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Cyclic nucleotide phosphodiesterase 7B mRNA: an unfavorable characteristic in chronic lymphocytic leukemia

Lingzhi Zhang[1], **Fiona Murray**[1], **Laura Z. Rassenti**[2,3], **Minya Pu**[4], **Colleen Kelly**[4], **Joan R. Kanter**[1], **Andrew Greaves**[3], **Karen Messer**[3,4], **Thomas J. Kipps**[2,3], and **Paul A. Insel**[1,2,3]

[1]Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093.

Department of Medicine, University of California, San Diego, La Jolla, CA 92093.

[3]Moores Cancer Center, University of California, San Diego, La Jolla, CA 92093.

[4]Division of Biostatistics, University of California, San Diego, La Jolla, CA 92093.

Abstract

A cost- and time-efficient means to define the prognosis of patients with chronic lymphocytic leukemia (CLL) is desirable but does not yet exist. Based on evidence that CLL cells have enhanced expression of the cyclic nucleotide phosphodiesterase isoform 7B (PDE7B), we hypothesized that PDE7B expression might provide such information. We assessed PDE7B mRNA expression using quantitative real-time PCR (QPCR) in peripheral blood mononuclear cells isolated from 85 patients and 30 normal subjects. We compared PDE7B mRNA expression with that of other disease features to determine if its expression correlates with the prognosis of patients with CLL. We found that CLL patients with PDE7B mRNA levels in the top quartile (>9-fold elevation relative to normal controls) have a several-year shorter median time-to-treatment (TTT, 36 months) compared to that of patients whose CLL cells express lower levels of PDE7B mRNA (TTT, 77 months, P=0.001). High PDE7B mRNA expression correlates with expression of Zeta-chain-associated protein kinase 70 (ZAP-70), unmutated immunoglobulin heavy chain variable (IGHV) region genes and β2 microglobulin (β2M), but use of a multivariate Cox model revealed that high PDE7B mRNA expression independently predicts a short TTT, even after adjusting for several other disease characteristics (ZAP-70 or CD38 expression, IGHV mutation status, Rai status). High expression of PDE7B is an unfavorable characteristic in CLL. Assessment of PDE7B mRNA expression thus appears to be a clinically useful biomarker to define the prognosis of patients with CLL.

Keywords

cyclic nucleotide phosphodiesterase 7B; chronic lymphocytic leukemia; prognostic factor; quantitative reverse transcriptase polymerase chain reaction (QPCR)

Correspondence to: Dr. Paul A. Insel, Departments of Pharmacology and Medicine, BSB 3076, Mail code 0636, 9500 Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0636. Tel: (858) 822-1006; Fax: (858) 822-1007; pinsel@ucsd.edu.

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The novelty and impact of this study We used quantitative reverse transcriptase polymerase chain reaction (QPCR) to assess PDE7B mRNA expression in CLL cells and to compare its expression with other CLL-cell characteristics that have been implicated as markers of clinical outcome. Our results show that PDE7B expression is a novel, potentially useful biomarker in CLL and more generally, suggest the use of QPCR to expand the assessment of mRNAs as clinical biomarkers.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia and, as a consequence of decreased apoptosis, is typically characterized by the accumulation of B cells that are $CD5^+$, $CD19^+$, and $CD23^+$ [1-4]. Many factors contribute to decrease apoptosis of B cells in CLL such as high concentrations of anaerobic adenosine-triphosphate (ATP) in malignant B cells [2], the CLL microenvironment [5] and genetic changes [6]. The second messenger cAMP can promote apoptosis of malignant lymphoid cells by activating protein kinase A (PKA) [7-9] but cAMP concentrations and PKA activity are lower in CLL cells than in normal lymphocytes, suggesting disease-related defects in this pathway [10, 11]. Cellular cAMP levels are regulated via formation by adenylyl cyclases and degradation by cyclic nucleotide phosphodiesterases (PDEs). Eleven PDE families, which include multiple isoforms and splice variants, hydrolyze cAMP and cGMP [12, 13]. The differential expression of PDE isoforms has the potential to influence cell physiology, including apoptosis [7, 9, 14]. Indeed, we have found that CLL cells express high levels of PDE7B, which is a potential target for treatment of the disease [14].

The current studies tested the hypothesis that PDE7B expression relates to the severity of CLL and thus may be a prognostic biomarker for this disease. CLL shows a highly variable clinical course: patients with aggressive CLL require early treatment while those with indolent CLL can have a long-lived disease without need of therapy [1]. Currently used staging systems (e.g., those of Rai and Binet) are unable to determine an individual patient's clinical course and most importantly, to identify patients who require early treatment after diagnosis [15]. Several prognostic markers of CLL cells have been proposed to help identify such patients, e.g., chromosomal aberrations [16], high-level expression of CD38 [17, 18], Zeta-chain-associated protein kinase 70 (ZAP-70) [19-23], or unmutated immunoglobulin heavy chain variable (IGHV) region genes, IGHV-3.21 [24, 25]. β2 microglobulin (β2M) level is an independent predictor for survival [26, 27]. However, such markers are neither universally accepted nor part of the standard care of CLL patients.

In the current study, we used quantitative reverse transcriptase polymerase chain reaction (QPCR) to assess PDE7B mRNA expression in CLL cells and to compare its expression with other characteristics implicated as markers of clinical outcome. Our results show that PDE7B expression is a novel, potentially useful biomarker in CLL and more generally, suggest the use of QPCR to expand the assessment of mRNAs as clinical biomarkers.

Materials and Methods

Patient characteristics

Written informed consent was obtained from all patients at the time of enrollment in the Chronic Lymphocytic Leukemia Research Consortium (CRC; UCSD). Blood was obtained from 85 CLL untreated patients evaluated at the UCSD Moores Cancer Center and 30 age-matched healthy subjects. All patients' blood samples were obtained on or before the first date of treatment. The patients (51 males, 34 females) had a median age at diagnosis of 59 years (range, 40-79 years). We obtained blood for PDE7B expression analysis at a median of 24 months (first quartile, 12 months; third quartile, 52 months) after diagnosis; the median time from diagnosis to initial treatment (TTT) was 57 months (first quartile, 33 months; third quartile 146 months). Serial blood draws were available from 12 subjects; the median time from the first blood draw available to the last follow-up was 46 months (range, 2-221 months). Nineteen patients whose PDE7B expression was only available on the day treatment was started (n= 8) or had not received treatment by the last follow-up in this study (n=11) were excluded in analyses using delayed-entry Cox models. All patients received

treatment if they developed symptomatic and/or progressive disease, according to established criteria [28].

Sample processing and clinical database

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation using Ficoll-Paque (Amersham Biosciences). The isolated cells were washed and suspended in fetal-calf serum containing 10% DMSO for storage in liquid nitrogen for subsequent use. Samples were analyzed for ZAP-70 and CD38 expression level, IGHV mutational status, and fluorescent in situ hybridization (FISH) (e.g., 11q deletion, 17p deletion and 13q deletion and trisomy 12). Data for each patient (including dates of diagnosis and initiation of therapy [if administered]) were collected using the CRC Information Management System, a 128-bit encrypted password-secured Web application [22].

Analysis of expression of ZAP-70 and CD38, IGHV mutation status and cytogenetic abnormalities

Flow cytometric detection of ZAP-70 and CD38, and sequence analysis of IGHV were performed as described [22, 23]. ZAP-70-positivity was defined as ≥20% CLL cells expressing ZAP-70; CD38-positivity was defined as ≥30% of CLL cells expressing CD38 [23]. Mutated sequences were identified as those with <98% homology with the corresponding germ-line IGHV. FISH was performed on interphase nuclei of blood lymphocytes; the FISH panel (Vysis) detected alteration at 11q22, 17p13, 13q14 and centromere 12 [29, 30].

Quantitative reverse transcriptase PCR (QPCR)

We used the Versagene RNA Cell Kit (Gentra, VWR) to isolate RNA and the Superscript III First Strand Synthesis System (Invitrogen) to synthesize cDNA. Real-time PCR (BioRad Opticon 2 using Eurogentec QPCR Mastermix Plus SYBR Green Kit) was performed using PDE7B primers (forward 5′-aaggctgcttcgtggaatta-3′ and reverse 5′-tccatttgtcaagcgatcaa-3′) and 28S rRNA primers (forward 5′- gcctagcagccgacttagaa-3′ and reverse 5′-aaatcacatcgcgtcaacac-3′). The cycle threshold (C_t) method was used to assess changes in PDE7B mRNA expression in CLL vs. normal cells: fold-change=$2^{-\Delta\Delta Ct}$ where ΔC_t is the difference between C_t values of PDE7B and 28S rRNA, and $\Delta\Delta C_t=[\Delta C_{t,cll}$ - mean($\Delta C_{t,normals}$)]/Std.dev($\Delta C_{t,normals}$). Lower C_t value indicates higher expression level of mRNA.

Statistical Analysis

Receiver-operator characteristic (ROC) analysis assessed if the level of PDE7B mRNA can discriminate CLL cells from PBMC of normal subjects. Mann-Whitney U-tests compared PDE7B expression levels of the patient groups. We plotted Kaplan-Meier curves for TTT and tested for a statistically significant difference using the score test from a delayed-entry Cox model. The delayed entry was used to adjust for the differing times from diagnosis to blood draw. To be conservative, 19 patients with no follow-up time after blood draw were excluded from the Cox models. The relationship between the fold-change of PDE7B and time from diagnosis to blood draw was assessed with Spearman rank correlation. In addition, we modeled the change of PDE7B over time with a mixed effects model with a random subject intercept using the subset of 12 subjects with longitudinal data. PDE7B mRNA expression was first categorized by quartiles and then used as a continuous variable if the effect appeared linear across quartile groups, or dichotomized at a corresponding quartile if a threshold effect was observed. All tests were two-sided (0.05 significance level), using Prism (version 4.03 GraphPad) or R (version 2.5.1, ***www.r-project.org***) statistical software.

Results

Relationship between PDE7B mRNA expression and known prognostic markers in CLL

CLL cells have increased expression of PDE7B mRNA that relates to the malignancy of the B cells; increased PDE7B expression in CLL cells correlates with increases in PDE7B protein expression and in PDE7 catalytic activity[14]. To test if PDE7B mRNA expression is a prognostic biomarker in CLL, we compared its expression in CLL cells with that of other putative prognostic factors. In initial studies we found that PDE7B expression is similar in PBMC and purified B cells (the malignant cell type in CLL) from CLL patients (data not shown) and confirmed that PBMC from CLL patients have higher PDE7B expression than do PBMC from healthy subjects (Fig. 1A, ΔC_t values, mean ± Std.dev: 20.13±1.77 [CLL] versus 23.35±1.38 [normals])[14]. ROC curve analysis of PDE7B mRNA expression levels gave an area-under-the-curve of 0.94 (95% confidence interval, 0.9 to 0.98 [P<0.0001]) (Fig. 1B), indicating that CLL PBMC have a 94% chance of expressing more PDE7B mRNA than do normal PBMC.

We assessed other features that have been proposed as determinants of prognosis in CLL patients: IGHV mutational status (n=85 patients), Rai clinical stage (n=72), ZAP-70 expression (N=84), CD38 expression (n=83), Serum β2M (n=63) and several chromosomal abnormalities: 17p deletion (n=53), 11q deletion (n=53), 13q deletion (n=53) deletion and trisomy 12 (n=53) that were detected by fluorescence in situ hybridization (FISH) analysis [22, 23, 31, 32]. We dichotomized subjects based on their expression of each feature and tested the relationship of such expression with that of PDE7B: mutated vs unmutated IGHV; Rai 0-II vs Rai III-IV clinical stage; normal vs elevated β2M; negative or positive ZAP-70 expression; and negative or positive CD38 expression. A Mann-Whitney test showed that CLL cells from Rai III-IV patients or cells that had elevated serum β2M, unmutated IGHV or ZAP-70 had significantly higher PDE7B mRNA levels than did CLL cells lacking those characteristics (Figs. 2A-D). In order to test the impact of PDE7B mRNA expression on clinical outcome, we classified the patients as having either indolent or aggressive CLL, based on whether they received treatment within 3 years of diagnosis [33]. CLL cells from patients with aggressive CLL had higher PDE7B expression (Fig. 2E). Expression of PDE7B did not significantly correlate with that of CD38 (Fig. 2F) or with FISH data, i.e., 11q deletion, 17p deletion and 13q deletion and trisomy 12 (Table 1). A logistic model (comparing patients with aggressive or indolent CLL) was built using $\log_2$ fold-changes of PDE7B categorized by quartiles and revealed that fold-change in PDE7B has a linear effect on outcome [p=0.013, odds ratio=1.87 for each unit increase on the $\log_2$ scale, 95% CI=(1.17, 3.19)]. After we adjusted for ZAP-70 and IGHV, PDE7B mRNA expression still significantly associated with outcome [p=0.016, odds ratio=2.26 for each unit increase on the $\log_2$ scale, 95% CI= (1.22, 4.64)]. Thus, PDE7B mRNA expression appears to be an independent means to distinguish patients with indolent vs. aggressive CLL.

PDE7B mRNA expression can predict TTT

An important potential use of a prognostic factor in CLL is to predict TTT. Previous studies indicate that compared to CD38 and IGHV, ZAP-70 is more strongly associated with a shorter TTT [22, 23]. We used TTT or time-to-last-follow-up (a censored observation) as an outcome and the Kaplan-Meier method to estimate the TTT distribution in all 85 patients (follow-up time from 2 to 221 months). We found that patients whose leukemia-cell PDE7B mRNA levels were at least 5-fold (N=45), 7-fold (N=31), 9-fold (N=23), or 11-fold (N=16) greater than those of normals (N=30) had respective median TTT of 52, 52, 36, or 31 months and significantly shorter TTT than did patients whose cells had lower PDE 7B mRNA levels. A higher level of PDE7B expression predicted a shorter median month of TTT. Significant differences in median TTT were assessed using the score test in a delayed-

entry Cox model. The delayed entry was used to adjust for the differing times from diagnosis to blood draw; to be conservative, we restricted the analysis to patients for whom blood was drawn prior to censoring or treatment (n=66). The delayed-entry Cox model revealed that $\log_2$ PDE7B mRNA expression fold-change (compared to normal controls) has a threshold effect on TTT; therefore it was dichotomized according to the 9-fold-increase in PDE7B mRNA expression in the upper quartile. CLL patients (N=23) in this upper quartile had a median TTT of 36 months compared to 77 months for patients whose cells had lower PDE7B levels (p=0.001) (Fig. 3A). A multivariate delayed-entry Cox model showed that >9-fold increase in PDE7B mRNA is significantly associated with shorter TTT (p=0.003, hazard ratio=3.86, 95% CI on HR= (1.58, 9.53) (Table 2.). The ability to predict a shorter TTT was not improved if CLL cells with >9-fold increase in PDE7B mRNA also expressed ZAP-70 or CD38 (Figs. 3B-C). For ease of presentation, we express PDE7B as fold-change, but we obtained similar results if we use the Ct values (data not shown).

PDE7B mRNA expression levels do not change significantly over time

By plotting the time at which blood was drawn after diagnosis of CLL vs. PDE7B expression level (Fig. 4A), we found that a later time of blood draw after diagnosis of CLL was not associated with higher levels of PDE7B mRNA expression (Fig. 4A, n= 85, Spearman rank correlation=−0.17, p=0.12). Moreover, mean PDE7B mRNA expression levels did not significantly change as a function of time in CLL cells isolated from patients in 2-3 serial samples over intervals of 6 to 54 months (mean increase per month from a random intercept model: 0.04, 95% CI=−0.02, 0.09 p=0.16) (Fig. 4B). Because PDE7B mRNA levels in CLL patients are relatively stable for many months, assessment of such levels appears to identify CLL patients who would benefit from treatment at a relatively short time after diagnosis.

Discussion

PDEs catalyze the hydrolysis of cAMP and cGMP, thereby controlling their intracellular levels and ability to regulate functions that include cell growth and death [9]. The differential expression of PDE isoforms in diseased cells can influence cell physiology [12, 34]. Patients with CLL have a highly variable clinical course, which can span from indolent to rapidly aggressive. This study focused on the use of PDE7B mRNA expression as a marker of CLL severity, since higher PDE7B expression is thought to result in less cellular accumulation of cAMP, thus making such cells more resistant to cAMP-PKA-promoted apoptosis [14]. Assay of PDE7B mRNA expression is much easier than that of PDE7B protein expression or PDE7 activity, which are also increased in CLL patients [14], and is potentially feasible (using QPCR) in routine diagnostic settings. The current data show that PDE7B mRNA expression is a prognostic factor in CLL that relates to the presence of the malignant B cells and that higher level of expression of PDE7B mRNA associates with unmutated IGHV and ZAP-70 expression. Quantitation of ZAP-70 mRNA in CLL B cells is prognostically useful [35] but because ZAP-70 is mainly expressed in T cells, it needs to be assessed in purified CLL B cells. We also previously found[14] that CLL cells express lower levels of PDE3B, 4D, 5A and 9A, however the contribution of these other PDEs to clinical features of CLL is unclear and will require future study.

We find that patients whose CLL cells have high-levels of PDE7B expression have shorter TTT than do those whose cells have lower PDE7B levels. Use of a Cox model to predict TTT from the $\log_2$ of PDE7B mRNA expression revealed a threshold effect, with a 9-fold increase (relative to normal controls) defining the upper quartile. Patients in this upper quartile had a >3 years shorter median TTT, 36 months vs. 77 months for those with lower PDE7B levels. We conclude that patients whose CLL cells have PDE7B levels >9-fold greater than controls have more severe disease and are more likely to require treatment.

Moreover, if PDE7B expression is high, knowing the levels of CD38 and ZAP-70 expression does not improve one's prognostic ability (Figs. 3B-C). In a multivariate Cox model, both PDE7B expression and Rai clinical stage predict TTT, even if one adjusts for other prognostic factors (Table 2). Higher PDE7B expression thus appears to identify more aggressive CLL and may aid in predicting TTT. PDE7B mRNA expression significantly correlates with expression of β2M, an independent predictor for survival, although the reason for this correlation is not clear. In our current study survival data were not available for each CLL patient, however once such data become available, we intend to investigate if PDE7B is a predictor for overall survival.

Of note, the fold-change in PDE7B mRNA expression does not significantly correlate with the time of quantitation of PDE7B after diagnosis of CLL (Fig. 4A) and longitudinal analysis of samples from untreated CLL patients failed to reveal within-patient, time-dependent trends in PDE7B expression within 6 years. Use of delayed-entry Cox models (to adjust for the timing of the blood draw) yielded findings similar to those obtained with ordinary Cox regression, in terms of the association of variables with TTT (Table 2).

The assessment of the expression level of PDE7B by QPCR should be feasible in routine diagnostic settings. QPCR has become the benchmark for the detection and quantification of RNA targets and is increasingly used for clinical diagnostic assays [36, 37]. Quantitative QPCR results are more informative than qualitative data and simplify assay standardization and quality management [38]. QPCR methods are relatively simple, rapid, cost-effective, sensitive and specific. Thus, assessment of the PDE7B mRNA expression by QPCR may be feasible in diagnostic settings and for monitoring response to therapy of CLL. Survival might be increased by treating patients with markers of advanced disease and sparing those not likely to benefit from the toxic effects of such therapy. Since we find that PDE7B expression in CLL cells is relatively stable over time, patients whose CLL cells have a PDE7B expression level higher than 9-fold would seem likely to benefit from early treatment. Prospective studies with larger numbers of patients and from different populations are needed to confirm these conclusions. Nevertheless, our results imply that differences in PDE7B isoform expression, as assessed by QPCR, is a potentially useful biomarker in CLL.

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Abbreviations

CLL	chronic lymphocytic leukemia
PDE7B	cyclic nucleotide phosphodiesterase isoform 7B
TTT	time-to-treatment
ZAP-70	zeta-chain-associated protein kinase 70
IGHV	unmutated immunoglobulin heavy chain variable region genes
β2M	β2 microglobulin
ATP	adenosine-triphosphate
PKA	protein kinase A

FISH	fluorescence in situ hybridization
PBMC	peripheral blood mononuclear cells

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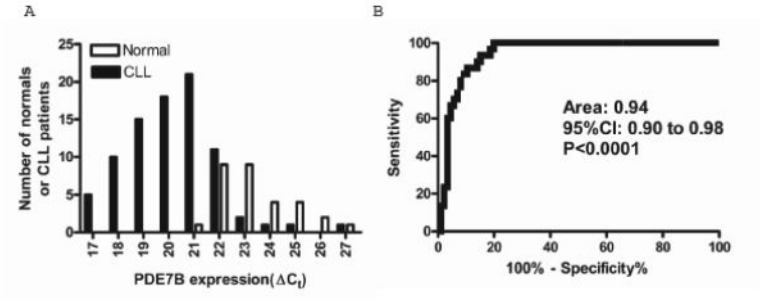

Figure 1.

CLL PBMC express higher levels of PDE7B than do normal PBMC. RT-PCR was used to quantify PDE7B mRNA in PBMC from 85 CLL patients and 30 healthy subjects. **Panel A):** Frequency distribution of PDE7B mRNA expression expressed as ΔC_t, the PCR cycle threshold value (C_t) of PDE7B relative to that of 28S rRNA. **Panel B):** Receiver operating characteristic (ROC) curve is shown for PDE7B mRNA levels expressed as ΔC_t, $\log_2$-fold or fold-change relative to the normal mean.

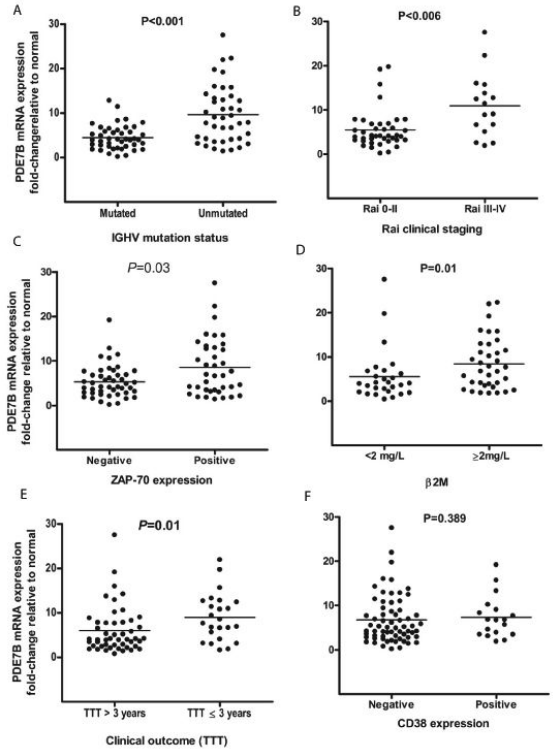

Figure 2.

High level expression of PDE7B mRNA expression in CLL PBMC is associated with unmutated IGHV, Rai III-VI, ZAP-70 expression, elevated β2M and clinical outcome. The relationship is shown between PDE7B mRNA expression (expressed as fold-change relative to the normal mean) and IGHV mutation status, Rai clinical staging, serum β2M level, ZAP-70 or CD38 expression and clinical outcome. **Panel A):** Mutated IGHV (M-IGHV) or unmutated IGHV (U-IGHV) is defined as ≥ 98% or < 98% homology to the germline sequence; **Panel B):** Clinical stage as Rai 0-II vs III-IV; **Panel C):** ZAP-70^{+} or ZAP-70^{-} is defined as ≥ 20% or < 20% level of expression; **Panel D):** Normal or elevated serum β2M levels are defined as < 2mg or ≥ 2mg; **Panel E):** Clinical outcome is based on 3 years treatment [33], i.e. TTT > 3 years vs. TTT ≤3 years (73 of the 85 CLL patients had a follow-up period of at least 3 years) and **Panel F):** $CD38^{+}$ or $CD38^{-}$ is defined as ≥ 30% or < 30% level of expression and Nonparametric (Mann-Whitney test) analysis yielded the p-values shown.

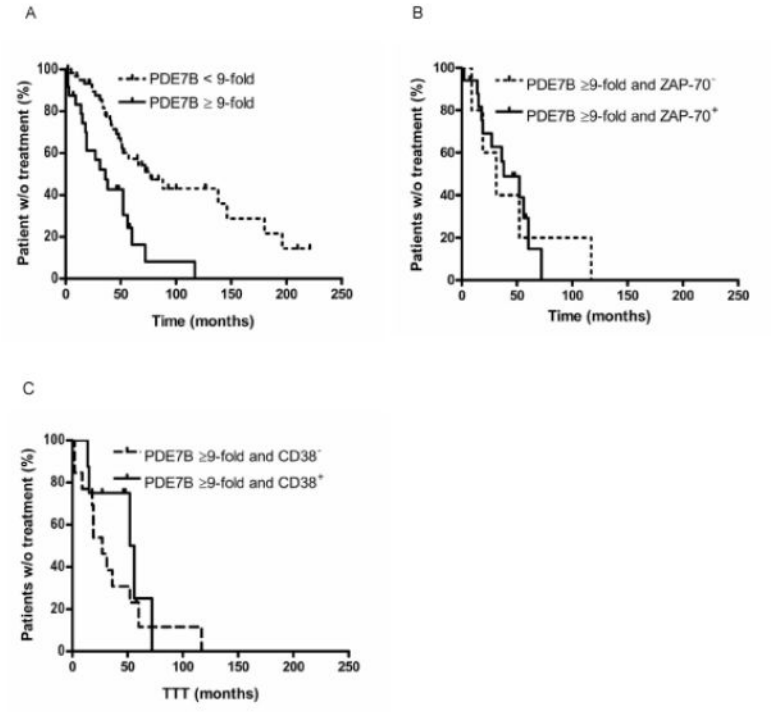

Figure 3.

PDE7B expression can predict time from diagnosis to initial therapy (TTT). Kaplan-Meier curves depict the proportion of CLL patients who did not receive therapy, as per IWCLL guidelines, as a function of time from diagnosis. **Panel A):** Patients (N=23) whose CLL PBMC-PDE7B mRNA levels were at least 9-fold greater than those of normal PBMC had a median TTT of 36 months, compared to 77 months for patients with lower levels. **Panel B):** Patients (n=17) whose CLL PBMC expressed ≥ 9-fold higher PDE7B and were ZAP-70^{+} had a median TTT of 31 months while those who were ZAP-70^{-} (n=5) had a median TTT of 38 months. **Panel C**): Patients whose CLL PBMC expressed ≥ 9-fold higher PDE7B and were CD38^{+} (n=13) and CD38^{-} (n=8) had a median TTT of 27 and 54 months, respectively. Statistical significance was assessed using a delayed entry Cox model to account for varying times from diagnosis to blood draw; 19 patients with no follow-up time after blood draw were excluded from these models. Data are not shown for 2 patients who had mutated IGHV and ≥ 9 fold higher PDE7B.

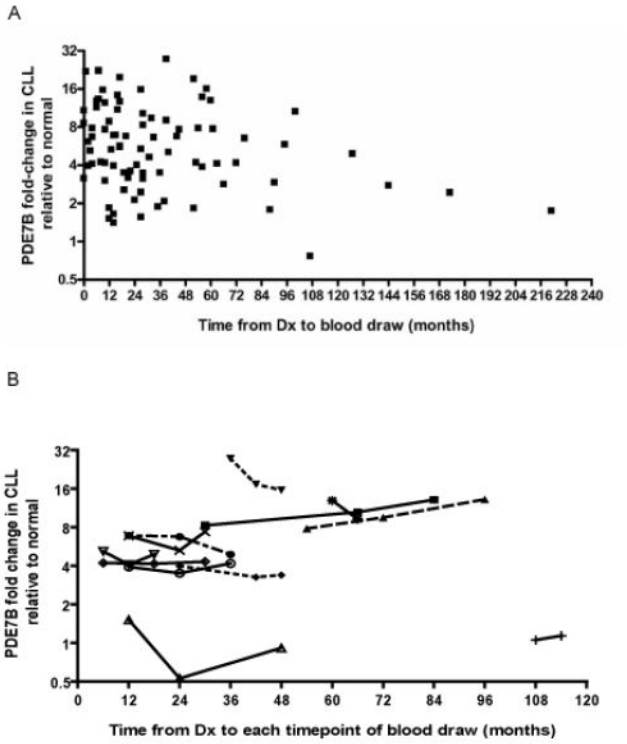

Figure 4.

PDE7B mRNA expression levels do not significantly change over time. Spearman rank correlation tests were used to evaluate the association between PDE7B fold-change and months from diagnosis to the first blood draw available for this study; longitudinal trend was tested using a random intercept regression model. **Panel A):** shows that PDE7B expression level does not correlate with time from initial diagnosis to blood draw between 0 and 221 months. **Panel B):** shows data for 12 untreated CLL patients assayed in 2-3 serial samples separated by intervals of 6 to 54 months with data expressed as fold-change in PDE7B in CLL relative to normal PBMC.

Table 1

Lack of correlation between PDE7B expression and chromosome aberration

FISH Variable	PDE7B mRNA expression				P-value
	Low expression		High expression		
	Mean	N	Mean	N	
Del(11q22)	7.5	47	9.8	6	0.45
Trisomy 12	7.7	45	8.2	8	0.33
Del(13q)	8.4	25	7.1	28	0.25
Del(17p13)	7.8	47	7.5	6	0.92

Table 2

Multivariate Cox regression analysis for TTT

Variable	Regular Cox models		Delayed-entry Cox models	
	HR (95% CI)	P	HR(95% CI)	P
PDE7B≥9-fold	3.39(1.43-8.03)	0.006	3.86(1.58-9.53)	0.003
IGHV Unmutated	0.38(0.11-1.28)	0.12	0.40(0.05-3.50)	0.41
ZAP-70 positive	2.75(0.99-7.64)	0.05	1.96(0.94-16.13)	0.53
CD38 positive	2.63(1.24-5.56)	0.01	2.08(0.92-4.69)	0.08
Rai III-IV	2.24(1.13-4.47)	0.02	4.44(1.94-10.15)	<0.001
Rai Unknown	1.05(0.42-2.61)	0.91	1.17(0.44-3.12)	0.42