyzed by in situ immunofluorescence using anti-PRAME and anti-CD19 MoAbs. As shown in Fig. 3, we could demonstrate that PRAME was co-localized with CD19 in the cell membrane.

3.3. Flow cytometry analysis of PRAME protein

We analyzed the PRAME protein expression by quantitative flow cytometry in the PB of 15 healthy individuals and of 33 patients with CLD (26 CLL and seven MCL cases). The median percentage of PRAME+ cells in the PB of CLL and MCL patients was 65% (range: 16–95%) and 88% (12–94%), respectively ($P > 0.05$, Kruskal–Wallis test). In contrast, in the PBMCs of healthy subjects ($n = 15$) PRAME+ cells cor-

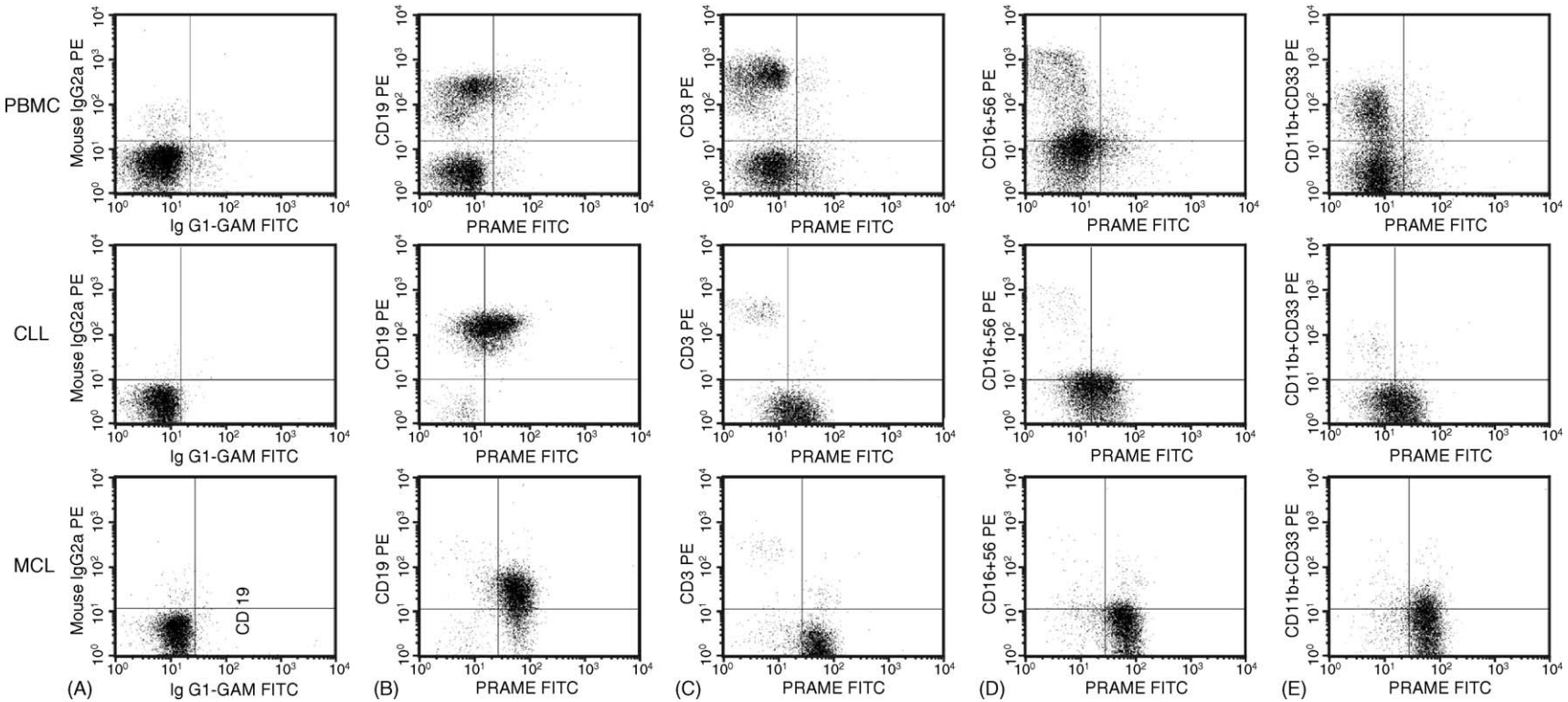

Fig. 1. Flow cytometric analysis of PRAME expression in PB cells subpopulations of normal PBMC (1st row), CLL (2nd row) and MCL (3rd row). Cells were labeled with anti-PRAME and the co-expression of CD19 (column B); CD3 (column C); CD16 + CD56 (column D) and CD11b + CD33 (column E) was analyzed.

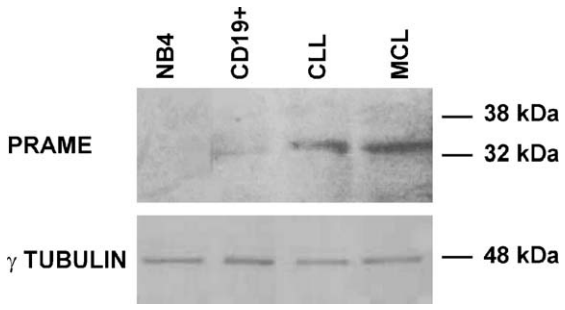

Fig. 2. PRAME protein expression in NB4 (human promyelocytic leukemic cell line), normal CD19, CLL and MCL cells were analyzed by Western blot using total protein extracts. Molecular weights markers are indicated.

responded predominantly to a fraction of the B lymphocytes and monocytes (Fig. 1 columns B and E), representing only 7% (3–18%) of the PB cells. PRAME was expressed by higher than the maximal observed in normal PB in 25 out of 26 CLL and in six out of seven MCL cases. T and NK cells did not express PRAME. In order to determine whether the intensity of PRAME expression would also differ in normal, CLL and MCL lymphoid cells, we performed a quantitative flow cytometry. The median SABC for PRAME was of 1659 (range: 1628–1781); 9364 (3075–24,665) and 14,044 (5059–20,679) sites per cell in normal PBMC, CLL and MCL cells, respectively. To confirm that PRAME was aberrantly expressed in B cells of CLL and MCL, we determined PRAME SABC values in these cells as well as in normal CD19+ cells. Fig. 4A shows that the median SABC value was 2098 sites per cell (range: 543–6402) in normal B cells and was significantly lower ($P < 0.001$, Kruskal–Wallis test) than in CLL and MCL cells. In order to establish if PRAME intensity of expression could discriminate between normal and malignant B cells, we adopted the highest SABC value observed in normal CD19+ cells as a cut off. Eighteen out of 26 CLL and six out of seven MCL cases presented SABC values above this limit ($P < 0.0001$ and $P = 0.0002$, respectively, by Fisher's exact test). Furthermore, we performed QFC analysis of PRAME expression in limiting dilution experiments, in which we were able to distinguish one MCL cell in 1000

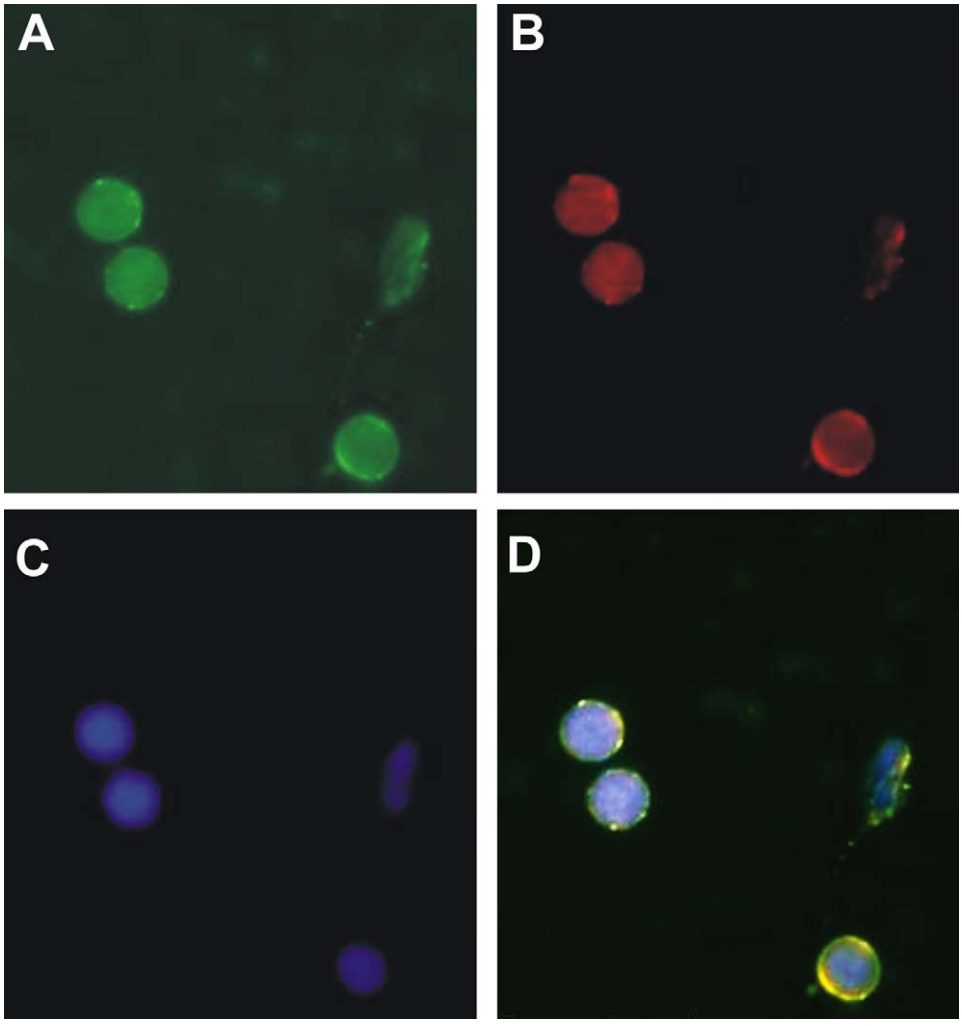

Fig. 3. Indirect immunofluorescence staining indicating the co-localization of PRAME (green fluorescence, A) and CD19 (red fluorescence, B). Nuclei were stained with DAPI (C). The superposed image is illustrated in D.

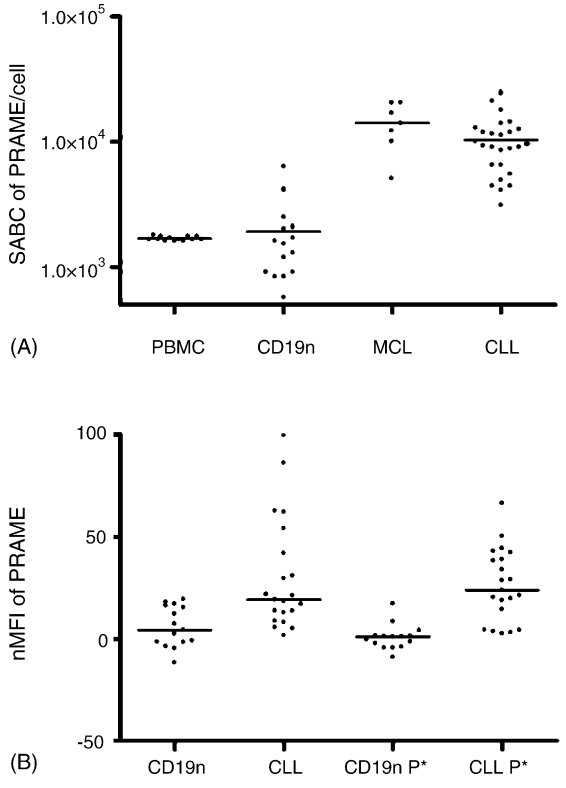

Fig. 4. (A) Quantitative analysis of PRAME protein expression. PRAME SABC values ($\times 10^3$ sites per cell) in normal PBMC; normal CD19+ cells; MCL and CLL cells. Horizontal lines represent the median values. (B) Normalized mean fluorescence intensity (nMFI) of 21 CLL leukemic cells and 15 normal healthy blood donors CD19+ cells. P* represent the permeabilized cells.

normal PB lymphocytes based exclusively on PRAME SABC values (data not shown).

There was a positive correlation between the PRAME protein expression and the number of total peripheral blood lymphocytes (Pearson's correlation $P < 0.0001$, Pearson $r = 0.9163$ and $R^2 = 0.8395$). There was no correlation between PRAME protein expression and Binet stage.

Permeabilizing the CLL and the healthy B lymphocytes did not change significantly the normalized mean fluorescence intensity (nMFI) as compared to non-permeabilized cells (Fig. 4B, $P > 0.05$, Kruskal–Wallis test), thus confirming our results by in situ immunofluorescence. This supports the view that PRAME expression in normal cells and its overexpression in lymphoid neoplasia is not predominantly cytoplasmic.

3.4. Real-time RT-PCR for PRAME

To evaluate the correlation of protein and RNA approaches, we performed a real-time relative quantification of PRAME from 33 CLL, 7 MCL patients and 15 normal healthy blood donors. The median ΔCt for all CLD samples was 15.04 and ranged from 0.4 to 19.7. In 30 CLD patients and in all 15 normal samples the cycle threshold for PRAME amplification was 40, thus indicating lack of amplification. In four cases of MCL (57%) and six of CLL (18%), PRAME mRNA was detected, demonstrating a frequency similar to that previously reported in CLD [4,9]. When compared the ΔCt values between the nMFI of PRAME, including permeabilized and non-permeabilized cells, there was no statistical correlation ($P > 0.05$, rank sum test) either when the 10 PRAME positive cases by RT-PCR or when all 40 patients were analyzed.

4. Discussion

The aberrant expression of *PRAME* gene has been demonstrated in acute leukemias, multiple myeloma and chronic lymphoproliferative disorders, and it has been suggested that the quantitative analysis of PRAME transcripts by real-time PCR may be a useful tool for diagnosis and treatment monitoring [7,10]. Moreover, higher levels of PRAME expression were associated with advanced stages of multiple myeloma and more aggressive subtypes of lymphoproliferative diseases [3,7]. Nevertheless, the PRAME expression at protein level has never been assessed and therefore its clinical value in hematological malignancies remains unknown. In the present study, PRAME MoAb generated a single band of approximately 33 kDa in the Western blot analysis of CLL and MCL samples, a weaker similar band was detected in B lymphocytes, but was absent in NB4 cells. These results corroborate the findings by immunophenotyping and demonstrate the specificity of the MoAb. PRAME was expressed by more than 20% of PBMCs in 93.9% of CLD patients, whereas in the PB of normal subjects only a small fraction of B lymphocytes and monocytes was PRAME+. Although very low levels of PRAME mRNA expression have also been reported in adrenals, ovaries and endometrium, possibly these normal cells do not present enough PRAME peptide to ensure recognition by CTL [7,11]. Here we describe by flow cytometry analysis very low intensities of PRAME antigenic expression in normal B lymphocytes that probably would not be sufficient to induce a CTL antigenic recognition. Nevertheless, PRAME was expressed in significant higher intensities by CLL and MCL cells as demonstrated by QFC analysis. There was no difference in SABC values between CLL and MCL ($P > 0.05$, Kruskal–Wallis test). Therefore, we can attest that PRAME protein is aberrantly expressed in the majority of CLD cases. Furthermore, the analysis of PRAME intensity expression may be useful for monitoring treatment response, since QFC could detect one MCL amongst 1000 normal PBMCs.

The physiologic role of PRAME and its effects in neoplasia remain elusive. Interestingly, PRAME expression correlates with a favorable prognosis in childhood acute leukemias [12,13]. Recently, Tajeddine et al reported that KG-1 leukemic cells stably transfected with PRAME present a significant decrease of expression of the heat-shock protein Hsp27, the cyclin-dependent kinase inhibitor p21, and the calcium-binding protein S100A4, suggesting that over-

expression of PRAME is associated with molecular changes of a better prognosis of leukemias [5]. Conversely, Epping et al. recently identified that PRAME is a dominant repressor of RAR signaling. PRAME binds to RAR in the presence of RA, preventing ligand-induced receptor activation and target gene transcription through recruitment of Polycomb proteins. This data would suggest that overexpression of PRAME frequently observed in human cancers confers growth or survival advantages by antagonizing RAR signaling [6]. Accordingly, the association of PRAME overexpression with poor outcome in breast cancer and neuroblastoma was previously reported [9,14].

Several types of cancer/testis-associated antigens, as PRAME, are recognized by T cytotoxic lymphocytes in vitro and in vivo. Importantly, several groups have demonstrated that effective anti-tumor immune response can be elicited against these proteins [1,2,15]. Here we demonstrated by in situ immunofluorescence and flow cytometry that PRAME is an antigen localized in the cell membrane, therefore, potentially able to elicit an immunogenic response and mediate cell lysis by T cytotoxic lymphocytes. Nevertheless, PRAME protein was also observed in cell cytoplasm of CLD malignant cells. By using real-time PCR to quantify the mRNA of PRAME we demonstrate the lack of RT-PCR amplification after 40 cycles on 30 out of 40 CLD patients and in none of 15 normal PB samples. In contrast, 20 out of 26 CLL and six out seven MCL patients present PRAME SABC values above the cut off. Therefore, the analysis of protein expression may represent a useful tool for MRD analysis.

In conclusion, our results demonstrated that PRAME is a transmembrane and a cytoplasm protein aberrantly expressed in CLL and MCL lymphoid cells, thus making this molecule an interesting target for diagnosis, minimal residual disease detection and therapeutic approaches either by immunotherapy or silencing *PRAME* gene expression using small interfering RNAs.

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