Research Article

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Clinical importance of PCA3 lncRNA aberrant expression in chronic myeloid leukemia patients: a comparative method

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Abstract

Objectives: Long noncoding RNAs (lncRNAs) plays important role in disease spread and its invasion. Overexpression of prostate cancer antigen 3 gene (PCA3gene) is reported in prostate cancer. To analyze the PCA3 lncRNA expression in chronic myeloid leukemia (CML) patients.

Methods: The study included clinically confirmed 100 CML patients and 100 healthy subjects. Relative quantification using Sybr Green dye was used to calculate the PCA3 lncRNAs expression. Total RNA was extracted by TRIzol method and quantitative real-time polymerase chain reaction.

Results: In CML patients, 9.96 ± 4.77-folds increased noncoding PCA3 lncRNA expression was observed compared to healthy subjects. Patients of chronic phase, accelerated phase, and blast crisis phase had 4.46 ± 1.36, 7.31 ± 3.10, and 12.91 ± 4.85-fold PCA3 lncRNA expression compared to healthy subjects ($p<0.0001$), respectively. CML patients who have a complaint of splenomegaly had higher PCA3 lncRNA expression than those who did not complain splenomegaly compared to healthy subjects (12.04 ± 5.02-fold vs. 6.09 ± 3.39-fold, $p<0.0001$). Patients who had ≤20,000 TLC showed fewer PCA3 lncRNA expression than those who had >20 thousand of TLC (4.45 ± 1.84 vs. 11.25 ± 5.05, $p<0.0001$). Receiver operating characteristic showed correlation of PCA3 lncRNA expression with severity of cancer.

Conclusions: lncRNA PCA3 expression to be linked with different stages of the disease and a prognostic indicator for disease in CML patients.

Keywords: chronic myeloid leukemia; long noncoding RNAs expression; quantitative real-time polymerase chain reaction; receiver operating characteristic curve; relative quantification

Introduction

Chronic myeloid leukemia (CML) is recognized as a hematological disorder of hematopoietic stem cells. Leukemia patients significantly represent myeloid hyperplasia of bone marrow and their peripheral blood samples showed an increased level of platelets, myeloid cells, erythroid cells [1]. CML is caused by the main translocation of fusion gene of chromosome number 9 and chromosome number 22 (ABL-BCR) and the product gives fused protein with uncontrolled tyrosine kinase activity that is implicated in the malignant transformation of normal cellular functions [2]. Wright et al. in [3] reported that only 2 % of the human genome coded for protein and the rest of the genome does not code any proteins. In eukaryotic transcribed nonprotein coding RNAs are more than 200 nucleotides without open reading frames called long noncoding RNAs (lncRNAs) [4]. It has been revealed that lncRNAs can play important role in disease spread and invasion of disease [5]. The prostate cancer antigen 3 gene (PCA3gene) is situated on locus 9q21.2 of the long arm, 23 kilobases long, and comprises 4 exons. Studies have shown that a repetitive number of stop codons and the presence of open reading frames makes it noncoding [6]. The PCA3gene function is still unknown but studies have shown that PCA3 lncRNA controls cell survival, interferes with cell signaling via targeting androgen receptors, which can increase the chances to use this as a marker for cancer therapies [7]. It has been shown that PCA3 was overexpressed in all samples of prostatic carcinoma and causes

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metastasis [6]. However, zero or lower PCA3 lncRNA up-regulation was found in normal tissue of the prostate and benign prostatic tissue [8, 9]. PCA3 gene targets multiple genes such as N-cadherin, inversin, and noggin [10]. PCA3 targets noggin and inhibits its function which has an inhibitory effect on the bone morphogenetic protein (BNP) [11]. BMP is upregulated due to noggin being suppressed, and unusual levels of BMP may have a consequence on primitive CML cells spread out [12].

The objective of the study was to compare PCA3 gene level expression in CML patients with healthy subjects. Also, to find out whether the PCA3 gene level expression in CML patients is increased according to the study hypothesis.

Materials and methods

Patients' selection and sample collection

The present study included 100 clinically confirmed CML patients in different stages and 100 healthy subjects. A total of 3 mL of peripheral blood sample were collected in ethylene diamine tetra acetate (EDTA) vials from all study subjects after informed consent and sample were immediately stored at −20 °C for further downstream process. CML diagnosis was done by physician and pathologist based on diagnostic criteria such as complete blood count/bone marrow cell counting. The molecular detection method was used to confirm the CML diagnosis by detection of BCR/ABL fusion transcripts.

The procedure for determination of total leukocyte counts (TLC)

A total of 0.38 mL Turk's solution (Thermo Fisher Scientific, USA) and 20 µL blood sample were mixed in a test tube. This mixture was subjected to shanking with thumb and figure. Using counting chamber on the microscope stage white blood cells were calculated in four large corner squares. Cells which touched left-hand line and upper line were included and cells which touched lower and right margin were excluded. Then TLC was calculated as per Eq. (1).

$$\text{TLC} = \text{Total cells of 4 squares} \times 50 \quad (1)$$

Splenomegaly

The computed tomography images of spleen were taken. If splenic index was more than 480, then it was considered as splenomegaly. Splenic index was calculated from Eq. (2) [13].

$$\text{Splenic index} = \text{Length} \times \text{Width} \times \text{Thickness (cm)} \quad (2)$$

Thrombocytopenia

Fewer than 150,000 platelets/µL of circulating blood was considered as thrombocytopenia.

Total RNA extraction and cDNA synthesis

Total RNA isolation was done by using TRIzol reagent (Thermo Fisher Scientific, USA) following manufacturer instructions from blood samples collected in EDTA vials of patients as well as from healthy subjects. The concentration of RNA was measured by absorbance at 260 nm by Nanodrop. Complementary DNA (cDNA) was synthesized by using a cDNA synthesis kit (Verso, Thermo Fisher Scientific, USA) following the manufacturer's protocol. Briefly, total RNA of 100 ng, 2 µL oligo (dT) primer, and 9 µL of DEPC treated H_2O and reaction mixture was kept in a thermal cycler for 10 min at 65 °C and further stored at 4 °C for the next step reaction process. The reaction mixture, 5X RT buffer (4 µL), 5 mM dNTPs (2 µL), RNAse inhibitor (1 µL), and reverse transcriptase enzyme (1 µL) were then added in a total volume of 20 µL, and the final reaction mixture was incubated at 45 °C for 1 h and then heated to 95 °C for 5 min to stop the enzymatic action and cooled at 4 °C to complete the cDNA synthesis process.

Expression study of PCA3 lncRNA by quantitative real-time PCR

Real-time PCR was used to analyze the expression levels of PCA3 lncRNA and comparative method (delta-delta CT) was used for analysis. Quantitative real-time polymerase chain reaction (QRT-PCR) was performed in Quant Studio 6 to evaluate the PCA3 lncRNA expression using Sybr Green dye (Thermo Scientific, USA) and β-actin was used as a reference control. The primers sequences were used as follows: PCA3 forward primer sequence (5′-GAGAACAGGGGAGGGAGAG-3′), reverse primer sequence (5′-ACGTTCTGGGATACATGTGC-3′), β-actin forward primer sequence (5′-TGAAGATCAAGATCATTGCTCCC-3′), and reverse primer sequence (5′-AGTCATAGTCCGCCTAGAAGC-3′). QRT-PCR programmers were programmed to amplify the PCA3 and β-actin QRT-PCR cycling parameters were for 10 min at 95 °C, followed by 40 cycles of 40 s at 94 °C, 40 s at 58 °C, and 40 s at 72 °C.

Splenomegaly and/or thrombocytopenia were evaluated by consulting physician on the basis of pathology. Loss of appetite was self-reported.

Statistical analysis

SPSS version 20.0 and Graph Pad Prism version 6.05 were used to analyze the collected data. Parametric (for normally distributed data) and nonparametric tests (for not normally distributed data) were used to analyze the data based on observation among the different groups. Visual expression was used to check whether data were normally distributed or not. Expression data analysis was done by the comparative cycle threshold (Ct) method and each sample was performed in duplicate. MicroRNA expression alteration levels were calculated by relative quantification using the $2^{-(\Delta\Delta Ct)}$ method. Results more or less than 1 were taken as a reference to indicate up-regulation or down-regulation of expression pattern. For statistical significance, the p-value <0.05 was regarded as significant. The comparative method based on comparisons of the test sample with control and indicates the difference as folds. Therefore, control values are taken as 1 and test sample values are indicated as folds compared to control.

Results

Demographic and clinical characteristics

All the demographic characteristics of research subjects were depicted in Table 1. In a brief total of 200 subjects were included in the study, 100 were CML cases and 100 were healthy controls. A total of 70 (70 %) males and 30 (30 %) females were in CML cases, 67 (67 %) were males 33 (33 %) were female among healthy controls. Patients in ≤45 years of age group were 48 (48 %) and >45 years age group were 52 (52 %) in CML cases while among healthy controls 50 (50 %) were in ≤45 years of age and 50 (50 %) were in >45 years of age group. A total of 19 (19 %), 24 (24 %), and 57 (57 %) of patients were in chronic phase, accelerated phase, and blast crisis phase, respectively in CML cases. Among CML patients 19 (19 %) were treated with imatinib therapies and 81 (81 %) were not.

Association of noncoding PCA3 lncRNA expression in CML patients

In CML patients, more than 9 mean folds increased non-coding PCA3 lncRNA expression was observed. In CML patients, PCA-3 expression between male and females was not significant (p=0.63) (Table 2). A statistically significant difference was observed in PCA3 lncRNA expression in different stages of CML patients (p<0.0001, Figure 1). Patients in the chronic phase showed more than 4-fold up-regulation while accelerated and blast crisis patients had more than 7-and 12-fold increased PCA3 lncRNA expression. Patients who received therapy (imatinib) among them developed thrombocytopenia, showed more than 4-fold increased PCA3 lncRNA expression while non-thrombocytopenic patients, mostly not received any therapy showed more than 10-fold on PCA3 lncRNA expression, and differences among them were found to be significant (p<0.0001, Figure 2).

Table 1: Demographic and clinical characteristic of CML patients and healthy subjects.

Variables	CML cases, n	Healthy controls, n
Total	100	100
Gender		
Males	70	67
Females	30	33
Age, years		
≤45 years	48	50
>45 years	52	50
CML stages		
Chronic phase	19	
Accelerated phase	24	
Blast crisis	57	
Thrombocytopenia		
Yes	14	
No	86	
Loss of appetite		
Yes	74	
No	26	
Loss of weight		
Yes	71	
No	29	
Splenomegaly		
Yes	65	
No	35	
TLC count		
≤20 thousand	19	
>20 thousand	81	
Imatinib treatment		
Yes	19	
No	81	

Table 2: Non-coding PCA3 lncRNA expression in CML patients with different variables.

Variables	Mean + SD	p-Value
Over all PCA-3 expression	9.96 ± 4.77	–
Gender		
Males	9.74 ± 5.10	0.63
Females	10.47 ± 5.89	
Age, years		
≤45	9.81 ± 5.26	0.83
>45	10.10 ± 5.44	
CML stages		
Chronic phase	4.46 ± 1.36	<0.0001
Accelerated phase	7.31 ± 3.10	
Blast crisis	12.91 ± 4.85	
Thrombocytopenia		
Yes	4.33 ± 1.76	<0.0001
No	10.88 ± 5.15	
Loss of appetite		
Yes	10.56 ± 5.41	0.02
No	8.0 ± 4.64	
Loss of weight		
Yes	9.83 ± 5.25	0.81
No	10.28 ± 5.59	
Splenomegaly		
Yes	12.04 ± 5.02	<0.0001
No	6.09 ± 3.39	
TLC count		
≤20 thousands	4.45 ± 1.84	<0.0001
>20 thousands	11.25 ± 5.05	
Imatinib treatment		
Yes	4.45 ± 1.84	<0.0001
No	11.25 ± 5.05	

A significant association was observed with the appetite status of patients, patients who showed loss of appetite showed more than 10-fold of increased PCA3lncRNA expression while those who did not report loss of appetite had only 8-fold of PCA3 lncRNA expression (p=0.02, Figure 3). Patients who have a complaint of splenomegaly showed more than 12-fold increased PCA3 lncRNA expression while patients who did not complain splenomegaly showed more than 6-fold increased PCA3 lncRNA expression and differences were shown to be significant (p<0.0001, Figure 1 Supplementary). Patients who had ≤20 thousand TLC showed more than 4-fold increased PCA3 lncRNA expression. However, patients who had >20 thousand of TLC showed more than 11-fold of increased PCA3 lncRNA expression, and differences among groups were found to be significant (p<0.0001, Figure 2 Supplementary). Among all the included patients few of them received imatinib treatment and showed only 4.45-fold PCA3 lncRNA expression while those who did not receive any treatment showed more than 11-fold of PCA3 lncRNA expression and differences among them was found to be significant (p<0.0001, Figure 3 Supplementary).

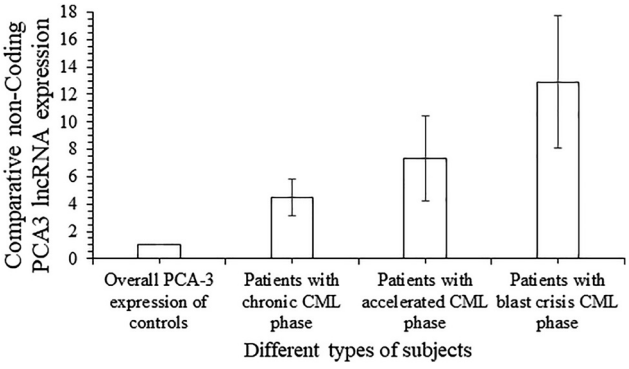

Figure 1: Comparative non-coding PCA3 lncRNA expression for different stages of CML patients. CML, chronic myeloid leukemia.

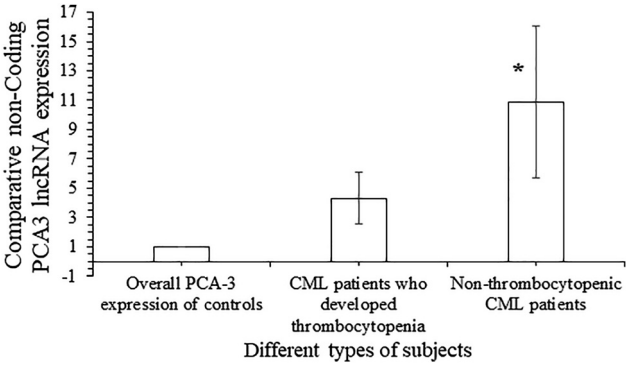

Figure 2: Comparative non-coding PCA3 lncRNA expression for thrombocytopenia of CML patients. CML, chronic myeloid leukemia. *Significantly higher.

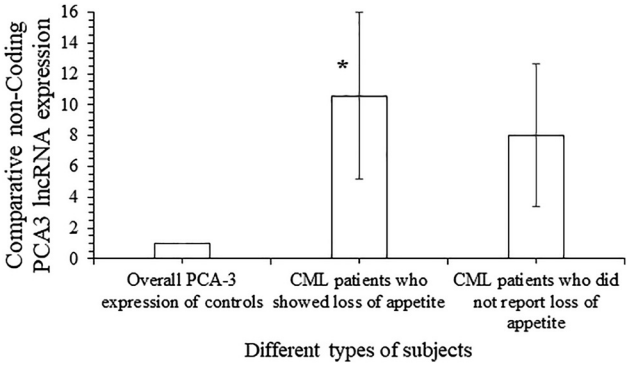

Figure 3: Comparative non-coding PCA3 lncRNA expression for the appetite status of CML patients. CML, chronic myeloid leukemia. *Significantly higher.

Prognostic significance of noncoding PCA3 lncRNA expression in CML phases

Receiver operating characteristic (ROC) curve was plotted to evaluate the prognostic importance of PCA3 lncRNA expression in CML patients. Chronic phase patients were taken as a reference against the accelerated and blast crisis patients to evaluate the data. It was found that the area under the curve was 0.87 and at 5.83 mean cutoff value of PCA3 lncRNA expression could be used to predict the stage of patients. However, sensitivity and specificity were 82 %, 85 %, respectively (area under curve=0.81, p<0.0001; Figure 4 Supplementary), (Table 3).

Discussion

The present study found remarkable higher PCA3 lncRNA expression among CML patients than those of counter parts of controls. The results of the current study were consistent with the previous report of Sajjadi et al. [14]. However, Sajjadi et al. [14] is small study done with small sample size (30 subjects).Transcriptome investigation by tiling arrays and RNA sequence analysis is given revealed that only 70–90 % of genome transcribed and only 2 % have coding sequence to produce protein [15]. It has been said that several lncRNAs serve as oncogenic functions or suppressors for tumors [16]. It is accepted that non-coding RNAs such as

Table 3: ROC curve with respect to chronic phase vs. accelerated and blast crisis phase.

AUC (95 % CI)	Sensitivity	Specificity	Cutoff value (fold change)	p-Value
0.87 (0.81–0.94)	82 %	85 %	5.83	<0.0001

miRNAs serve a revolutionary role in cell biology [17]. lncRNAs could have very specific importance as a diagnostic marker and can have a very specific expression in tissues or cells and specific expression patterns in cancers types [18]. PCA3 contains 4 exons and is present in the BMCC1 gene intron, which regulates the oncogenic signaling of cells [19]. There is higher expression of PCA3 in CML patients.

PCA-3 expression of male and females were statistically same. These effect is not performed in the previous report Sajjadi et al. [14]. In the current study, the ratio of males to female patients is 70 to 30 % in CML patients. These values were close to discard the effect of gender on PCA3 expression in CML patients.

The current study found that patients in accelerated phase, blast crisis phase showed higher PCA3 lncRNA expressions than those of chronic phase patients. Also, these effect is not performed in the previous report Sajjadi et al. [14]. Advances stage of CML may have higher up-regulation or abnormal expression pattern of BMP causes primitive CML cell expansion [12]. PCA3 lncRNA expression was linked with stages of CML and differences in expression among them were significantly associated. Accelerated phase of disease have higher expression of PCA3 lncRNA.

It has been found that CML patients received imatinib treatment therapy and showed thrombocytopenia had low expression of PCA3 lncRNA. However, those were newly diagnosed (no thrombocytopenia) had higher PCA3lncRNA expression. There is also higher expression due to advanced stage(s). This also expressed that treatment decreases expression of PCA3 lncRNA.

Patients who reported loss of appetite had more PCA3lncRNA expression compared to those who did not report any loss of appetite. People with advanced cancer often notice changes in their appetite and advanced stage has higher expression of PCA3 lncRNA. However, many other factors including the ones based on molecular and even psychological conditions have effects on loss of appetite. Further study is required on loss of appetite in evaluation of the advance in cancer through discriminating the overall PCA3lncRNA expression.

Patients with splenomegaly showed more than 5-fold higher PCA3 lncRNA expression compared to patients without splenomegaly. Splenomegaly shows severity of cancer. Therefore, it obvious that expression of PCA3 lncRNA is higher with patients of splenomegaly.

Patients without any treatment and patients who had >20 thousands total TLC showed more than 6-fold higher PCA3 lncRNA expression compared to patients who had receive therapy and patients who had ≤20 thousands of TLC. Therapies decreases severity of cancer and expression of PCA3 lncRNA.

In receiver operator curve analysis with respect to CML stages (chronic phase vs. accelerated and blast crisis phase) at best possible cut-off value of 5.83-fold upregulated in PCA3 lncRNA expression, sensitivity and specificity were 82 %, 85 %, respectively. This indicates direct correlation of PCA3 lncRNA expression with severity of cancer.

However, growing research suggested that a great number of lncRNAs are associated with several human malignancies, mainly in diverse types of carcinomas, including leukemia, colorectal, breast, prostate, liver carcinoma, and glioblastoma [20, 21]. PCA3 could be a good target for genetic manipulation for its expression and associated genes.

Conclusions

The present study revealed that lncPCA3 alteration was established to be linked with different stages of the disease, other variables included in disease and ROC curve analysis showed that it can be used as a prognostic indicator for disease. Current study revealed that unusual lncRNA PCA3 expression in CML patients had promising implications in terms of prognostic significance and monitoring of lncPCA3 expression which could be helpful in CML patients' management. There is need for more research towards mechanisms of molecular pathways related to PCA3 that could help to achieve better treatment.

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Research ethics: The study was approved by the Institutional Review Board of the First Affiliated Hospital of Chengdu Medical College, Chengdu, Sichuan, China, with Approval No.2016-CMC-026. The study reporting adheres to the law of China and the V2008 Declarations of Helsinki.

Informed consent: The study was conducted after obtaining the written informed consent from every patient, the study was conducted.

Authors contributions: The authors have read and approved the manuscript for publication. LH and JZ were project administered and contributed equally to the literature review, resources, visualization, and supervision of the study. YP contributed to the investigation, the literature review, resources, software analysis, and data curation of the study. HW contributed to the methodology, conceptualization,

literature review, resources, formal analysis, and data curation of the study. ZS contributed to the literature review, resources, and software analysis of the study, draft, review, and edited the manuscript for intellectual content. The authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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