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PRAME expression in Hairy Cell Leukemia

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Abstract

PRAME has been proposed as a useful marker for solid tumors and acute B-cell malignancies. Several studies demonstrate expression in CLL. To further examine its B-cell tumor distribution, we studied PRAME in both CLL and hairy cell leukemia (HCL). While by conventional PCR only 8% of 37 HCL and 27% of 22 CLL patients were positive, nearly all patients and normal donors expressed PRAME by real-time quantitative (TaqMan) PCR. We conclude that HCL and CLL differ in PRAME overexpression, and that basal normal expression of PRAME may limit its usefulness for following patients with minimal residual CLL or HCL.

Keywords

Hairy cell leukemia; Real-time PCR; chronic lymphocytic leukemia; PRAME; TaqMan; minimal residual disease

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Contributions:

EA and TS performed PCR assays, CY and MS interpreted flow cytometry assays, IM worked with cell lines, and EA and RJK analyzed all data and wrote the manuscript.

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1. Introduction

PRAME (Preferentially Expressed Antigen of Melanoma) was originally identified as a tumor antigen recognized by HLA-A24- and HLA-A2-restricted cytotoxic T cells against a melanoma surface antigen (1,2). It is considered a melanocyte differentiation antigen which is overexpressed in both solid and hematologic tumors. In normal tissue, a very low level of PRAME expression is found in normal testis, adrenals, ovary and endometrium (1). A high level of PRAME expression has been reported for several solid tumors, including ovarian cancer (3), breast cancer (4), lung cancer and melanomas (1), medulloblastoma (5), sarcomas (6), head and neck cancers (7), neuroblastoma (8), renal cancer (9), and Wilms' tumor (10). Hematologic malignancies reported to overexpress PRAME include acute lymphoblastic and myelogenous leukemias (ALL and AML) (1,11–18), chronic myelogenous leukemia (CML) (18–20), Hodgkin's disease (21), multiple myeloma (22,23), chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) (12,24–26).

PRAME gene is located on chromosome 22 (22Q11.22) and encodes a protein consisting of 509 amino acids. As a nuclear protein, it binds to retinoic acid receptor α , thereby inhibiting retinoic acid induced differentiation, growth arrest, and apoptosis (27,28). Overexpression of PRAME is associated with hypomethylation in its regulatory regions (29), and in pediatric acute leukemias confers a favorable prognosis, possibly by inhibiting tumorigenicity and enhancing apoptosis (28).

The strong expression of PRAME in acute B-cell malignancies compared to normal blood or bone marrow samples has suggested real-time quantification of PRAME as a possible method for monitoring minimal residual disease (MRD) (17,30). Conventional RT-PCR studies with CLL showed PRAME expression in 3–16% patients (12,26). By quantitative real-time PCR studies, 18–28% of CLL patients were positive vs 0% of normal donors (24,25). These studies concluded that PRAME may be a potential target for therapy and a useful marker for MRD detection in CLL.

Recent molecular studies with hairy cell leukemia (HCL) show important differences compared to CLL (31,32). PRAME expression has not been reported in patients with HCL. To determine if PRAME might be a useful marker for therapy or MRD detection in HCL, and to determine whether its expression in this disease would be different than in CLL, we assessed PRAME expression by conventional and real-time quantitative PCR in patients with CLL and HCL, and in normal donors.

2. Materials and Methods

2.1 Cell Lines

The HCL cell lines HC1, Bonna-12, and Eskol, the CML line K562, the Burkitt's lymphoma line Raji, and the murine K1 cell line were maintained in RPMI 1640 medium (Biosource, Camarillo, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) at 37°C in a humidified 5% CO₂ atmosphere.

2.2 Patients and controls

Blood collected in heparin and EDTA tubes was obtained as part of sample acquisition protocols with informed consents approved by the NCI Investigator's Review Board (IRB). Complete peripheral blood mononuclear cell (PBMC) immunophenotypic analysis was performed as part of their standard evaluation. Flow cytometry was performed as described (33). The diagnosis of HCL by flow cytometry required demonstration of the typical immunophenotypic profile of monoclonal lambda or kappa light chain, CD19, CD103, and

abnormally high levels of CD20, CD22 and CD11c. Classic HCL expressed CD25 while the variant (HCLv) did not.

2.3 RT-PCR

1 µg total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) and an oligo(dT)₂₀ primer (Invitrogen). The resulting cDNAs were used as template to amplify PRAME transcripts in a 50-µl reaction containing 0.4 µM forward primer (5'-GTCCTGAGGCCAGCCTAAGT-3') and reverse primer (5'-GGAGAGGAGGAGTCTACGCA-3') (14). These primers were specific for human PRAME. Included in the PCR reaction was 1.5 mM MgCl₂, 200 µM of each dNTP, and 2.5 U of GoldTaq polymerase in supplied buffer from Applied Biosystems (Foster City, CA). The 7-min initial denaturation at 95°C was followed by 35 cycles of PCR, each consisting of 30 sec denaturation at 94°C, 30 sec annealing at 64°C, and 1 min extension at 72°C. The PCR finished after a further 10-min extension at 72°C. Under similar conditions, GAPDH was amplified as an internal control for each human cDNA using the primers 5'-AGCCACATCGCTCAGACACC-3' (forward) and 5'-GTACTCAGCGCCAGCATCG-3' (reverse) (34) and murine cDNA using the primers 5'-TGCAGTGCCAGCCTCGTCCC-3' (forward) and 5'-CATACTCAGCACCGGCCTCAC-3' (reverse)".

2.4 Real Time quantitative PCR assay (TaqMan™)

The level of PRAME expression was determined using PRAME specific primers and probe as follows: forward primer, 5'-TCTTCTACATTTCCCCGGA-3'; reverse primer, 5'-GCACTGCAGACTGAGGAACTGA-3'; and fluorescent probe, 5'-AAGGAAGAGCAGTATATCGCCCAGTTCACC-3'; amplicon boundaries, 1052–1126 nt; amplicon size 75 bp (18). The probe was labeled with the 5'-reporter dye FAM and the 3'-quencher BHQ1. All primers and probes were human-specific and purchased from Integrated DNA Technologies (Coralville, IA). Real Time PCR was performed with a IQ5 thermal cycler (BioRad Laboratories, Hercules, CA) using a QuantiTect™ Probe PCR kit (QIAGEN Inc., Valencia, CA). Briefly, cDNA was amplified in a 25 µl total volume per reaction using 10 nM PRAME specific primers, 5 nM TaqMan probe, and QuantiTect Probe PCR Master Mix. Reaction conditions were as follows: 95°C for 15 min followed by 40 cycles of 94°C for 15 s and 60°C (universal conditions) for 60 s. Data relative to PRKG1 expression were analyzed using IQ5 Optical System Software (BioRad Laboratories, Hercules, CA). IgH expression level was determined relative to PRKG1 gene expression level amplified using human PRKG1 gene specific primers and probe: PRKG1-F: GGGAAAAGATGCTTCTGGGAA, PRKG1-R: TTGGAAAGTGAAGCTCGGAAA, PRKG1-P: AAGGTTAAAGCCGAGCCA (35). Data was presented as $2^{-\Delta C_t}$, where C_t is the threshold cycle and ΔC_t is the C_t value of target amplification minus that of reference amplification (36).

3. Results

3.1 PRAME expression in HCL cell lines by conventional PCR

PRAME expression was studied by conventional PCR using PRAME specific primers in three HCL cell lines, HC1, Eskol and Bonna12, as well as in the CML cell line K562 used as a positive control (14). The Burkitt's lymphoma cell line Raji and murine K1 cell line were used as negative controls (17). As shown in Figure 1 and Table I, the 835 bp PRAME-specific PCR product was expressed only by K562 cells. In contrast, all HCL cell lines, Raji and K1 cells were negative.

3.2 PRAME expression by Real Time quantitative TaqMan PCR in HCL lines

PRAME expression was quantified using the TaqMan system, and intensity was calculated relative to that of PRKG1 gene expression (37). The positive control K562 cell line demonstrated the highest level of relative PRAME expression, 2.1. Mouse K1 cells were completely negative. All 3 HCL cell lines showed measurable PRAME expression, at $1.2\text{--}4.8 \times 10^{-4}$, about 4 logs lower than the positive control K562 (Table 1).

3.3 Clinical characteristics of patients

PRAME expression was assessed in 37 patients with classic HCL, 6 with HCLv, 4 with HCL without evidence of HCL cells in the peripheral blood (HCL-CR), 22 with CLL, 2 with follicular lymphoma (FL) and 12 normal donors. Flow cytometry and PCR results are listed in Table II. The percentage of circulating mononuclear cells which were malignant in HCL patients ranged from 0.03 to 93% (median 6%), compared to 5.4–97% (median 48%) for HCLv and 1–99% (median 93%) for CLL. The absolute numbers of leukemic cells per microliter (mm^3) are also listed. By rank order, the percentages and absolute concentrations were lower for HCL than for either HCLv ($p=0.017$ for percentages, $p=0.004$ for concentrations) or CLL ($p < 0.0001$). Also, circulating cell concentrations but not percentages were higher for CLL than for HCLv ($p=0.019$).

3.4 PRAME expression in peripheral blood samples of patients with HCL and CLL by conventional RT-PCR

To detect PRAME expression by conventional PCR, patients samples were subjected to RT-PCR using PRAME-specific primers and the products were analyzed by agarose gels. As shown in Table II, A PRAME-specific PCR product was detected in 3 (8%) out of 37 HCL samples, in 6 (27%) out of 22 CLL samples, and in 1 (8%) out of 12 normal controls. All HCL-CR, HCLv and FL samples were negative for PRAME expression by conventional RT-PCR. There was a trend for higher incidence of PRAME positivity in CLL than HCL ($p=0.066$, Fisher's exact). There was no correlation between PRAME expression and clinical or laboratory features, such as number of leukemic cells in the peripheral blood, disease length, and degree of organ enlargement.

3.5 PRAME expression in peripheral blood samples of patients with B-cell malignancies by quantitative real-time PCR

PRAME expression using the quantitative TaqMan assay was studied in the same group as conventional PCR, using PRKG1 gene expression as reference (37). The assay was performed in triplicate and relative PRAME expression is presented in Figure 2. For each group of patients, the ranges, medians, means and standard deviations are listed in Table II. Samples were considered to overexpress PRAME if their expression level was at least three standard deviations above the mean level of expression reported for the normal group. Four (11%) of the HCL patients showed overexpression, including all 3 of the patients for which PRAME expression was demonstrated by conventional RT-PCR, and 1 additional patient for which conventional RT-PCR results were equivocal. Six (27%) of the CLL patients overexpressed PRAME, including 5 of the 6 patients positive by conventional RT-PCR. One patient positive by conventional RT-PCR had a PRAME level of 0.108, which was 2.85-fold higher than the mean of normal controls, and thus did not meet the definition for overexpression of 3-fold above the mean of normal controls. Median PRAME relative expression levels were 0.033 in HCL, 0.039 in HCL-CR, 0.009 in HCLv, 0.019 in FL, 0.056 in CLL, and 0.016 in the control group. PRAME was not overexpressed among any HCLv, HCL-CR or FL patients. By rank order (Wilcoxon) analysis, real-time quantitative PRAME expression was significantly higher than normal controls in CLL ($p=0.0077$), and there was a trend for higher expression than normal in HCL ($p=0.06$), but not in HCLv ($p=0.35$). PRAME expression was higher in CLL

than in either HCL ($p=0.02$) or HCLv ($p=0.004$), and higher in HCL than in HCLv ($p=0.02$). There was no correlation between the level of PRAME expression and clinical features, including disease prognosis or response to therapy.

3.6 PRAME expression during HCL treatment

The correlation between PRAME expression and tumor burden (assessed by number of HCL cells in peripheral blood) was studied in 4 patients before and during treatment with BL22, a recombinant anti-CD22 immunotoxin with clinical activity in HCL (38,39). Only 1 (25%) of the patients had PRAME activity considered elevated prior to BL22, defined as 3 standard deviations above the mean of controls (Table II). Pretreatment PRAME levels were 0.040, 0.032, 0.109 and 0.15 for patients BH02, BH05, BH07 and BH11 respectively. As shown in Figure 3B for patient BH05, PRAME expression was strongly and directly correlated ($r^2=0.961$, $p < 0.0001$) with circulating HCL cells, although at one point (i.e. day 250) an increase in HCL count was associated with a decrease in PRAME expression. Patient BH02 had an increase in PRAME expression with a decrease in HCL count, but without significant correlation ($r^2=0.080$, $p=0.46$). Patients BH07 and BH11 also had no correlation between PRAME expression and HCL counts. Thus PRAME can decrease significantly with decreasing tumor burden in HCL, even if PRAME expression was not elevated prior to treatment. However, correlation between PRAME and HCL tumor burden was not a consistent finding.

4. Discussion

PRAME expression has been characterized for a wide variety of solid tumors and hematological malignancies (1,3–26), but has not yet to be reported for HCL. To determine if PRAME is expressed in HCL, and like in other tumors, may be a marker for following MRD, we studied HCL cell lines and patient samples using conventional and real-time quantitative PCR. We found by conventional PCR that PRAME was expressed by 8% of HCL samples compared to 27% of CLL and 8% of normal controls, and that the level of PRAME expression by quantitative real-time PCR was higher in CLL than in either HCL or normal controls. Moreover, 1 out of 4 HCL patients achieving CR had a decrease in PRAME levels significantly correlating with disease burden, while the other HCL patients achieving CR did not.

4.1 PRAME expression on malignant and normal cells

PRAME was reported to be expressed in 35–41% of cases of pediatric AML and both pediatric and adult ALL (17). PRAME expression was much lower in normal controls, suggesting its utility in following MRD (30). RT-PCR studies with CLL showed PRAME expression in 6 (16%) of 38 patients (12) and 1 (3%) of 30 patients (26). By quantitative real-time PCR, 6 (18%) of 33 CLL patients were positive (defined as cycle threshold < 40) versus 0 of 15 normal donors (24). In another study, 5 (28%) of 18 CLL cases were positive (defined as copy number $> 2.5/10,000$ cells), versus none of 42 normal donors (25). Our study also required a cycle threshold (Ct) of < 40 to be considered positive and we did not determine PRAME copy number/cells. Our finding that samples from nearly all normal donors express PRAME was previously observed by flow cytometry in 15 normal controls (24) and by RQ-PCR in 25 normal blood and bone marrow samples (17). PRAME positivity by flow cytometry was associated with PRAME negativity by RQ-PCR in one study, possibly due to the RQ-PCR method and to the primer sequences used (24). It is certainly possible that normal cells alone were responsible for PRAME expression in our samples. PRAME expression from the malignant cells likely occurred in at least some samples, since PRAME was overexpressed in patients with $>90\%$ HCL cells and in several CLL patients with 95–99% CLL cells.

4.2 PRAME as a target for following MRD in HCL

A good target for following MRD must be detectable in the presence of a low number of malignant cells and also be specific for tumor versus normal cells. We presented both conventional and RQ-PCR data to determine if either method would provide a difference between normal and malignant cells significant enough to justify PRAME as a target for MRD in this disease. In the present study, the incidence and degree of PRAME expression in HCL appears lower than in CLL, and normal PBMCs expressed PRAME at a level which was similar to that of the HCL patients. In fact, the one HCL patient with significant correlation ($r^2=0.96$, $p < 0.0001$) between PRAME level and tumor burden (Figure 3B) had a peak (pretreatment) PRAME level (0.03) which was essentially identical to the mean for normal controls. This suggests the possibility that in this patient, PRAME was expressed by the normal rather than the HCL cells, and that the decrease with treatment was due to a decrease in normal cells. Because BL22 is specific for B-cells and because normal cells in this patient did not decrease during achievement of CR, that explanation for this patient is unlikely. Nevertheless, because pretreatment PRAME expression in HCL patients is not very high compared to normal expression, it is unlikely to be of value in following MRD. In fact, in leukemias like HCL, the normal PBMCs would be expected to significantly increase several months after achievement of CR, and this might lead to increases in PRAME expression despite loss of the HCL cells. This might have been the case in patients BH02 (Figure 3A) and BH11 (Figure 3D). Using the patient-specific immunoglobulin rearrangement as a target, recently published real-time PCR methods can detect one HCL cell in as many as 10^6 normal cells (40). Our results indicate that PRAME cannot add to these previously reported methods for detecting MRD in HCL.

4.3 PRAME as an MRD target for other leukemias

We speculate that PRAME expression would be of limited use in following MRD in other leukemias, particularly CLL. For example, in our study, even as little as a 1-log reduction of CLL would in nearly all patients reduce the PRAME expression (if due to CLL) to levels at or below that found in normal controls. However, there may be patients with CLL expressing high levels of PRAME for which disease could be followed by monitoring PRAME expression. For example, it was reported that CLL cells express a median of 9364 sites/cell and range up to 24,665 sites/cell of PRAME, compared to a median of 1659 sites/cell on normal cells (24). Thus, for some CLL patients, 1–2 logs of disease reduction could be monitored. In contrast, some patients with pediatric AML by RQ-PCR expressed PRAME in excess of 13-logs more than the mean of normal samples, and even low expressers of pediatric AML and ALL had 6 logs more than the mean of normal controls. (17). Thus PRAME may be a poor MRD target for chronic leukemias but an excellent one for the acute leukemias, particularly for patients with very high pre-treatment expression.

4.4 Differences in PRAME expression between CLL and HCL

It is notable that significant differences are found between CLL and HCL in terms of level of PRAME expression. It is possible that these differences were simply due to the higher tumor burden in CLL compared to HCL patients, but in neither disease was there a correlation between tumor burden (concentration of circulating leukemic cells) and PRAME expression. Moreover, the tumor burden in most of the HCL patients overexpressing PRAME was lower than that of >95% of the CLL patients, most of whom did not overexpress PRAME. We have recently reported a number of differences in these 2 diseases with respect to immunoglobulin rearrangements, in terms of VH gene usage and mutation frequency (31) and also in light chain kappa/lambda usage ratios and light chain gene usage (41). We would speculate that PRAME expression is different in these 2 leukemias either because at the time of malignant transformation the precursor HCL and CLL cells express different levels of PRAME, or

because certain characteristics of differentiated HCL and CLL cells lead to different tendencies for PRAME overexpression.

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Fig 1. Conventional RT-PCR of PRAME in cell lines

PRAME and the housekeeping gene GAPDH were amplified using unlabeled primers. Shown are agarose gels stained with ethidium bromide.

Fig 2. PRAME expression relative to PRKG1

Relative expression is shown for the samples listed in Table II. The housekeeping gene PRKG1 was used as a reference.

Fig 3. PRAME expression and circulating HCL cells

HCL patients BH02 (A), BH05 (B), BH07 (C), and BH11 (D) were treated with BL22. PRAME expression, determined by real-time quantitative PCR (●), and concentrations of circulating HCL cells (○) in cells/mm³, determined by flow cytometry, are shown at the indicated time points after beginning the first cycle of treatment.

Table I

PRAME expression* by TaqMan assay in B-cell malignancies derived cell lines.

Cell line	PRAME expression
Bonna12	$2.3 \times 10^{-4} \pm 9.9 \times 10^{-5}$
Eskol	$1.2 \times 10^{-4} \pm 5.3 \times 10^{-5}$
HC1	$4.8 \times 10^{-4} \pm 1.9 \times 10^{-5}$
K1	0
K562	2.1 ± 0.88

* PRAME expression in human cells is defined as the ratio of PRAME to PRKG1 expression and in murine K1 cells by the ratio of PRAME and GAPDH expression.

Table II

Flow cytometry and PCR results of patients and controls.

Samples	HCL	HCL-CR	HCLv	FL	CLL	Normal
% Circulating leukemic cells in peripheral blood	37	4	6	2	22	12
Range	0.03–93%	0	5.4–97%	0	1–99%	0
Median	6%	0	48%	0	93%	0
Number of circulating leukemic cells in peripheral blood (cells/mm ³)						
Range	0.4–20000	0	110–57000	0	25–510000	0
Median	30	0	1400	0	67000	0
Positive for PRAME by conventional RT-PCR (%)						
	3(8%)	0(0%)	0(0%)	0(0%)	6(27%)	1(8%)
PRAME expression by real-time quantitative PCR						
Positive (%)	37(100%)	3(75%)	6(100%)	2(100%)	22(100%)	12(92%)
Expression relative to PRKG1						
Range (low)	6.3×10^{-5}	0	5.2×10^{-4}	9.2×10^{-3}	6.2×10^{-3}	0
(high)	0.22	0.067	0.034	0.029	41	0.087
Median	0.033	0.039	8.5×10^{-3}	0.019	0.056	0.016
Mean	0.049	0.036	0.013	0.019	2.000	0.027
Standard deviation (SD)	0.051	0.036	0.014	0.014	8.8	0.029
PRAME overexpression by real-time quantitative PCR (%) [*]						
	4(11%)	0(0%)	0(0%)	0(0%)	6(27%)	1(8%)

^{*} PRAME overexpression is defined as ≥ 3 SD above the mean of normal controls.