

Epigenetic inactivation of PLCD1 in chronic myeloid leukemia

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Abstract. Phospholipase C δ 1 (PLCD1), is located at the important tumor suppressor locus 3p22. It encodes an enzyme that mediates regulatory signaling of energy metabolism, calcium homeostasis and intracellular movements. PLCD1 has been studied in some human solid tumors relating to the CpG island methylation of the gene promoter as a functional tumor suppressor. However, no such information is available in chronic myeloid leukemia (CML). In this study, we investigated PLCD1 expression in the CML K562 cell line (0/1) and 15% (2/13) of bone marrow mononuclear cells with CML by using semi-quantitative PCR. The CpG island (CGI) methylation status of the PLCD1 promoter was detected in K562 (0/1) and 56% (23/41) of CML patients by methylation-specific PCR (MSP), but not in the normal adult bone marrow mononuclear cells. Furthermore, the DNA demethylation agent 5'-aza-2'-deoxycytidine restored the expression of PLCD1 in K562 cells. Functional studies showed that ectopic expression of PLCD1 in K562 cells was able to dramatically inhibit their colony formation and induce cell cycle G1 arrest, suggesting that PLCD1 acts as a functional tumor suppressor and may serve as a biomarker for possible early detection and prognosis of CML.

Introduction

PLCD1 (phospholipase C δ 1) belongs to a mammalian phosphoinositide specific PLC superfamily, which is a key enzymatic superfamily in the phosphoinositide metabolism system and hydrolyses phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] to generate two second messengers, inositol 1,4,5-triphosphate

and diacylglycerol. To date, there are six major families of PLC enzymes (β , γ , δ , ϵ , ζ and η). Among these family members, PLCD is considered to be the basic isoform and to be composed of 3 isozymes, PLCD1, D3 and D4 (1). Deletion of 3P is one of the most common genetic alterations in multiple cancers (2,3). It is believed that multiple TSGs are located in this important region, such as BLU, DLEC1 and PLCD1 (4-6).

In the pathogenesis of leukemia, epigenetic silencing of TSGs is a frequent event (7-9). The methylation of several TSGs, such as estrogen receptor (ER), death-associated protein kinase (DAPK1) and protein tyrosine phosphatase receptor typer (PTPRG), have been confirmed to be associated with the development and progression of chronic myeloid leukemia (CML) (10-12). Multiple TSGs at 3p have been reported, such as BLU, DLEC1, PLCD1, FHIT, RASSF1A, CACNA2D2 (4-6,13-15) and these TSGs are involved in the pathogenesis of various tumors. Although these TSGs have been widely studied in many tumors, TSG PLCD1 with tumor-specific methylation has not been identified in CML, which would be worthy of further exploring to identify novel diagnostic biomarkers and therapeutic strategies for this tumor.

Here, we report that PLCD1 is frequently methylated in the CML K562 cell line and bone marrow samples with CML, resulting in loss of its expression at the mRNA level. Ectopic PLCD1 expression in K562 cells obviously suppressed colony formation ability and led to cell cycle arrest at G1 phase, confirming that PLCD1 is a novel functional tumor suppressor gene in CML.

Materials and methods

Cell culture and sample collection. The K562 CML cell line was obtained from the Clinical Laboratory of Chongqing Medical University (Chongqing, China) and routinely maintained in RPMI-1640 media (Gibco-BRL, USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL). We studied 41 patients [18 females, 23 males, of a median age of 46 years (range 17-80 years)] with CML and 15 healthy adults. Thirty-one patients were in chronic (CP), 3 in accelerated (AP) and 7 in blast phase (BP). The diagnosis was established according to standard morphologic and immunophenotypic criteria of WHO classification (16). The samples were collected by the Laboratory of Hematology (the

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Table I. Correlation between methylation of the PLCD1 gene and the clinical characteristics of CML patients.

Patient parameters	Patients with methylated PLCD1 (n=23)	Patients with unmethylated PLCD1 (n=18)	Total (n=41)	P-value
Staging, n				0.1378
CP	17	14	31	
AP	2	1	3	
BP	4	3	7	
Gender (male/female), n	14/9	9/9	23/18	0.486
Age (years) mean (range)	45.74 (17-80)	46.90 (18-75)	45.99 (17-80)	0.843
WBC ($\times 10^9$) mean (range)	38.00 (3.18-213)	67.46 (2.1-284)	49.43 (2.1-284)	0.243
Hemoglobin ($\times 10^9$) mean (range)	89.09 (43-155)	79.00 (10-133)	86.01 (10-155)	0.724
Platelet counts ($\times 10^9$) mean (range)	273.17 (11-873)	422.56 (21-913)	352.07 (11-913)	0.082

CP, chronic phase; AP, accelerated phase; BP, blast phase; WBC, white blood cells.

First Affiliated Hospital of Chongqing Medical University, Chongqing, China). Informed consent was provided according to the Declaration of Helsinki.

Drug treatment. K562 cells were treated with 10 μ mol/l 5'-aza-2'-deoxycytidine (Aza; Sigma, USA) for 3 days and further treated with 100 μ mol/l trichostatin A (TSA; Sigma) for 24 h as described previously (17,18).

Total-RNA isolation and semi-quantitative reverse transcription (RT-PCR). Total-RNA was reversely transcribed to first-strand cDNA, using Go-Taq (Promega, USA) and random hexamer primers. GAPDH served as a control for RNA integrity. PLCD1 expression was analyzed by PCR, primers used were PLCD1-F, 5'-TGTCGCTACTCAAGTGAGTC-3' and PLCD1-R, 5'-AGT CCTCTGAACCTGTAG-3'. GAPDH-F, 5'-ATCTCTGCCC CCTCTGCTGA-3' and GAPDH-R, 5'-GATGACCTTGCCCA CAGCCT-3'. The PCR thermocycling conditions included initial denaturation at 95°C for 10 min, followed by 35 cycles (for PLCD1) or 23 cycles (for GAPDH) of reaction (95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec), using Go-Taq polymerase (Promega), with a final extension at 72°C for 10 min.

DNA bisulfite treatment and methylation analysis. DNA was extracted from CML and healthy adult bone marrow samples by standard method (Zymo Research, USA). Bisulfite modification of DNA and methylation status in the CpG island of PLCD1 promoter were carried out as described previously (19,20). PLCD1 primers detecting methylation or unmethylation alleles of the promoter were PLCD1-m1, 5'-AATGATA GGGTTCGCGGTTTC-3' and PLCD1-m2, 5'-CCCGAACCAA CGAACGCG-3' for methylated; PLCD1-u1, 5'-GTAATGA TAGGGTTTGTGGTTT-3' and PLCD1-u2, 5'-TAACCCAAA CCAACAAACACA-3' for unmethylated. Methylation-specific PCR (MSP) was done for 40 cycles using Ampli Taq-Gold (methylation specific primer: annealing temperature 60°C, unmethylation specific primer: annealing temperature 58°C). MSP primers were tested previously for not amplifying any unbisulfite DNA and the specificity of MSP was further

confirmed by any direct sequencing of some PCR products. PCR reactions were resolved on a 2% agarose gel.

Cell transfection and colony formation assays. K562 cells were plated in a 6-well plate and transfected with PLCD1 expression plasmid pcDNA3.1 or the empty vector pcDNA3.1 (0.8 μ g each) respectively, using FuGENE® 6 (Roche Diagnostics, Mannheim, Germany). Forty-eight hours post-transfection, cells were re-plated into a 6-well plate with G418 selection (0.5 μ g/ μ l). The selective medium was replaced every 3 days. After 2 weeks, stable PLCD1 expression clones were selected for further study. Total-RNA was extracted and analyzed by RT-PCR to confirm the ectopic expression of PLCD1.

Stable PLCD1 transfectants and the vector cells were suspended in RPMI-1640 containing 1% methylcellulose, 10% fetal bovine serum (FBS) and 0.5 mg/ml G418 in a 6-well plate. After 2 weeks, surviving colonies (≥ 50) were counted under a light microscope. All the experiments were performed 3 times in triplicate wells.

Cell cycle analysis. The stable PLCD1 expression cells (PLCD1-K562) or Vector-K562 cells were cultured in RPMI-1640 medium and 10% FBS with G418 (0.2 mg/ml). These cells were harvested and fixed in ice-cold 75% ethanol for 1 h. The cell cycle profiles were assayed by the Elite ESP flow cytometer and data were analyzed with the CellQuest software (BD Biosciences, USA).

Protein extraction and western blot analysis. After washing the cells with PBS for 3 times, PLCD1-K562 or Vector-K562 cells were harvested and lysed in RTPA buffer containing 0.1 mg/ml of PMSF as final concentrations. Western blot analysis was performed according to the standard protocol as described (13). Anti-PLCD1 rabbit polyclonal antibody (1:700; GeneTex, Inc., USA) and anti-GAPDH mouse monoclonal antibody (1:1,000; Epitomics, Inc., USA) were used.

Statistical analysis. All statistical calculations were done using SAS version 9.1 for windows (SAS Institute, Inc., Cary, NC, USA). The correlation between the frequency of PLCD1

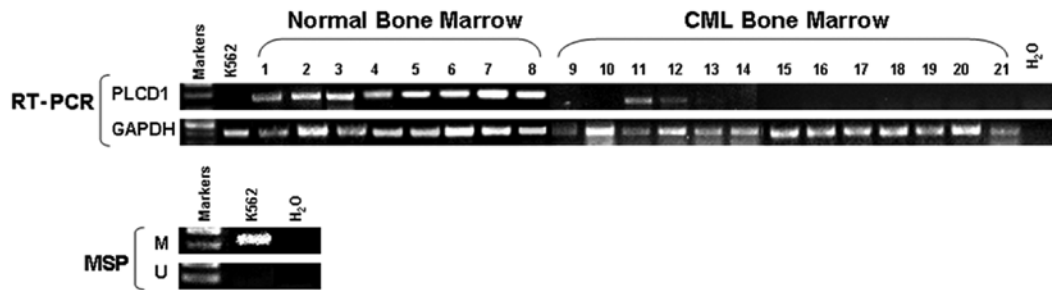


Figure 1. Downregulation of PLCD1 by promoter methylation in CML K562 cell line. PLCD1 expression and methylation status in K562 cells was determined by semi-quantitative RT-PCR and MSP. PLCD1 expression in human normal and CML samples was determined by semi-quantitative RT-PCR with GAPDH as a control. M, methylated; U, unmethylated.

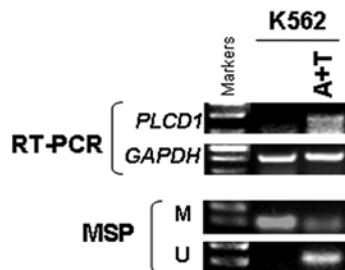


Figure 2. Pharmacological demethylation using Aza together with TSA reactivating the silenced PLCD1 in K562 cells. A+T, Aza and TSA treatment.

promoter methylation and age, white blood cells, hemoglobin level and platelet counts was analyzed with Student's t-test. Student's t-test was also used for the difference in subgroups (cell cycle analysis and colony formation assay). Chi-square test was carried out to compare the correlation between frequency of PLCD1 promoter methylation and clinical stages, correlation between the frequency and gender was analyzed with Fisher's exact test. P-value <0.05 was considered to be significant.

Results

PLCD1 downregulation and promoter hypermethylation in K562 cells. To examine if PLCD1 is downregulated in K562 cells, we first examined the expression in K562 cells, 8 normal adult bone marrow samples and 13 CML samples with semi-quantitative RT-PCR. PLCD1 was silenced in K562 cells and 11/13 (85%) CML bone marrow samples while readily detected in normal adult bone marrow samples (0/8) (Fig. 1).

We further analyzed the methylation status of PLCD1 to determine whether downregulation in K562 cells is caused by hypermethylation in its promoter region. MSP using methylation- or unmethylation-specific primers was done to investigate the methylation status of PLCD1. PLCD1 CGI was methylated in K562 cells, while no methylation was detected in normal adult bone marrow (Fig. 1), showing that the silence of PLCD1 expression in K562 cells was correlated with its methylation status.

Pharmacological demethylation reactivates the silenced PLCD1. To evaluate the effect of promoter CGI methylation

on the expression of PLCD1, K562 cells were treated with a DNA methyltransferase inhibitor, 5'-azacytidine for 3 days together with a histone deacetylase inhibitor, TSA for 1 day. As shown in Fig. 2, PLCD1 mRNA expression was dramatically induced after the treatment, this reactivation was associated with an increase of unmethylated alleles and decrease of methylated alleles of the PLCD1 promoter, as assessed by MSP. These results show a direct link between CGI methylation and PLCD1 silencing.

Frequent PLCD1 methylation in various phases of CML.

In view of the effect of promoter region methylation on the expression of PLCD1 in K562 cells, we proceeded to determine the methylation status of CML and normal adult bone marrow samples. As summarized in Fig. 3, aberrant methylation was detected in 23/41 (56%) CML samples (17/41 in the stage of CP, 2/41 in the stage of AP, 4/41 in the stage of BP), but not in normal adult samples (0/15). The frequencies of PLCD1 promoter methylation in CML patients in CP, AP and BP are shown in Table I. Thus, promoter methylation of PLCD1 is a frequent and tumor-specific event in CML. The correlation between PLCD1 promoter methylation and clinical stage, age, gender, white blood cells (WBC), hemoglobin (Hb) and platelet counts (PLT) are shown in Table I.

Ectopic expression of PLCD1 inhibits tumor cell clonogenicity and induces G1 cell cycle arrest. To evaluate the role of PLCD1 as a TSG in CML, we thus sought to establish whether ectopic expression of PLCD1 could inhibit tumor cell clonogenicity. The expression vector encoding full-length PLCD1 or vector alone were transfected into K562 cells, in which PLCD1 was fully silenced by methylation. After G418 selection for 2 weeks, stable overexpression of PLCD1 as shown by RT-PCR and western blot analysis, was successfully obtained (Fig. 4C). Colony formation assay (Fig. 4A) was used to evaluate the suppressor function of PLCD1 in vector or PLCD1-transfected cells. Ectopic expression of PLCD1 dramatically reduced the colony formation efficiencies of K562 cells (Fig. 4B) down to 40-50% of vector controls, $P < 0.05$, indicating that PLCD1 was a functional TSG in K562 cells.

To explore the mechanism by which PLCD1 suppresses colony formation, we investigated the effect of PLCD1 on cell cycle distribution by flow cytometry. The percentage of cells in G1 phase was increased in PLCD1-transfected cells compared

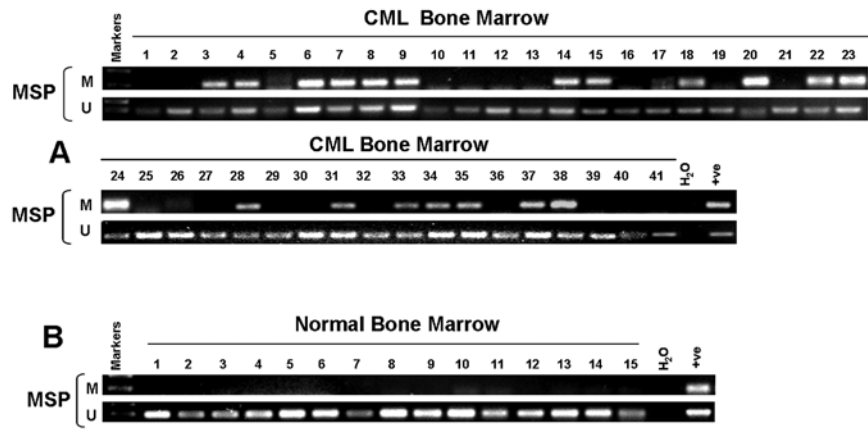


Figure 3. PLCD1 CpG methylation in CML and normal adult bone marrow samples. (A) CML bone marrow samples. (B) Normal adult bone marrow samples. M, methylation; U, unmethylation.

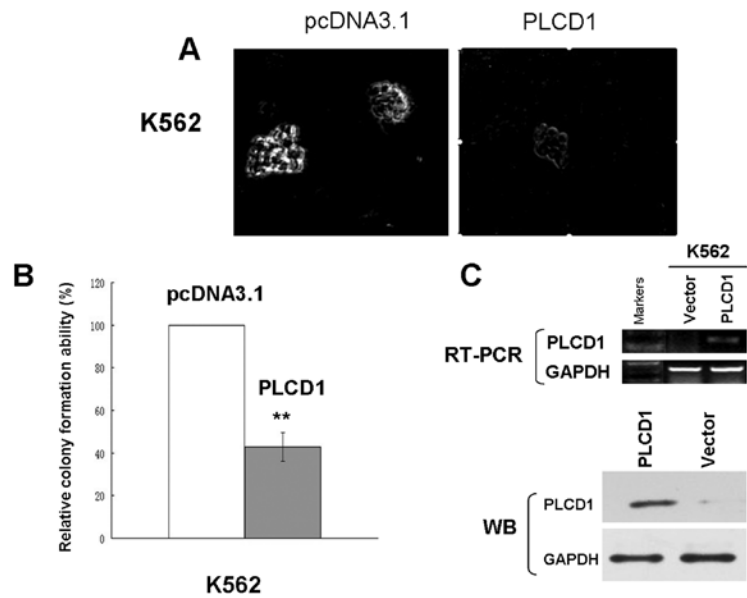


Figure 4. Ectopic expression of PLCD1 inhibited tumor cell clonogenicity. (A) Representative inhibition of colony formation in soft agar culture by PLCD1. (B) Quantitative analysis of colony formation. The numbers of G418 selection colonies in vector-transfected cell line were set to 100%, values are mean \pm SD, P-values were calculated using Student's-test. **Significant statistical difference ($P < 0.05$). Independent experiments were performed in triplicate wells 3 times. (C) Ectopic expression of PLCD1 in the transcript level in transfected K562 cells was confirmed by RT-PCR and WB. WB, western blot analysis.

to vector K562 cells ($P < 0.05$), indicating that the effect of PLCD1 was likely to be dependent on the cell cycle (Fig. 5). Thus, taken these results together, PLCD1 indeed has growth inhibitory activity and was a functional TSG in CML.

Discussion

Multiple TSGs at 3p have been reported, such as BLU, DLEC1, PLCD1, FHIT, RASSF1A, CACNA2D2 (4-6,13-15), and these TSGs are involved in the pathogenesis of various tumors such as gastrointestinal tract, lung, nasopharynx, esophagus, kidney, breast and cervix. Although these TSGs have been widely studied in many tumors, TSG PLCD1 with tumor-specific methylation has not been identified in CML.

In the present study, we found that PLCD1 was downregulated in K562 cells and in 85% CML bone marrow samples, but widely expressed in normal adult bone marrow samples.

Our study showed that promoter CGI methylation of PLCD1 in K562 cells and 56% CML samples, and pharmacological demethylation could restore PLCD1 expression, suggesting that promoter CGI methylation plays an important role in the inactivation of PLCD1 during the tumorigenesis of CML.

Our present study showed that ectopic expression of PLCD1 in K562 cells significantly suppressed their colony formation which was able to arrest tumor cells at cell cycle in K562. Fu *et al* (21) demonstrated that PLCD1 exerted its tumor-suppressive function through inhibiting cell cycle progression from G1 to S-phase in ESCC, which is consistent with our findings. Thus, PLCD1 is a functional TSG in CML.

PLCD1 methylation was found to be significantly correlated with lymph node metastasis in breast cancer (18), ESCC (21) and high-stage gastric tumor (6), indicating that PLCD1 has been identified as a TSG and can be a potential biomarker in these tumors. The high incidence of this tumor-specific

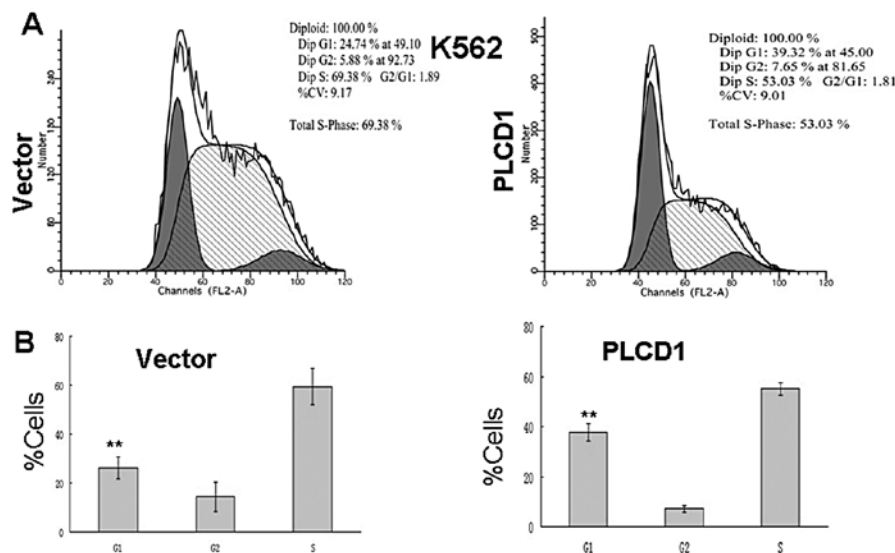


Figure 5. PLCD1 induces cell cycle arrest at the G1 phase in K562 cells. (A) Representative distribution of cell cycles in K562 cells transfected with or without PLCD1 by fluorescence-activated cell sorting analysis. (B) Analysis of K562 cells transfected with or without PLCD1 distributing in different phases of cell cycle. Results are presented as the mean \pm SD (n=4). Significant statistical difference, $P < 0.05$ (Student's t-test).

molecular marker in CML suggest that it might be a clinically useful marker of disease. PLCD1 is a relevant new player in the pathogenesis of CML and suggests that it as a potential target for diagnostic and therapeutic applications. There were no significant correlations between the methylation status of PLCD1 promoter and the clinical features, such as clinical stages, age, gender, hemoglobin level, white blood cells (WBC) and platelet counts ($P > 0.05$). Our results suggest that the methylation of PLCD1 promoter might occur in the early stage of CML development. However, the clinical impact of PLCD1 inactivation in CML is still unknown, further studies of possible clinicopathological relevance of PLCD1 methylation and its potential diagnostic and prognostic implications in patients with CML are needed, such as chromosomal abnormalities and BCR/ABL transcript. Other mechanisms besides DNA methylation such as histone modification might also be involved in the regulation of PLCD1 expression. More cases should be studied to determine the impact of PLCD1 methylation on the regulation of transcription.

In summary, we found that PLCD1 is frequently silenced by promoter methylation in CML in a tumor-specific manner, which could be activated by pharmacological demethylation. Ectopic expression of PLCD1 suppresses CML cells clonogenicity and leads to the disruption of cycle progression at G1 phase in CML, supporting that PLCD1 is a TSG in this tumor. It would be worthy of further exploring the possible use of PLCD1 methylation as an epigenetic biomarker for future molecular diagnosis.

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