

Programmed cell death 4 and BCR-ABL fusion gene expression are negatively correlated in chronic myeloid leukemia

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Abstract. Programmed cell death 4 (PDCD4) is a tumor suppressor that inhibits carcinogenesis, tumor progression and invasion by preventing gene transcription and translation. Downregulation of PDCD4 expression has been identified in multiple types of human cancer, however, to date, the function of PDCD4 in leukemia has not been investigated. In the present study, PDCD4 mRNA and protein expression was investigated in 50 patients exhibiting various phases of chronic myeloid leukemia (CML) and 20 healthy individuals by reverse transcription-quantitative polymerase chain reaction and western blot analysis. PDCD4 expression and cell proliferation was also investigated following treatment with the tyrosine kinase inhibitor, imatinib, in K562 cells. The results demonstrated that PDCD4 mRNA and protein expression was decreased in all CML samples when compared with healthy controls, who expressed high levels of PDCD4 mRNA and protein. No significant differences in PDCD4 expression were identified between chronic phase, accelerated phase and blast phase CML patients. In addition, PDCD4 expression was negatively correlated with BCR-ABL gene expression ($r=-0.6716$; $P<0.001$). Furthermore, K562 cells treated with imatinib exhibited significantly enhanced PDCD4 expression. These results indicate that downregulation of PDCD4 expression may exhibit a critical function in the progression and malignant proliferation of human CML.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the presence of the Philadelphia

(Ph) chromosome, which is formed by a t(9;22) (q34;q11) balanced reciprocal translocation (1). The Ph chromosome translocation generates the BCR-ABL oncogene that encodes for the BCR-ABL oncoprotein, which exhibits constitutively active tyrosine kinase activity that promotes the growth of leukemic cells (2). CML patients in the chronic phase (CP) that are treated with tyrosine kinase inhibitors (TKIs) achieve a significant effect (1). However, a significant percentage of patients develop TKI resistance and disease recurrence (3), which involves a variety of cellular mechanisms. Therefore, identifying molecules that are involved in the development and progression of CML may provide novel therapeutic targets for CML treatment.

Programmed cell death 4 (PDCD4) is a novel tumor suppressor that inhibits tumor growth via suppression of protein translation by binding to eukaryotic initiation factor (EIF) 4A via two MA-3 domains, which are highly homologous to eIF4G (4). PDCD4 also suppresses translation elongation by combining directly with target gene coding regions, such as c-myc (5) and A-myc (6). PDCD4-deficient mice exhibit a significantly reduced life span and develop spontaneous lymphomas with frequent metastasis to the liver and kidneys (7). PDCD4 transgenic mice exhibit resistance to tumor promotion and progression in response to a multi-stage carcinogenesis regimen (8). Additionally, decreased or absent PDCD4 expression has been identified in several types of human cancer, including lung (9), hepatocellular (10), colon (11), glioma (12) and ovarian cancers (13). Overexpression of PDCD4 suppresses tumor phenotypes by inhibiting activator protein-1 (AP-1) transactivation in JB6 cells (14). PDCD4 overexpression and has also been demonstrated to inhibit invasive capacity in colon RKO cells (15), inhibit tumor cell intravasation (16) and suppress malignant phenotypes of human ovarian cancer (13). Furthermore, PDCD4 knockdown significantly promotes invasion and activates both β -catenin/T cell factor (Tcf) and AP-1-dependent transcription (17). Additionally, PDCD4 knockdown leads to increased Snail expression and subsequent downregulation of E-cadherin resulting in the activation of catenin/Tcf-dependent transcription and the expression of c-Myc and urokinase receptor (18).

Taken together, these findings indicate that PDCD4 presents a potential target in the diagnosis and treatment of neoplasms. However, whether PDCD4 is involved in human hematologic neoplasms remains unclear. In the present study,

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PDCD4 expression levels in CML patients were evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Materials and methods

Patients and samples. A total of 50 bone marrow aspirate samples were obtained from patients diagnosed with CML, according to the World Health Organization guidelines (19), at Yuhuangding Hospital of Qingdao University (Yantai, China) between June 2012 and September 2014. The CML patient sample included 23 females and 27 males with a mean age (\pm standard deviation) of 41.94 years (\pm 14.13 years). Patients were divided into the following three groups depending on their clinical and laboratory data: into 30 CP (n=30), accelerated phase (AP; n=10) and blast phase (BP; n=10). The criteria for diagnosis of CML-CP was the presence of t (9;22) or the BCR-ABL fusion gene, <10% bone marrow blasts, and does not satisfy the diagnostic criteria of CML-AP or CML-BP. Peripheral blood samples were also obtained from 20 healthy individuals, which served as the control group. Patient characteristics are listed in Table I. The final protocol for the use of patient samples in the present study was approved by the local Institutional Review Board of Qingdao University and informed consent was obtained from all patients and controls.

Cell lines and treatment. K562 cells were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific), 1% penicillin and streptomycin at 37°C in a humidified incubator with 5% CO₂. A total of 2x10⁵ cells/well were seeded in 6-well plates and treated with 0.5 and 1 μ M imatinib (LC Laboratories, Woburn, MA, USA) for 6, 24 and 48 h. Next, cells were harvested and washed once with phosphate-buffered saline and subjected to RT-PCR and immunoblotting analyses. Experiments were performed in triplicate.

MTT assay. A total of 8x10³ cells/well were seeded into 96-well plates and treated with 0.5 and 1 μ M imatinib. After 6, 24 and 48 h of imatinib treatment, 20 μ l MTT reagent (5 mg/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to each well and incubated in the dark for 4 h at 37°C. After 4 h, 100 μ l dimethyl sulfoxide was added to each well and the absorbance was determined using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm. Cell proliferation of treated cells was calculated from the average optical density at 570 nm values compared to that of untreated cells. Experiments were performed in triplicate.

RNA isolation and RT-qPCR. RNA was extracted from isolated peripheral blood mononuclear cells using a modified TRIzol one-step extraction method (Invitrogen; Thermo Fisher Scientific). RNA concentrations were determined based on the absorbance at 260 nm. Total RNA (2 μ g) was reversely transcribed to cDNA using the Reverse-Transcribe Kit (Promega Corp., Madison, WI, USA). Quantitative PCR

was performed using SYBR® Selected Master Mix (Applied Biosystems; Thermo Fisher Scientific) and specific primer pairs (Invitrogen; Thermo Fisher Scientific Inc.). The sequences of the sense and antisense primers were as follows: Sense, 5'-TGT AAACCCTGCAGATCCTGATAA-3' and antisense, 5'-TGG AGGATGCTGAAATCCAA-3' for PDCD4; sense, 5'-GGA GCTGCAGATGCTGACCACC-3' and antisense, 5'-TCAGAC CCTGAGGCTCAAAGTC-3' for BCR-ABL; sense, 5'-AAC GGATTTGGTCGTATTGGG-3' and antisense, 5'-CCTGGA AGATGGTGATGGGAT-3' for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The samples were denatured at 95°C for 10 min, followed by 39 cycles of 95°C for 30 sec and 60°C for 1 min and 65°C for 30 sec to stop the reaction. Each experiment was conducted in triplicate. Relative PDCD4 expression was calculated using the $\Delta\Delta$ Cq model (20). All samples were normalized to GAPDH, which served as an endogenous control.

SDS-PAGE and western blot analysis. Cells were lysed in SDS sample buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the protein concentration of the homogenized lysates was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) and equal amounts of protein were separated on 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were then blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (Beyotime Institute of Biotechnology) for 1 h and incubated overnight at 4°C with rabbit monoclonal anti-PDCD4 (catalog no. 9535S; dilution, 1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA) and human polyclonal anti- β -actin (catalog no. sc-130656; dilution, 1:2,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies. The membranes were then incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (catalog no. sc-2004; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After washing with Tris-buffered saline with 0.1% Tween-20, signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). Quantification of PDCD4 protein was normalized to β -actin using Quantity One® Version 4.3.1 (Bio-Rad Laboratories, Inc.). Western blot analysis was performed at least three times for each sample.

Statistical analysis. Data were obtained from at least three independent experiments and are presented as the mean \pm standard error of the mean. All statistical analysis was performed using SPSS 10.0 statistical software (SPSS, Inc., Chicago, IL, USA). Data was analyzed using the Student's t-test or one-way analysis of variance. Pearson's coefficient was calculated to analyze the correlation between PDCD4 and BCR-ABL expression. P<0.05 was considered to indicate a statistically significant difference.

Results

Decreased PDCD4 mRNA expression is observed in CML patients. To investigate PDCD4 expression in primary CML, PDCD4 mRNA expression was analyzed in 50 CML patients

Table I. Clinical parameters and PDCD4 expression in 50 CML patients and 20 healthy individuals.

Parameter	Healthy controls	CML patients		
		CML-CP	CML-AP	CML-BP
Gender, n				
Male	10	13	5	4
Female	10	17	5	6
Age, years				
Median	36	49	49	51
Range	24-59	15-71	32-63	39-76
PDCD4/GAPDH (\pm SEM)	1.65 \pm 0.17	0.150 \pm 0.03	0.13 \pm 0.04	0.15 \pm 0.05

PDCD4, programmed cell death 4; CML, chronic myeloid leukemia; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CP, chronic phase; AP, accelerated phase; BP, blast phase; SEM, standard error of the mean.

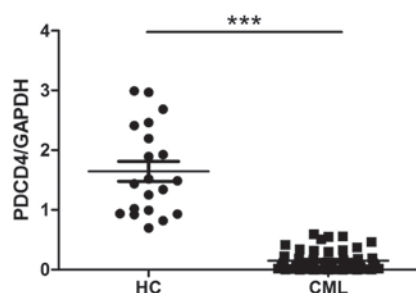


Figure 1. Expression of PDCD4 mRNA expression in 50 primary CML patients and 20 HCs was analyzed by reverse transcription-quantitative polymerase chain reaction. PDCD4 mRNA expression was significantly higher in HCs compared with CML patients. *** P <0.001. PDCD4, programmed cell death 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CML, chronic myeloid leukemia; HC, healthy controls.

and 20 healthy controls by RT-qPCR. As shown in Fig. 1, high levels of PDCD4 mRNA expression were observed in the 20 healthy control samples, however, all CML patients exhibited extremely low PDCD4 expression. Furthermore, the differences between PDCD4 expression in three phases of CML were analyzed, however, no significant differences in PDCD4 mRNA expression were identified in CML-CP, CML-AP and CML-BP patients (Table I).

Decreased PDCD4 protein expression is observed in CML patients. To evaluate PDCD4 protein expression levels in primary CML, PDCD4 protein expression in 50 CML patients and 20 healthy controls was analyzed by western blot. The results demonstrated that all healthy controls exhibited high PDCD4 protein expression, however, PDCD4 protein expression was markedly decreased or absent in the 50 CML samples, in accordance with that of PDCD4 mRNA (Fig. 2A). PDCD4 protein expression in CML patients was significantly decreased when compared with healthy controls (P <0.01; Fig. 2B).

PDCD4 expression is significantly associated with BCR-ABL expression. BCR-ABL exhibits an important function in the development of CML, therefore the association between

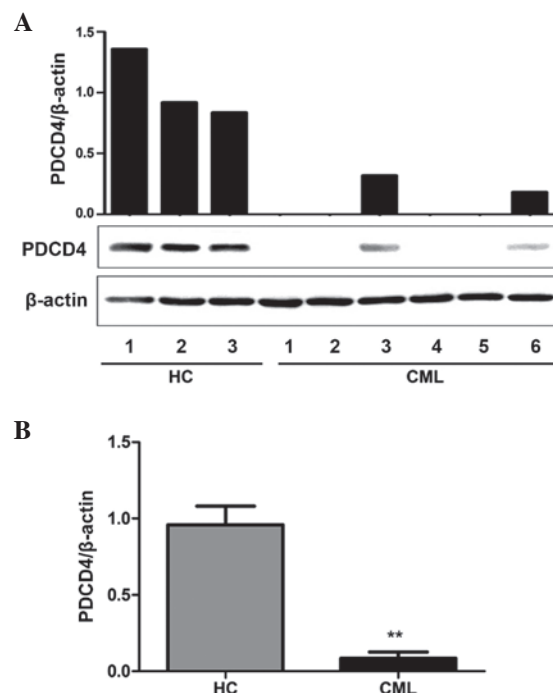


Figure 2. PDCD4 protein expression in 50 CML patients and 20 HCs was evaluated by western blot analysis. (A) PDCD4 protein expression was higher in HCs compared with CML patients. Data were normalized to β -actin (CML lanes 1-3, CML-chronic phase; lanes 4 and 5, CML-accelerated phase; lane 6, CML-blast phase). (B) Normalized PDCD4 protein levels in 50 primary CML and 20 HCs were analyzed. PDC4 expression in CML patients was significantly decreased compared with HCs. ** P <0.01. PDCD4, programmed cell death 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CML, chronic myeloid leukemia; HC, healthy controls.

PDCD4 expression and BCR-ABL expression was analyzed by Pearson correlation analysis. As PDCD4 expression was markedly decreased or absent in CML patients, 20 CML patients with relatively high levels of PDCD4 expression (PDCD4/GAPDH>0.1) were selected for the analysis. The results revealed that PDCD4 expression was negatively correlated with BCR-ABL expression ($n=20$; $r=-0.6716$; P <0.001; Fig. 3), which indicates that PDCD4 expression is associated with the development and progression of CML.

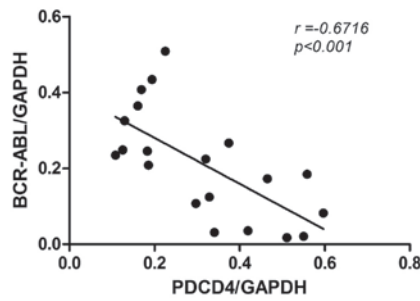


Figure 3. Association between PDCD4 and BCR-ABL expression. PDCD4 expression was negatively correlated with BCR-ABL expression ($r = -0.6716$; $P < 0.001$). PDCD4, programmed cell death 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Imatinib increases PDCD4 levels in the K562 cell line. Since PDCD4 expression was negatively correlated with BCR-ABL expression, PDCD4 expression in K562 cells after 6, 24 and 48 h treatment with 0.5 and 1 μM imatinib was analyzed by RT-qPCR and western blot analysis. The results revealed that the proliferation of K562 cells was significantly inhibited following 48 h of 0.5 and 1 μM imatinib treatment ($P < 0.05$; Fig. 4A). Furthermore, PDCD4 mRNA expression was significantly increased ($P < 0.05$; Fig. 4B) and BCR-ABL expression ($P < 0.05$; Fig. 4C) was significantly decreased after 24 and 48 h of treatment with 0.5 and 1 μM imatinib. Western blot analysis of PDCD4 protein expression demonstrated the same results (Fig. 4D and E). These results indicated that decreased BCR-ABL expression may enhance PDCD4 expression and inhibit malignant proliferation of K562 cells.

Discussion

CML is characterized by the presence of the Ph chromosome, which results from a balanced reciprocal translocation between the ABL gene on chromosome 9 and the BCR gene on chromosome 22 (1). A number of tumor suppressor genes are inactivated or downregulated by BCR-ABL in Ph^+ leukemia, including protein phosphatase 2 (21), p53 (22) and phosphatase and tensin homolog (23). The results of the present study revealed that the tumor suppressor, PDCD4, is also downregulated in CML patients and is negatively correlated with BCR-ABL expression. In addition, inhibition of BCR-ABL expression by imatinib induced PDCD4 expression in the CML K562 cell line.

PDCD4 was recently identified as a tumor suppressor gene and its expression is markedly decreased or absent in a number of solid tumors (9-13). However, the expression of PDCD4 in hematological neoplasms has rarely been reported. Recently, it was reported that miR-21 is frequently overexpressed in AML blasts, in association with a marked PDCD4 protein downmodulation in nucleophosmin-mutant acute myeloid leukemia (24). In the present study, PDCD4 mRNA and protein expression were significantly decreased in CML patients compared with healthy controls, which suggests that PDCD4 is involved in normal hematopoietic differentiation. PDCD4 is known to suppress protein translation by directly interacting with eIF4A to inhibit the formation of the translation-initiation complex (4) or by interfering with translation elongation via an RNA-binding domain (6), which indicates that PDCD4 inhibits cell proliferation

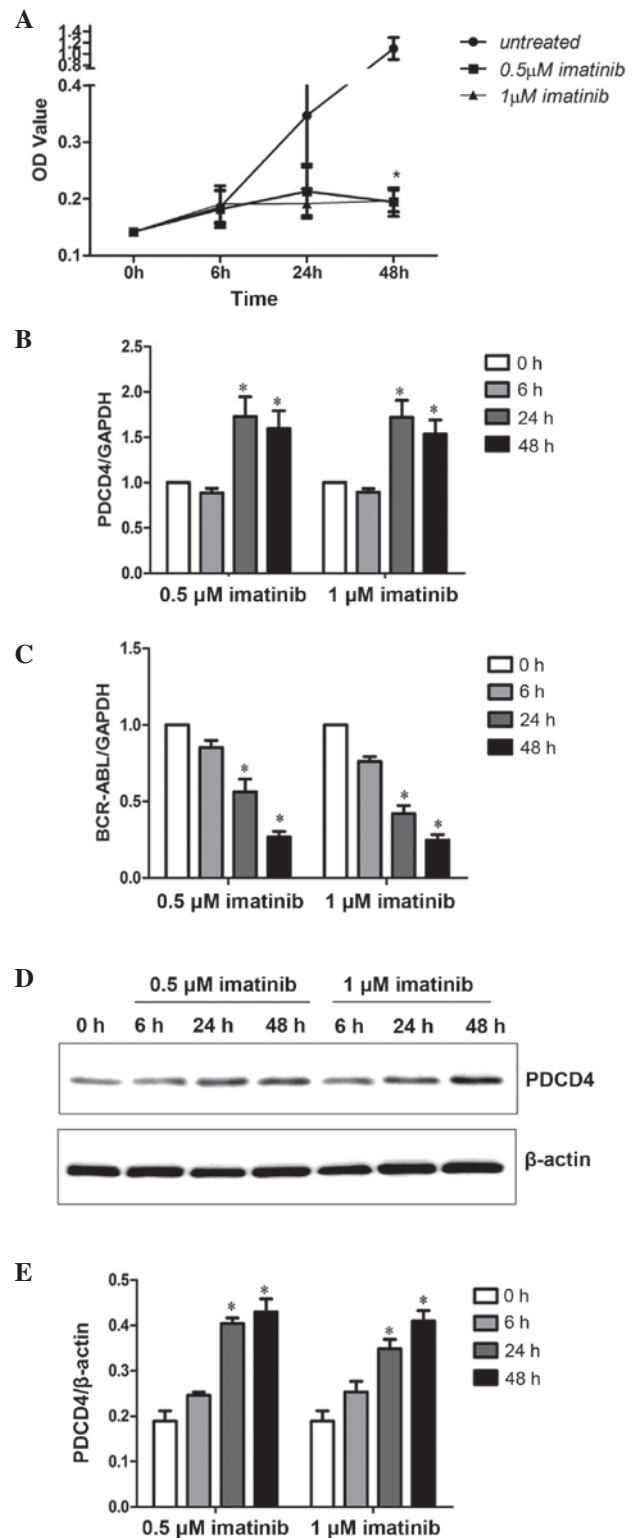


Figure 4. Imatinib increases PDCD4 expression in the K562 cell line. (A) MTT assay revealed that cell proliferation of K562 cells was decreased following treatment with 0.5 and 1 μM imatinib for 6, 24 and 48 h. (B) RT-qPCR revealed (B) PDCD4 mRNA and expression was significantly increased and (C) BCR-ABL mRNA expression was significantly decreased in K562 cells following 24 and 48 h treatment with 0.5 and 1 μM imatinib. (D) Western blot analysis revealed that PDCD4 protein expression was increased following treatment with 0.5 and 1 μM imatinib in K562 cells. (E) PDCD4 protein levels were normalized to β -actin, which revealed that PDCD4 protein levels were significantly increased following treatment with 0.5 and 1 μM for 24 and 48 h in K562 cells. All data are expressed as the mean of triplicate experiments \pm standard error of the mean. * $P < 0.05$ vs. untreated cells. PDCD4, programmed cell death 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and is involved in tumor development and progression. Furthermore, PDCD4 also suppresses autophagy and promotes cell differentiation (25). In addition, it has been reported that PDCD4 expression contributes to all-trans retinoic acid (ATRA)-induced granulocytic but not monocytic/macrophagic differentiation in acute myeloid leukemia, and ATRA induces PDCD4 expression via the inhibition of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway (26). PDCD4 is also involved in the germ line stem cell differentiation pathway: It is hypothesized to relieve the inhibition of Bam by eIF4A (27). These findings suggest that PDCD4 may contribute to cell differentiation of CML. However, in the present study, no significant difference in PDCD4 expression was observed between CML-CP, CML-AP and CML-BP patients, which indicates that PDCD4 does not promote the transition of CML from CP to BP. However, a recent study demonstrated that in the blast crisis of CML, RBP2 expression was decreased; moreover, RBP2 could induce cell differentiation and inhibit cell proliferation, which depend on inhibiting miR-21 and increasing PDCD4 expression (28). All these findings suggest that PDCD4 may play a role in the blast crisis of CML.

The BCR-ABL oncoprotein constitutively activates several downstream pathways (29). One important target is the PI3K/AKT/mTOR pathway, which is constitutively activated in BCR-ABL-transformed cells and is inhibited by imatinib mesylate (30). In this study, the expression of PDCD4 and BCR-ABL in primary CML patients was investigated, which revealed that PDCD4 expression was negatively correlated with BCR-ABL expression. In addition, following 24 and 48 h of imatinib treatment, PDCD4 expression was significantly upregulated in K562 cells. These results suggest that BCR-ABL negatively regulates PDCD4 expression in CML patients, which is consistent with the results of previous studies, which reported that inhibition of the BCR-ABL/mTOR/p70 S6K pathway results in enhanced protein expression of PDCD4 in K562 cells (31) and inhibition of p70 S6 kinase activity by fluvastatin results in the upregulation of expression of PDCD4 in renal cell carcinoma (32).

In conclusion, the results of the present study indicate that the downregulation of PDCD4 expression is associated with the development of human CML. Therefore, increased understanding with regard to the involvement of PDCD4 in CML progression may provide novel molecular targets for diagnosis and therapy of human CML.

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