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Research article

Attenuation of Programmed Cell Death 4 (PDCD4) in Peripheral Blood Mononuclear Cells (PBMCs) of Acute Myeloid Leukemia (AML) patients

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ABSTRACT

Inactivation or loss of tumor suppressor genes is known to be involved in development of acute myeloid leukemia (AML). As a tumor suppressor gene the programmed cell death 4 (PDCD4) protein has been shown to be implicated in several cellular processes including regulation of gene transcription and translation and also cellular signal transduction pathways. In the present study, we investigated the relative expression of PDCD4 gene in de novo AML patients with various French American British (FAB) sub groups' classification. Our study was investigated the relative mRNA expression of PDCD4 gene in peripheral blood of normal samples in compare to 100 newly diagnosed AML cases by Real Time Quantitative PCR. Expression of PDCD4 gene was significantly lower in AML patients comparing to normal samples ($p < 0.001$). PDCD4 expression was almost uniform among the particular FAB subgroups. We observed the highest expression of PDCD4 in patients with standard risk karyotype. We found no significant correlation between PDCD4 gene expression level and clinical parameters of patients. We showed a reduced expression of PDCD4 in AML patients which is similar to some types of other carcinomas such as lung and colon cancers and it seems that this reduction is important in AML and other cancer prognosis. Although, further studies are need to confirm this hypothesis.

Keywords Acute Myeloid Leukemia. Programmed Cell Death 4 Protein. Tumor Suppressor Genes. Polymerase Chain Reaction

[I] NTRODUCTION

Acute myeloid leukemia (AML) a heterogeneous neoplastic disorder is clinically, cytogenetically and molecularly and it is the most common types of leukemia in adults (1) . In AML, an accumulation of acquired genetic alterations and epigenetic modifications in hematopoietic precursor cells disrupts normal hematopoiesis (2, 3).The genetic abnormalities in AML include mutation of oncogenes and the loss of tumor suppressor genes. The epigenetic changes, including DNA methylation and histone modifications, are also critical to pathogenesis of AML (to disease pathogenesis). These alterations results in a differentiation arrest in progenitor cells, excessive proliferation of immature blasts and infiltration of blasts in the bone marrow and peripheral blood. AML accounts for approximately 30% of all leukemias in adults. In the United States and Europe, the annual incidence of AML is nearly 2-5 per 100000 populations (4-6). Cytogenetic is one of the most powerful prognostic factors in AML. However, about 40–50 % of AML cases have normal karyotypes [cytogenetically normal (CN)-AML] (7-9). So far, in CN-AML more than 100 gene mutations as well as deregulated gene expression have been identified. These alterations in CN-

AML can contribute in prediction of clinical outcome.

Inactivation or the loss of tumor suppressor genes is an important event contributing to the development of cancer. PDCD4(Programmed cell death) is a tumor suppressor gene and it has been identified to act via up-regulating during apoptosis (10, 11). PDCD4 is involved in several cellular processes including regulating gene transcription, translation, and cellular signal transduction pathways (12). The gene encoding PDCD4 is located on chromosome 10q24 in human (13). PDCD4 comprises 469 amino acids and have two conserved α -helical MA-3 domains (14). MA-3 domains are involved in protein–protein interactions and PDCD4 through interaction of these domains with eIF4A repress protein translation (15, 16). It seems that PDCD4 plays an important role as a tumor suppressor and it has been shown that PDCD4 could inhibit cellular transformation in the JB6 transform cells (17, 18). PDCD4 transgenic mice showed remarkably reduced tumor formation (19), while PDCD4 deficiency in mice results in tumor development especially spontaneous lymphomas (20). The role of PDCD4 in human cancers has also been investigated. In Ovarian cancer cells, PDCD4 inhibits the cell proliferation and cell cycle progression by up-regulation of cell cycle inhibitors of p27 and p21 (21). It has also been shown down-regulation PDCD4 promotes tumor invasion and progression by activating both beta-catenin/Tcf and AP-1 signaling pathways and reduction of E-cadherin expression in colon carcinoma cells (22). Reduction or the loss of PDCD4 expression has been detected in several types of human cancers (23-26). The mechanisms underlying down-regulation of PDCD4 are not completely understood. Decreased PDCD4 expression seems to be related to enhanced tumor progression, metastasis and prognosis. The role of PDCD4 and its expression levels in AML have not yet been studied. The aim of the present study was to investigate the PDCD4 expression levels as a prognostic factor in de novo AML patients by Real Time-PCR.

[II] MATERIALS AND METHODS

2.1. Patients and ethics

In this clinical trial peripheral blood (PB) samples were obtained from 100 newly diagnosed AML patients. Characteristics of patients were summarized in table 1. All AML patients were classified on the base of the French- American-British (FAB) study group classification. Patients' information, including age, WBC and Platelet count and Genetic Abnormalities were obtained from their medical records.100 patients were studied, 59 and 41 patients were male and female respectively. The age range of patients was between 4 to 79 years and their average age was 38 years old.

Immediately after getting the samples, mononuclear cells, including leukemic blasts, were separated by density gradient sedimentation using Ficoll-Hypaque; the cells were washed twice with phosphate-buffered saline (PBS) to remove platelets. The separated mononuclear cells (1×10^6 to 1×10^7) were dissolved in 1 mL of TRIzol (Sigma USA) and stored in -80°C until used for RNA extraction.

Table: 1. Characteristics of patients and treatment outcome

Total number of samples(n)	100
Age (median, range)	38 (4-79)
Female/male(n)	41/59
French-American-British subtypes(n)	
M0	1
M1	8
M2	29
M3	10
M4	28
M5	17
M6	6
M7	1
cytogenetic risk groups(n)	
good risk karyotype or favorable	12

standard risk karyotype or intermediate	32
poor risk karyotype or unfavorable	39
unknown	17
WBC count, cell/µL (median, range)	39005(400-208000)
Plt count, cell/µL (median, range)	77795(2000-814000)

2.2. RNA extraction

Total RNA was isolated with the acid-guanidine-phenol-chloroform method and dissolved in diethylpyrocarbonate-treated water (DEPC water). In order to make cDNA, total RNA (2 µg) mixed up with 4 µL 5X MMLV-first strand buffer (250 mmol/L Tris-HCl [pH 8.3], 375 mmol/L KCl, 15 mmol/L MgCl2), 1 µLdNTP Mix, 1 µLoligo-dT primer, 1 µL 200U/µLMoloney murine leukemia virus reverse transcriptase (MMLV-RT), 1 µL 20U/µLRNase inhibitor and the volume adjusted to 20 µl with diethylpyrocarbonate-treated water. The reaction mixture (20 µL) was incubated at 37°C for 1 hour and then was stopped by heating to 75°C for 10 minutes. The mixture (cDNA) was diluted to 50 µL with ddH2O and then stored at -20°C until use.

The cDNA was amplified by quantitative real-time polymerase chain reaction (RT-PCR) to evaluate total PDCD4 relative expression. The primers for PDCD4 and GAPDH (as an internal control) genes was designed via using AlleleId7 software (table 2).

The Reactions (15 µL for each reaction) were prepared using 7.5 µL SYBR® Green master mix, 0.8 µL of 10µM primer mix, 3 µLof diluted cDNA and 3.7 µL ddH2O. Amplifications were performed with 40 cycles under the following conditions : 95°C for 30 seconds, 95°C for 10 seconds, 60° C for 30 seconds, steps 2 and 3 were repeated for 40 cycles, followed by melt curve analysis. Individual melt curves were analyzed to assure PCR product purity. Relative expression was calculated using the $2^{\Delta\Delta ct}$ method (13) using GAPDH as an internal control.

Table: 2. Primers for PDCD4 and GAPDH genes

Primer	Gene symbol	Sequence (5´ to 3´)
PDCD4	Forward	GAPGAPGACCAGGAGAAC
	Reverse	TAAGGATACTGCCAACAC
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAAGATGGTGATGGGATTTC

2.3. Statistics

All data were analyzed using the SPSS software Version 21. Patients were categorized into two groups of PDCD4 expression based on median value. Non parametric tests including Mann-Whitney U and Kruskal-Wallis were used to compare PDCD4 expression differences among two different groups and more than two groups respectively.

[III] RESULTS

3.1. Different levels of PDCD4 expression in normal peripheral blood samples and AML samples

Relative mRNA expression of PDCD4 gene in 10 normal samples (peripheral blood samples) and 100 newly diagnosed AML samples were analyzed by Real Time Quantitative PCR. GAPDH gene was amplified as an internal control gene. PDCD4 mRNA expression significantly was lower in patients with AML (median 0.16, interquartile range 0.07-0.35) in comparison with that of in normal samples (median 0.80, interquartile range 0.74-1.51) (Fig. 1; $p<0.001$). According to the figure 1, more than 77% of AML patients had twofold decrease in the expression of PDCD4 compared to the median expression in normal samples of peripheral blood.

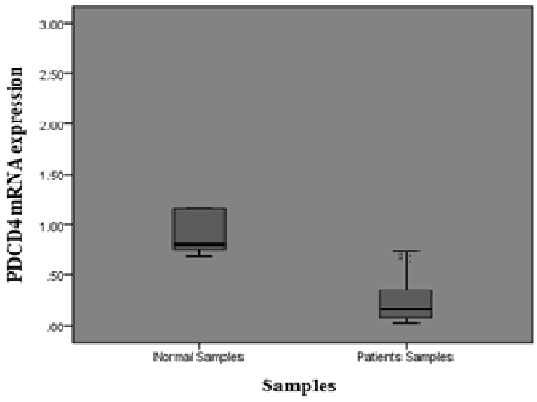

Fig: 1. Relative expression of the PDCD4 gene in PB of normal healthy donors and AML patients in

diagnosis. The bars represent medians and the boxes represent 25 and 75 percentiles of WT1 expression. The results demonstrate that PDCD4 mRNA expression significantly was lower in patients with AML (median 0.16, interquartile range 0.07-0.35) in compare with normal samples (median 0.80, interquartile range 0.74-1.51)($p < 0.001$).

3.2. PDCD4 expression in different sub-groups of FAB-AML

According to the FAB classification, maximum and minimum distribution of patients was related to the FAB-M2 sub-groups (29%) and FAB-M0/M7 (1%) respectively. PDCD4 expression among specific FAB-AML sub-groups was approximately uniform and no difference between FAB sub-groups in terms of PDCD4 expression was observed (Fig. 2; $p>0.05$).

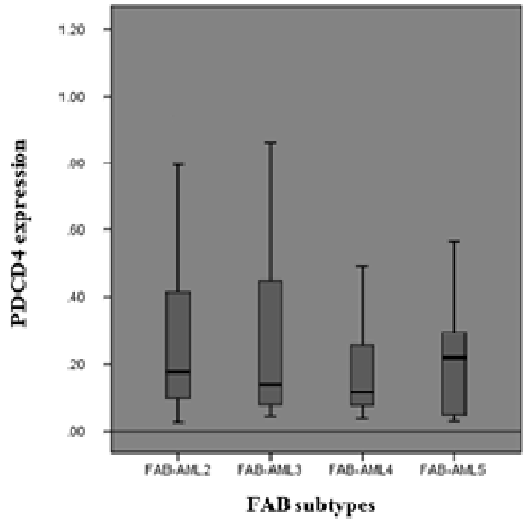

Fig: 2. Relative expression of the PDCD4 gene in FAB subtypes of AML patients at diagnosis. The bars represent medians and the boxes represent 25 and 75 percentiles of WT1 expression. PDCD4 expression among specific FAB-AML sub-groups was approximately uniform.

3.3. PDCD4 expression levels between cytogenetic risk groups

Cytogenetic analysis was performed in patients BM cells at diagnosis time. According to several studies, a classification system based on cytogenetic risk for AML patients has been suggested that certain karyotype abnormalities divide into three groups: (good risk karyotype or favorable), (standard risk karyotype or intermediate) and (poor risk karyotype or unfavorable) (27). A significant correlation between the cytogenetic risk groups in terms of PDCD4 expression was found. According to Figure 3, the highest and lowest PDCD4 expression was observed in patients with standard risk karyotype and poor risk karyotype respectively.

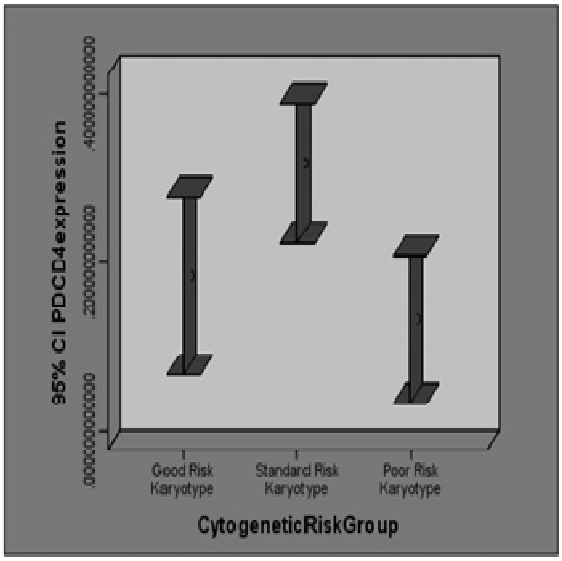

Fig: 3. Relative expression of the PDCD4 gene in cytogenetic risk groups. The bars represent medians and the boxes represent 25 and 75 percentiles of WT1 expression. The highest expression of PDCD4 was observed in patients with standard risk karyotype.

3.4. Correlation of PDCD4 expression with clinical parameters

Of the one hundred investigated patients, 59 (59%) were male and 41 (41%) were female. The age of patients ranged from 4 to 79 years and the median age of the patients was 38 years. White blood cell and platelets count ranged from 400 to 208000 and 2000 to 814 000 in respects and the median count was 39005 and 77795 cells per microliter respectively (Table 1). No significant correlation was observed between PDCD4 expression and sex, age, WBC and platelet (data was not shown). According to the expression levels of PDCD4, patients were divided into two groups: PDCD4 expression below or equal to median (<=0.16) and above to median (>0.16). No significant correlation was observed between patients with higher PDCD4 expressions

compared to the patients with PDCD4 expressions lower than the median (data was not shown).

[IV] DISCUSSION AND CONCLUSION

In addition to conventional balanced chromosomal translocation, mutation of oncogenes and the loss of tumor suppressor genes play an important role in the pathogenesis of AML. For example, abnormality in certain genes such as FLT3, c-KIT, and RAS and inactivation or decreased expression of tumor suppressor genes including p53 are commonly found in AML cells (28, 29).

PDCD4 is a tumor suppressor gene that involves in the gene transcription and translation (12). It is thought that PDCD4 in both mice and humans plays an important role in tumor development and metastasis (17, 20, 22).

In the present study, we found that decreased expression of PDCD4 occurs in newly diagnosed AML patients compared to normal peripheral blood samples. It is suggested that PDCD4 may play an important role in the pathogenesis of AML. These results are consistent with findings that indicate the reduction or loss of PDCD4expression occurs in lung or hepatocellular carcinoma cancer in compared with normal tissues (23, 26).Moreover, PDCD4 expression is also reduced in many other human tumors.

Ding et al. studied the PDCD4 expression in a total of 63 gastrointestinal stromal tumor samples at both mRNA and protein levels by RT-PCR, western blot, and immunohistochemistry. They showed that the expression of PDCD4 mRNA was decreased in 68% of the cancer samples, and the level of PDCD4 protein clearly to be diminished in 66.7% of the samples, as compared to near normal gastrointestinal tissues (30).

It has been shown that the expression of PDCD4 was significantly increased in AML cell lines such as HL60 and NB4 and in primary human promyelocytic leukemia cells (AML-M3) following all-trans retinoic acid (ATRA)–induced granulocytic differentiation (31). These data suggest PDCD4 that may play an important role in granulocytic differentiation, is down-regulated during leukemogenesis.

Cytogenetic analysis is an important factor in determining disease prognosis and selection of appropriate therapy in patients with AML (32). Three cytogenetic categories were defined by The Medical Research Council (MRC): favorable risk, intermediate risk and unfavorable rick (33). The favorable risk group included t(8;21) without either a del(9q) or being part of a complex karyotype, t(15;17) and t(16;16)/inv(16)/del(16q). Patients with favorable cytogenetic abnormalities have a better prognosis and response to therapy. The intermediate risk group included patients who have abnormalities of del(12p), +8, +6, -Y or normal karyotype. The unfavorable risk group was characterized by the presence of one or more of del(9q), -5/del(5q), -7/del(7q), inv(3q), abn 11q, 20q, or 21q, t(6;9), t(9;22), abn 17p, and complex karyotype defined as 5 or more abnormalities. In our study, expression of PDCD4 was higher in patients with intermediate and favorable risk compared with unfavorable rick, although, the importance of this issue remain to be studied.

It seems that down-regulation of the PDCD4 gene expression in various human cancers is associated with accelerated tumor progression, metastasis and poor prognosis. Yuan Chen and colleagues demonstrated that the loss or reduction of PDCD4 expression is a common molecular abnormality in lung cancer and is associated with tumor progression and decreased survival (23). In the present study, significant correlation between PDCD4 gene expression levels and clinical parameters of patients such as sex, age, white cell count and platelets were not observed.

The PDCD4 expression is regulated by multiple mechanisms (12) including DNA methylation at the chromatin level, phosphorylation by Akt and S6K1 at the protein level and down-regulation by increased level of miR-21 at mRNA level. Therefore, extensive studies on the molecular levels are necessary to be examined altered expression of PDCD4 in de novo AML. Nevertheless, the regulatory mechanisms of

PDCD4 in AML and its role in normal hematopoiesis as well as its probable association with the outcome of patients require further study.

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