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Expression Level of the PPP2R5C Gene in Patients With Acute Myeloid Leukemia and Chronic Myeloid Leukemia

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Abstract

Background: One of the most important B-subunits of protein phosphatase 2A (PP2A) is PPP2R5C, which is involved in both the suppression and promotion of cell growth.
Objectives: With regard to this vital role of PPP2R5C, we will examine whether alteration in expression of some proteins is involved in signal transduction, for example the B-subunit of protein phosphatases can be observed in patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML).
Materials and Methods: We recruited 50 patients with AML and 31 patients with CML, together with 23 individuals in the control group. The expression levels of PPP2R5C and the reference gene B2-MG were determined by SYBR green I real-time PCR. The $2^{(-\Delta\Delta CT)}$ method was used to present the data of the genes of interest relative to an internal control gene.
Results: In this study, significantly higher expression of PPP2R5C was found in the AML (4.13 ± 4.85) compared with healthy individuals. The expression level of PPP2R5C significantly decreased (0.469 ± 0.34) in CML patients compared with healthy individuals.
Conclusions: With regard to the critical role of PPP2R5C B-subunit on regulation of PP2A, the change in the expression pattern of PPP2R5C may be a critical finding in AML and CML patients, because it could be a cause of abnormal cell proliferation, differentiation, and transformation in these two groups of patients.

Keywords: Acute Myeloid Leukemia, Chronic Myeloid Leukemia, PPP2R5C, PP2A, Expression

1. Background

Both acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) are heterogeneous diseases of hematopoietic stem cells (HSCs) and progenitor cells (HSPCs) (1). In many cases, the cause of leukemia is ambiguous; as such, the pathogenesis of leukemia involves cytogenetic abnormalities, genetic mutations, epigenetic anomalies, alterations in gene expression, and eventually dysregulation of various signaling pathways (2). Therefore, assessment of molecular genetic aberrations in different types of leukemia is needed for further understanding the causes of leukemia.

Protein phosphorylation and dephosphorylation by protein kinases and phosphatases is the more important mechanism for the regulation of various signaling pathways, and the aberration in proteins involved during this mechanism is a vital reason behind cancer (3). Reversible protein phosphorylation is needed for a well-balanced network of kinases and phosphatases to take care of the normal physiological condition of cells, and the dysregulation of signaling pathways that ends up altering this balance results in the development of diseases such as cancer (4-7). With regard to phosphorylation in the regulation of proliferation, differentiation, and programmed cell death, each of the kinases and phosphatases is known as an oncogene or tumor suppressor in several different cancers (8). Though abundant is thought concerning kinase role alterations in many diseases like cancer; but, the operation of specific phosphatases in cancer and other diseases is poorly understood (9).

Protein phosphatases are broadly classified into two major families: The protein serine/threonine (Ser/Thr) phosphatases (PSPs) and the protein tyrosine phosphatases (PTPs) (10). PSPs are characterized by the way they operate in the taking away of phosphate groups from the phosphorylated Ser/Thr residue of a protein (10). The bulk of PSPs fall under two major families, consisting of the type-1 (PP1) and type-2 (PP2) family phosphatases. One among the foremost Ser/Thr phosphatases concerned

within several cellular mechanisms, including control of various signaling pathways, cell-cycle control, DNA replication, transcription, and translation (11-15), is protein phosphatase 2A (PP2A). A heterotrimeric holoenzyme, PP2A consists of catalytic (C) and structural (A) subunits (core enzymes) and regulatory B-subunits. The B-subunits consist of several distinct families (11-16). The regulatory B-subunits are encoded by four unrelated gene families: PR55/B (PPP2R2A, PPP2R2B, PPP2R2C, and PPP2R2D), PR56/61/B' (PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, and PPP2R5E), PR130/72/48/59/G5PR/B" (PPP2R3A, PPP2R3B, and PPP2R3C), and PR93/110/B"' (STRN and STRN3) (17). Observations demonstrated the B56 (PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, and PPP2R5E) family of B-subunit proteins is the more important family with impact on cancer transformation (18, 19).

One of the most important B-subunits of PP2A is PPP2R5C, the function of which mainly affects the phosphorylation position of many proteins (20).The functional PPP2R5C gene locus resides at 14q32.2, while a nonfunctional B56γ1 pseudogene PPP2R5C is present at 3p21.3 (20, 21). PPP2R5C plays a critical function in cell proliferation, differentiation, and transformation based on its induction of dephosphorylation of P53 at various residues (22). B56γ3 regulatory subunit in nuclear may be responsible for the tumor-suppression function of PP2A (21).

2. Objectives

With regard to the vital role of PPP2R5C, we tend to examine whether alteration in expression of some proteins is involved in signal transduction; for example B-subunit of protein phosphatases can be observed in patients with AML and CML. To assess this supposition, we analyzed the expression level of PPP2R5C in peripheral blood mononuclear cells from patients with CML and AML, compared with healthy individuals by SYBR-green real-time PCR.

3. Materials and Methods

This study enrolled 50 newly diagnosed and untreated patients with acute myeloid leukemia (25 males, 25 females, median age 34 years old), and 31 patients with chronic myeloid leukemia (12 males, 19 females, median age 45 years old), together with 23 healthy individuals as the control group (11 males, 12 females, median age 16 years old). They were individuals brought to our center and their peripheral blood, which had no abnormalities, was gathered. Patients were included in FAB subtypes of AML that had the characteristics of the patients presented in Table 1. Diagnosis was according to the WHO criteria. All patients had clinical features, complete blood count (CBC), peripheral blood smear, and bone marrow patterns of AML and CML. Exclusion criteria were consistent with samples not having enough RNA, and patients who had received treatment in the past.

The bone marrow samples were collected and sent to the laboratory of molecular hematopathology in Ghaem hospital, Mashhad University of Medical Science in 2014 - 2015, for cytomorphology and cytogenetic and cytoimmunolgical study. First samples were analyzed for identification of t (15; 17), t (8; 21), inv 16 and t (9; 22), followed by respective immunophenotyping, and positive samples were then selected. The medical history and laboratory finding of patients were perused and two pathologists approved this data. All data related to the patients, such as lymphadenopathy, splenomegaly, hepatomegaly, and socio-demographic data, such as ages and gender, were extracted from the files of the hematopathology Department of Ghaem hospital. After informed consent, samples were tested for PPP2R5C expression and that study was approved by the ethics committee of the Mashhad University School of Medicine.

3.1. RNA Extraction and cDNA Synthesis

Two milliliters of blood were drawn from all patients in tubes containing ethylenediaminetetraacetic acid (EDTA-K2) as an anticoagulant. We used the Ficoll-Hypaque gradient centrifugation to isolate mononuclear blood cells. Using the Trizol kit (Invitrogen, Carlsbad, CA, USA), RNA was extracted from these cells according to the manufacturer's recommendations. RNA quality was approved by conformation of intact 28 S and 18 S ribosomal bands by means of agarose gel electrophoresis and ethidium bromide staining. Also, we determined the sample's RNA concentration by thermo scientific NanoDrop 2000 spectrophotometer. Specimens with low RNA content ($<$ 20 ng/μL) were excluded from the study.

After RNA extraction, 1 μg of total RNA was used as a template in a 12 μL volume cDNA synthesis reaction containing 1 μL random hexamer and DEPC water. This complex was denatured at 65°C for 5 minutes and immediately chilled on ice. Subsequently, the mixture of the 4 μL 5 $\times$ buffer, 2 μL 10 mM dNTPs, 1 μL ribonuclease inhibitor (RiboLock™) and 1 μL RevertAid™ H minus reverse transcriptase (Fermentas Inc.), was added to the previous mixture and this mixture) final volume = 20 μL) was incubated in a thermocycler (BIO RAD) at 42°C for 60 minutes, and at 70°C for 5 minutes afterwards. We also included two negative control reactions, without any RNA and without RevertAid™ H minus reverse transcriptase, accompanied by each reaction. Eventually, we used the B2MG primer to

confirm the quality of cDNA. Strict precautions were pursued to hinder any contamination, including the use of specific PCR-designed UV flow cabinets, pipettes, and filtered tips. For the prevention of contamination, all pre-PCR steps (RNA isolation, cDNA synthesis, and PCR set-up) were performed in a room isolated from the post-PCR processing room.

3.2. PPP2R5C Expression Analysis

Methods of SYBR® green (TAKARA) were applied to detect the PPP2R5C expression level in this study and relative expression was calculated from the $2^{-\Delta\Delta CT}$ method (23).

We used the 5'-GTAATAAAGCGGGCAG-CAGG-3' (forward) and 5'-CAAAGTCAAAGAGACGCAACA-3' (reverse) primers for gene of interest. According to protocol instruction, we used a master mix with 6.6 μL deionized distilled water free nuclease, 10 μL TAKARA Pre-mix, 0.4 μL from Rox, 0.5 μL of each primer, and 2 μL of patient cDNA for PCR reaction. PCR was performed in a 7500 real time PCR system (ABI-ONE STEP-USA), with a program consisting of an initial preheating at 95°C for 10 minutes, followed by denaturation at 95°C for 40 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for a total of 40 cycles, and a final extension at 72°C for 5 minutes. We also normalized the expression data (PPP2R5C expression) against internal β-2-microglobolin (B2M) by specific primers including: forward: 5'-CAGCAAGGACTGGTCTTTCTAT-3' and reverse 5'-GCGGCATCTTCAAACCTC-3'. All real-time analyses were performed at least in duplicate for each sample in an isolated room. Samples were always accompanied simultaneously with positive and negative controls.

3.3. Statistical Analysis

Analysis of data was performed using SPSS V. 19 (statistical software for social analysis, version 11.5). We applied the Kolmogorov–Smirnov test for assessment and distribution pattern of data (the equality of continuous). We also used the chi-square independent sample t-test to compare the categorical variables and mean of continuous variables, respectively, while the Mann–Whitney U-test and Spearman's rank correlation analyses were used for data distributed non-normally. $P < 0.05$ was considered for the statistically significant relationship.

4. Results

Specificity of RT-PCR products was confirmed by high resolution gel electrophoresis. In addition, we applied melt curve to confirm the specificity of reaction, which resulted in single product-specific melting temperature, and the efficiency for the gene of interest (PPP2R5C) and control gene (B2MG) was determined at 90.1 and 90.5, respectively.

Significantly higher expression of PPP2R5C was found in the AML (4.13 ± 4.85) compared with healthy controls. Thirty-four (69.4%) out of 49 patients had upregulation of PPP2R5C. Fold change of PPP2R5C in FAB subtypes of AML was included in Table 1 and Figure 1, and there was no significant difference in the fold change of PPP2R5C among FAB subtypes of AML (P value > 0.05). We also assessed CBC indexes in AML patients with different expression of PPP2R5C (upregulated and downregulated), for which there was no significant relationship (P value > 0.05) (Table 2).

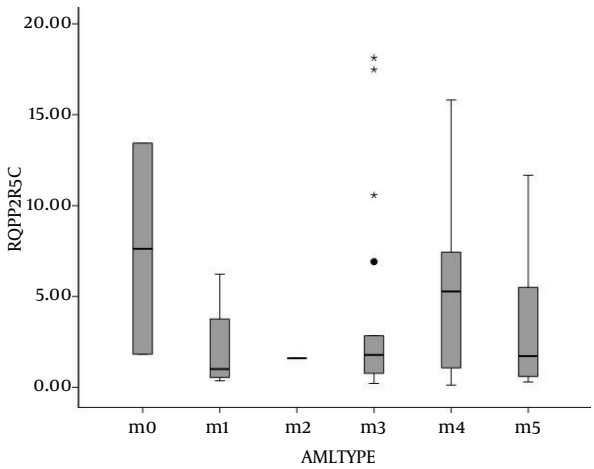

Figure 1. Differences in Expression Level of PPP2R5C Between FAB Subgroups of AML

Table 1. Differences in Expression Level of PPP2R5C Between FAB Subgroups of AML[a]

Fab Subtypes Of AML	Number	Mean ± SD
M_0	2	7.62 ± 8.21
M_1	4	2.14 ± 2.74
M_2	2	1.60 ± 1.20
M_3	18	4.00 ± 5.64
M_4	12	5.34 ± 5.08
M_5	12	3.40 ± 3.62

[a] P value = 0.71.

The expression level of PPP2R5C significantly decreased (0.469 ± 0.34) in CML patients compared with healthy individuals, 29 (93.5%) out of 31 patients with CML. Assessment of the relationship of PPP2R5C fold change with CBC, a finding by Mann–Whitney U-test, did not show

Table 2. Difference in Mean of CBC Indexes Between AML Patients With Different PPP2R5C Expression Level (Down-Regulate and Up-Regulate)[a]

CBC-Index	Up Regulate	Down Regulate	P Value
RBC, 10^{12}/L	2.61 ± 0.53	2.73 ± 0.65	0.52
WBC, /μL	545 ± 765	355 ± 271	0.20
PLT, /μL	692 ± 690	763 ± 102	0.79
HCT, %	24.5 ± 5.41	34.6 ± 40.1	0.34
HGB, gr/dL	8.0 ± 1.91	7.7 ± 2.09	0.69
MCV, fL	92.4 ± 6.96	89±7.43	0.13
MCH, pg	30.7 ± 4.57	28.8±3.45	0.15
MCHC, gr/dL	33.3 ± 4.12	31.9 ± 2.37	0.23

Abbreviations: HCT, hematocrite; HGB, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RBC, red blood cell; WBC,white blood cell.
[a]Values are expressed as mean ± SD.

any significant differences (P value > 0.05) (Table 3).

Table 3. Difference in Mean of CBC Indexes Between CML Patients With Different PPP2R5C Expression Level (Down Regulate And Up Regulate)[a]

CBC Index	Up Regulate	Down Regulate	P Value
RBC, 10^{12}/L	3.39 ± 3.75	3.83 ± 0.71	0.89
WBC, /μL	107 ± 219	101 ± 794	0.91
PLT, /μL	295 ± 211	274 ± 186	0.88
HCT, %	25.0 ± 25.1	34.0 ± 6.1	0.7
HGB, gr/dL	8.2 ± 7.9	10.8 ± 1.85	0.72
MCV, fL	84.6 ± 19.7	89.2 ± 8.09	0.48
MCH, pg	28.8 ± 8.9	28.2 ± 3.44	0.94
MCHC, gr/dL	33.5 ± 2.89	31.9 ± 2.80	0.43

Abbreviations: HCT, hematocrite; HGB, hemoglobin; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RBC, red blood cell; WBC, white blood cell.
[a]Values are expressed as mean ± SD.

Table 4. Differences in Expression Level of PPP2R5C in AML Patients With Different Clinical Manifestation[a]

Clinical Manifestation	Fold Change of PPP2R5C Gene	P Value
Lymphadenopathy		0.656
Positive	3.29 ± 2.47	
Negative	4.25 ± 5.11	
Splenomegaly		0.757
Positive	4.46 ± 4.62	
Negative	3.98 ± 5.02	
Hepatomegaly		0.807
Positive	3.81 ± 3.77	
Negative	4.22 ± 5.16	
Petechiae-Purpura		0.466
Positive	3.46 ± 4.67	
Negative	4.52 ± 4.99	

[a]Values are expressed as mean ± SD.

Table 5. Differences in Expression Level of PPP2R5C in CML Patients With Different Clinical Manifestation[a]

Clinical Manifestation	Fold Change of PPP2R5C Gene	P Value
Lymphadenopathy		0.766
Positive	0.46 ± 0.36	
Negative	0.47 ± 0.34	
Splenomegaly		0.591
Positive	0.44 ± 0.26	
Negative	0.50 ± 0.42	
Hepatomegaly		0.872
Positive	0.424 ± 0.26	
Negative	0.49 ± 0.38	
Petechiae-purpura		0.114
Positive	0.54 ± 0.24	
Negative	0.42 ± 0.38	

[a]Values are expressed as mean ± SD.

5. Discussion

The greater importance of PP2A than the Ser/Thr phosphatases has implications in several cellular mechanisms, including control of various signaling pathways, cell cycle control, DNA replication, transcription, and translation (11, 15). Tumor-suppressor genes are commonly affected by mutation, deletion, or abnormal expression in leukemia (2, 24, 25).

The role of the PP2A by in vitro activation of pro-apoptotic and inhibiting anti-apoptotic proteins by dephosphorylation in mammalian cell lines, becomes obvious (26). In addition, tumor suppressor P53 is tightly regulated by phosphorylation. The phosphorylated P53 promotes cell growth arrest and/or programmed cell death through P53-related genes (27). PP2A by phosphorylation of amino acid serine 15, 20, 33 and 37 of the amino terminal domain P53 once DNA harm, is involved in the genomic stability of cells (28). Also, PP2A through BV/PR61a regulatory subunit by dephosphorylation and inactivation of the anti-apoptotic Bcl-2 protein, is obviously involved in programmed cell death (26). The effects of PP2A on regulation

of the signaling pathway, for instance PP2A by phosphorylation events on Thr308 or Ser473, tightly modulated the function of Akt and PP2A is the major phosphatase targeting these residues in vitro (29). The role of PP2A in Wnt/β-catenin signaling exerted either positive or negative effects (30). PP2A also has a prominent role in controlling the accumulation of the protoncoprotein, c-Myc (31).

Over expression of BCR-ABL in lymphoid precursors causes over expression of SET by BCR-ABL product, as such, suppressed PP2A participated in leukemogenesis (32), and suppression of BCR-ABL by kinase inhibitory drugs, such as imatinib, decreased SET expression. This observation intimated the role of the PP2A enzyme in CML pathogenesis (9, 15). Thus, inhibition of tumor-suppressive activity of PP2A by over expression of SET could affect CML progression (33). In addition, the results recommend the fusion gene of SET-Nup214 in acute myeloid leukemia could suppress the normal activity of PP2A and result in leukemia (34).

PPP2R5C is one of the regulatory B-subunits of PP2A, and has a prominent role in its regulation; it also plays a crucial role in cell proliferation, differentiation, and transformation (35).

In lung cancer, F395C mutation, and A383G in intestinal cancer, could have happened in B56γ that disrupted B56γ-P53 interaction (36).In the other study, it showed ataxia telangiectasia mutated (ATM) phosphorylates and regulates B56γ3, B56γ2, and B56δ after DNA damage, and phosphorylation of B56γ3 by ATM leads to upregulation by blocking MDM2-mediated B56γ3 ubiquitination (37). Nobumori et al. showed B56γ mutation can lead to loss of tumor-suppressor activity of PP2A by disrupting interactions with the PP2A AC core (38). This revealed that in some case of myelodysplastic syndrome (MDS), over expression of a cancerous inhibitor of PP2A (CIP2A) might play a role in the proliferation of the blast and progression of the disease (39). Another study revealed the proliferation rate of PPP2R5C-si RNA-treated Molt-3 and Jurkat T-cell, was significantly decreased. It has been shown that the suppression of PPP2R5C by RNAi can inhibit the proliferation of the Molt-4 and Jurkat T-cell; however, the suppression could not significantly induce apoptosis in Molt-4 and Jurkat T-cells (40). It also showed that the proliferation of the PPP2R5C-siRNA-treated CML cell line was decreased in the K562 cell line and apoptosis was significantly increased (41).

To the best of our knowledge, few studies have been reported that have examined the expression of PPP2R5C in leukemia. In our study, significantly higher expression of PPP2R5C was found in the AML (4.13 ± 4.85), compared with healthy individuals. The expression level of PPP2R5C significantly decreased (0.469 ± 0.34) in CML patients compared with healthy individuals.

The experiments of others by Affymetrix method indicated the expression of PPP2R5C decreased in B-CLL, which led to the conclusion this event can be a marker of progressive disease in B-CLL. These experiments have compared expression of PPP2R5C between stable and progressive B-CLL without a control group (42). Another study by Zheng et al. demonstrated significantly higher expression of PPP2R5C in AML, CML, T-ALL, and B-CLL groups in comparison with healthy controls. In addition, the expression level of PPP2R5C in the CML-CR group decreased significantly compared with that in the de novo CML group (35). This finding is in accordance with our results, but we studied more patients than in the respective study to confirm over expression of PPP2R5C. In addition, we examined, in the expression level of PPP2R5C among FAB subtypes of AML, that there was no significant difference in the expression level between them. We also assessed differences in CBC indexes with regard to the expression level of PPP2R5C, where there was no significant difference in AML and CML patients.

PP2A has a controversial role involved in both suppression and promotion of cell growth. Therefore, these observations that have indicated abnormal expression of the PPP2R5C gene in CML and AML might be related to the pathogenesis of respective diseases.

5.1. Conclusions

The present results identified abnormality in the expression levels of PPP2R5C in AML and CML patients. With regard to the importance of PP2A in cancer, as has been demonstrated in several studies, and the critical role PPP2R5C B-subunit has on regulation of it, the change in the expression pattern of PPP2R5C might be a critical finding in AML and CML patients because it could be a cause of abnormal cell proliferation, differentiation, and transformation in these respective patients. Definitive results should be demonstrated by a larger number of samples than our study used, and more study is needed.

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