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ORIGINAL ARTICLE

Genetic variation in phosphodiesterase (PDE) 7B in chronic lymphocytic leukemia: overview of genetic variants of cyclic nucleotide PDEs in human disease

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Expression of cyclic adenosine monophosphate-specific phosphodiesterase 7B (PDE7B) mRNA is increased in patients with chronic lymphocytic leukemia (CLL), thus suggesting that variation may occur in the *PDE7B* gene in CLL. As genetic variation in other PDE family members has been shown to associate with numerous clinical disorders (reviewed in this manuscript), we sought to identify single-nucleotide polymorphisms (SNPs) in the *PDE7B* gene promoter and coding region of 93 control subjects and 154 CLL patients. We found that the *PDE7B* gene has a 5′ non-coding region SNP −347C>T that occurs with similar frequency in CLL patients (1.9%) and controls (2.7%). Tested *in vitro*, −347C>T has less promoter activity than a wild-type construct. The low frequency of this 5′ untranslated region variant indicates that it does not explain the higher PDE7B expression in patients with CLL but it has the potential to influence other settings that involve a role for PDE7B.
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INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs), a superfamily of enzymes divided into 11 families (PDE1–11), several with multiple isoforms,[1,2] hydrolyze cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), and thereby have a crucial role in termination of cyclic nucleotide-mediated signaling, in particular signaling by drugs that regulate the generation of cAMP and cGMP.[3] In addition, PDEs, including specific PDE isoforms, have been used as drug targets. Several PDEs, including PDE4, PDE7 and PDE8, preferentially hydrolyze cAMP.[4] In spite of their importance in the regulation of cyclic nucleotides, the mechanisms that regulate the expression of PDE genes are not well defined. For example, regions in the promoter that regulate activity have only been identified for a limited number of PDE genes[5] and details regarding events involved in PDE gene expression are not well understood. Increases in cAMP can regulate the expression of certain PDEs, in particular via action of the cAMP-response element-binding protein or the inducible cAMP early repressor,[6–9] and in addition, glucocorticoids can transcriptionally inhibit PDE expression.[10,11]

Our interest in PDE gene expression arose from studies of patients with chronic lymphocytic leukemia (CLL), the most common leukemia in North American and European adults.[12] We found that mRNA and protein expression of PDE7B, a cAMP-specific PDE predominantly expressed in brain and lymphocytes,[13–15] are increased >10-fold in peripheral blood mononuclear cells of patients with CLL.[16] We thus set out to determine if the increased expression of PDE7B in CLL patients might result from genetic variation in the coding sequence or 5′ untranslated region (5′ UTR) of the *PDE7B* gene. We began these studies with the knowledge that genetic variation, in particular single-nucleotide polymorphisms (SNPs), had been identified in certain PDE family members, and suggested to contribute to clinical disorders, such as schizophrenia (for example, PDE4B),[17–19] stroke (for example, PDE4D)[20] and retinitis pigmentosa (for example, PDE6A and PDE6B),[21] but evidence regarding such genetic variation for other PDEs is limited. We thus set out to identify genetic variants in the *PDE7B* gene and to test the hypothesis that genetic variation might contribute to the increased PDE7B mRNA expression in CLL and to the pathophysiology of CLL with implications for the possible use of inhibitors of PDE7B in the treatment of CLL. By sequencing the 5′ UTR of the *PDE7B* gene, we identified a variant that decreases promoter activity. In addition, in this article, we place our findings for PDE7B in the context of genetic variation of other human PDEs.

MATERIALS AND METHODS

Patient selection and sample preparation

We evaluated samples from 154 CLL patients followed by the CLL Research Consortium and 93 healthy donors at the University of California, San Diego. Following informed consent, blood was collected from the healthy donors and

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Table 1 Demographic data of CLL patients and control subjects who have WT PDE7B or those with the −347C>T SNP

	Control			*CLL*		
	SNP (n=4)	*WT (n=89)*	*Total (n=93)*	*SNP (n=6)*	*WT (n=148)*	*Total (n=154)*
Male:female	4:0	73:16	77:16	4:2	92:56	96:58
Age (years)	42±8	55±13	55±13	64±12	62±10	62±10
IgVH (%)	—	—	—	95±4	95±9	96±4
ZAP-70 level (%)	—	—	—	12±18	24±28	23±27
WBC (cells ml^{-1} ×1000)	—	—	—	105±50	87±121	88±119

Abbreviations: CLL, chronic lymphocytic leukemia; IgVH, immunoglobulin heavy chain variable; PDE7B, phosphodiesterase 7B; SNP, single-nucleotide polymorphism; WBC, white blood cell; WT, wild type; ZAP-70, 70-kDa zeta-associated protein.
Data for age, IgVH%, Zap70 and WBC are shown as mean±s.d.

from patients who satisfied diagnostic and immunophenotypic criteria for CLL. The University of California, San Diego, Institutional review board approved these studies and the CLL Research Consortium approved the procurement of the samples, with all approvals in accordance with the Declaration of Helsinki.

Diagnosis of CLL was based on morphological and immunophenotyping criteria.[22] Patients with CLL were also categorized by the expression of 70-kDa zeta-associated protein.[23–25] Mononuclear cells were isolated using a Ficoll gradient and then frozen in fetal calf serum plus 10% dimethyl sulfoxide before storage in liquid N_2. The 70-kDa zeta-associated protein expression was determined by flow cytometry of mononuclear cells. PCR was used to assess the immunoglobulin heavy chain variable gene family. Table 1 shows demographic data, heavy chain variable and the 70-kDa zeta-associated protein status.

Genomic DNA and RNA extraction, PCR and sequencing

Genomic DNA and RNA were prepared using isolation kits for blood cells (Qiagen, Valencia, CA, USA). The complementary DNA (cDNA) was generated using the Superscript III cDNA synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We sought to identify genetic variants within the putative PDE7B (NC_000006.11) promoter and coding regions as follows: (a) *PDE7B* gene promoter, the region 700 bp upstream and 300 bp downstream of the transcription start site of PDE7B was amplified; this resulted in a 1-kb PCR product (P1; forward primer: 5′-CACCATAGCCTTGCTTCTTGA-3′; reverse primer, 5′-TGGCAGTTCTGTG ACCTTTG-3′). (b) *PDE7B* coding region (304 to 1656 nt), the full-length cDNA of PDE7B was amplified in two PCR products, P2 from 276 to 1165 bp (forward primer: 5′-CTGGAGAAGTTGCTGGATTCT-3′; reverse primer: 5′-ATT CATTCTGCCTGTTGATGTCT-3′) and P3 from 1074 to 1870 bp (forward primer: 5′-CTCTGGACATCATGCTTGGA-3′; reverse primer: 5′-CTCCCACG TTACTGAATGGAG-3′). PCR amplifications were performed (AccuPower PCR Premix; Bioneer, Alameda, CA, USA) using standard procedures with 50 ng of genomic DNA product (P1) or 200 ng of cDNA (P2–3), in a total volume of 20 μL containing 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 1 U DNA polymerase, 1.5 mM deoxyribonucleotide triphosphate and 0.5 μM each specific primer. PCR cycling conditions were: initial denaturing at 95 °C for 5 min (P1) or 2 min (P2–3) followed by 35 cycles of denaturing at 94 °C for 40 s; annealing at 59 °C (P1), 55 °C (P2) or 57 °C (P3) for 40 s; extension at 72 °C for 57 s (P1), 59 s (P2) or 58 s (P3); and a final extension step at 72 °C for 5 min. DNA Clean & Concentrator TM-5 kit (D4003 lt 0940 BW) and Centrisep columns (Zymo Research, Orange, CA, USA) were used to purify PCR products. Sequence was determined on a 3130×l Genetic Analyzer (Applied Biosytems, Carlsbad, CA, USA), with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Polymorphisms were manually confirmed. The data were validated visually and the files were inspected to identify heterozygotes. Each sequence was imported into the Biology Workbench (http://workbench.sdsc.edu/) for analysis. SNPs were confirmed by re-sequencing from the reverse direction.

Dual luciferase assays

To investigate the functional impact of −347 C>T polymorphism on *PDE7B* promoter activity, we constructed reporter plasmids with 5′-flanking regions that contained either wild type (−347C) or SNP (−347T). The 1-kb fragment encompassing the *PDE7B* transcription start site (from −700 to +300) was amplified from patients with the −347 CC or −347 TT genotype, and inserted into the firefly luciferase reporter plasmid pGL4-basic (Promega, Madison, WI, USA). HEK293 cells were transfected with 3 μg of pGL4-Luc containing PDE7B promoter regions of either the wild-type or SNP variants and 0.1 μg of pRL-TK-Luc using the Optifect transfection reagent (Invitrogen, Carlsbad, CA, USA). pRL-TK-Luc encodes the Renilla luciferase under the control of an

Table 2 Gender-allele frequency analysis of expression of the −347C>T variant of PDE7B

Gender	*Number*	*Alleles*	*SNP/total alleles*
Male			
CLL	96	4	4/192 (2%)
Control	77	5	5/154 (3.3%)
Total	173	9	9/346 (2.6%)*
Female			
CLL	58	2	2/116 (1.7%)
Control	16	0	0/32 (0%)
Total	74	2	2/148 (1.4%)*

Abbreviations: CLL, chronic lymphocytic leukemia; PDE7B, phosphodiesterase 7B; SNP, single-nucleotide polymorphism.
**P*-values were >0.1 for differences in expression of the variant between control males and males with CLL and between males and females.

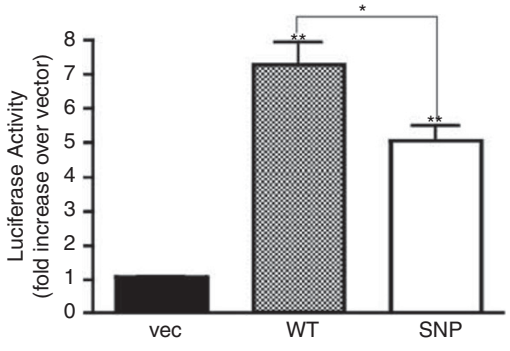

Figure 1 Impact of the −347C and −347T genotypes on PDE7B promoter function. pGL4 luciferase reporter constructs containing a 1-kb phosphodiesterase 7B promoter with the −347C (WT) or −347T (SNP) genotype were transfected into HEK293 cells. Vec, the promoter-less pGL4, was used as a control and its luciferase activity was defined as 1. The data represent the mean±s.d. of three independent experiments. $^{**}P<0.001$ compared with vec alone and $^{*}P<0.001$ between WT and SNP. SNP, single-nucleotide polymorphism; vec, vector; WT, wild type.

herpes simplex virus thymidine kinase promoter and serves as an internal control to normalize for transfection efficiency. After 48 h, cells were harvested and assessed using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly and Renilla luciferase activities were measured using a TD-20/20 Luminometer (Turner Design, Sunnyvale, CA, USA). The results shown were found in three independent experiments.

Statistical analyses

All determinations were performed in duplicate or triplicate. Values are expressed as mean ± s.d. Comparison between groups was based on 2×2 contingency table and use of the χ^2 statistic; $P<0.05$ was considered to be statistically significant.

RESULTS

CLL study cohort

To determine whether the *PDE7B* promoter possesses genetic variants, we sequenced samples prepared from 154 CLL patients and 93 control subjects. This cohort represents a typical population of CLL patients (that is, 62% male and 38% female subjects; mean age at diagnosis,

Table 3 Genetic variation (cSNPs) in human PDE isoforms, contigs that contain the SNPs and disorder/phenotype/disease associations of the variants

PDE	*Total*	*cSNPs*	*Contig*	*Disorder/phenotype/disease*
1A	2045	0	NT_005403.16	Major depression: association with remission produced by antidepressants drugs[34] Depression: no association with treatment response[33] or to citalopram[35] Autism: no significant association[49]
1B	212	7	NT_029419.11	None
1C	1574	11	NT_007819.16	Catecholaminergic polymorphic ventricular tachycardia: association[50]
2A	651	12	NT_033927.7	None
3A	1963	6	NT_009714.16	None
3B	652	8	NT_009237.17	Type 2 diabetes mellitus: no association in Japanese subjects[51]
4A	126	14	NT_011295.10	None
4B	276	11	NT_032977.8	Associated with female-specific protection against schizophrenia[19] Associated with schizophrenia and bipolar disorder[17] also in Japanese population[18] Not associated with risk for schizophrenia[52] Associated with strokes subtypes, intracranial large artery atherosclerosis and small artery occlusion and with stroke risk factors such as diabetes and smoking[53] Associated with control of prostate smooth muscle[54]
4C	248	10	NT_011295.10	None
4D	5442	5	NT_006713.14	Associated with greater risk of ischemic stroke[20,53,55–58] in hypertensive patients[59] and also in Korean,[60] Chinese Han[61] and Moscow[62] populations Not associated with greater risk of ischemic stroke,[20,63–65] including in a Sardinian population[66] Associated with chronic obstructive pulmonary disease[67] Associated with sleep and circadian phenotypes[68] and a subtype of neuroticism[69,70] Associated with chronic kidney disease in low risk subjects[71] Associated with carotid atherosclerosis[72] Associated with basal metabolic index and asthma[73]
5A	892	5	NT_016354.18	Heart rate and blood pressure: not associated with sildenafil response in men with erectile dysfunction[36,74] Associated with pulmonary hypertension in patients with advanced liver disease[75] Associated with progression of childhood immunoglobulin A nephropathy[76]
6A	464	14	NT_029289.10	Associated with retinitis pigmentosa[21,77] also in consanguineous Pakistani families[78]
6B	294	7	NT_037622.5	Associated with congenital stationary night blindness:[79] associated with retinitis pigmentosa[80,81] and Usher syndrome[82]
6C	443	15	NT_030059.12	Not associated with retinitis pigmentosa[83]
7A	253	3	NT_008183.18	None
7B	1616	2	NT_025741.14	Associated with fetal hemoglobin levels in sickle cell anemia[84] Chronic lymphocytic leukemia: no significant association (current study)
8A	971	5	NT_010274.16	Not associated with polycystic ovary syndrome and androgen levels in women[85]
8B	1196	7	NT_006713.14	Associated with serum TSH levels and thyroid function[86] and with subclinical hypothyroidism in pregnancy[87]
9A	1072	8	NT_030188.4	Associated with susceptibility to major depressive disorder and antidepressant treatment response[34] Depression: no association with treatment response[49]
10A	2267	11	NT_007422.13	Associated with serum TSH levels and thyroid function[88]
11A	2051	7	NT_005403.16	Associated with susceptibility to major depressive disorder and antidepressant treatment response[34] Not associated with treatment or citalopram response in depression[49] Associated with predisposition to adrenocortical tumors[88] Associated with risk of familial and bilateral testicular germ cell tumours[89] and marginally contributory to the development of somatotropinomas in a subset of acromegalic patients[90] Associated with asthma[91]

Abbreviations: cSNPs, coding single-nucleotide polymorphisms; PDE, phosphodiesterase; SNP, single-nucleotide polymorphism; TSH, thyrotrophin-stimulating hormone.
Contigs of PDE family members, their total number of SNPs and cSNPs listed on the National Center for Biotechnology Information dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/) are shown.

62 years). We also evaluated patient samples for expression of the immunoglobulin heavy chain variable mutation and 70-kDa zeta-associated protein, both of which are prognostic markers in CLL.[26,27] (Table 1).

Identification of a variant in PDE7B and comparison of its frequency in control and CLL subjects

In initial studies designed to identify genetic variants in PDE7B, we sequenced ~1.3 kb of its coding region in 43 CLL patients and 7 normal subjects and found limited genetic variation in this region: a SNP in the 5′ upstream region, −347C>T, occurred with ~2% frequency (1.9% in CLL patients 6/308 alleles (all heterozygotes) and 2.7% in 5/186, (3 heterozygotes and 1 homozygote) in controls). Thus, the overall SNP frequency was 2.2% (11/494 alleles). Subgroup analysis of CLL patients classified as having either aggressive or indolent CLL[28,29] revealed a comparable frequency of SNP expression in the two groups of patients (data not shown). Males had a slightly higher, statistically nonsignificant ($P>0.1$) SNP frequency (2.6%; 9/346) than did females (1.4%; 2/148; Table 2). Expression of the SNP was not associated with other CLL markers (Table 1).

Use of the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) to predict transcription factor binding sites revealed that the *PDE7B* promoter containing the SNP has putative binding sites for multiple transcription factors, including the sex determination region Y, which encodes a sex-determining transcription factor of the high-mobility group box family and two members of the forkhead box (FOX) family, FOXA2 and FOXD3.[30,31] The TFSEARCH program predicted that the −347C>T SNP abolishes the binding site for sex determination region Y and FOXA2 but not FOXD3. Although binding of FOXD3 was not altered by the SNP, this transcription factor is down-regulated in CLL and in a mouse model of CLL through a nuclear factor-κB p50:histone deacetylase 1 co-repressor complex 3.[32]

Functional assessment of the PDE7B promoter SNP

On the basis of possible impact of the PDE7B promoter SNP on transcription, we assessed its activity using a dual luciferase assay and found that the 347C>T variant had decreased transcriptional activity compared with the wild-type construct ($P<0.001$; Figure 1).

DISCUSSION

Our results indicate that the coding sequence and 5′ upstream region of the *PDE7B* gene has a SNP (−347C>T) that occurs with low frequency (that is, $P<3\%$), is located in a possible putative binding sites for sex determination region Y, FOXA2 or FOXD3 and decreases transcriptional activity. Thus, although relatively rare, the SNP has the potential to contribute to inter-individual differences in PDE7B expression.

On the basis of information available at the dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), PDE7B is predicted to contain 1616 SNPs within the 345 718-bp contig reported in Build 129. Only two of these are coding SNPs (found in exons 8 and 12), both synonymous, and the rest are found in regulatory regions and introns. There are nine SNPs identified between −473 and −1756 located in the 5′ UTR that are deposited in dbSNP. Thus, the −347C>T SNP discovered in this study is novel, although overall, PDE7B has a relative dearth of potentially functional polymorphisms. Such conservation may imply that the function of PDE7B is important, such that it has undergone minimal genetic change during evolution.

Table 3 summarizes data for genetic variation in PDEs. Genetic variants of PDE6, a retina-specific PDE, occur in certain retinal diseases while other data implicate associations between PDE polymorphisms and certain central nervous system, metabolic, cardiovascular or neoplastic disorders. Inter-subject variability in response to PDE-interacting drugs associates both positively and negatively with genetic variations in PDEs. For example, genetic variations in PDE1A, PDE9A and PDE11A associate with the response to antidepressants but apparently only in certain populations.[33–35] Polymorphisms in PDE5A appear unable to explain differences in cardiovascular effects in subjects who are administered sildenafil or other PDE5 inhibitors, implicating other mechanisms besides such genetic variation as contributing to variable responses.[33,34,36]

Could our results that fail to show an association of genetic variants for PDE7B in CLL be falsely negative? Might copy number variation explain the prominent increase in gene expression of PDE7B in CLL patients?[16,37] Copy number variation (1000 bp segments of genomic DNA with inter-individual variability in copy number[38,39]) has been associated with numerous diseases.[40–42] Copy number variations may affect gene expression[43] and thus might increase expression of PDE7B transcripts in patients with CLL. An additional mechanism for the increase in PDE7B expression in CLL could be non-coding RNAs, including miRNAs, expression of certain of which is increased in CLL and may contribute to the difference in aggressive and indolent forms of the disease.[44] Further studies will be needed to assess gene structure in CLL[45] and also whether the SNP alters methylation of the PDE7B promoter, thereby changing chromosomal structure and transcriptional activity.[35,46–48]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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