Prognostic Impact of PPP2R5C Gene Expression in Adult Acute Myeloid Leukemia Patients with Normal Cytogenetics

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Abstract Protein Phosphatase 2A (PP2A) is a crucial regulator of the cellular signalling pathways, proliferation, cell cycle checkpoints and apoptosis. The PPP2R5C gene encodes PP2A regulatory B56 γ subunit. Malignant transformation may occur, if mRNA of PPP2R5C is functionally deregulated, structurally altered, decreased or overexpressed. Therefore, the purpose of the study was to examine PPP2R5C mRNA expression, evaluate its association with the different clinical and haematological parameters and determine its prognostic impact in Egyptian adult acute myeloid leukaemia patients with normal cytogenetics (CN-AML). Peripheral blood samples of 50 de novo CN-AML patients and 20 age- and gender-matched healthy controls were examined for PPP2R5C expression by Quantitative Real Time-Polymerase Chain Reaction. The expression levels of PPP2R5C mRNA were significantly higher in the CN-AML samples than in the control samples ($P \leq 0.001$). There was a statistical significant difference between the low and high expression levels of PPP2R5C with regard to age ($P = 0.005$, $r = -0.447$, $P = 0.001$). The patients with an unfavourable response to induction chemotherapy had significant higher PPP2R5C expression levels than those with a favourable response ($P = 0.002$). There was a significant influence of high

PPP2R5C expression levels on the overall survival and progression free survival ($P = 0.03$, 0.026), respectively. PPP2R5C overexpression is an adverse prognostic factor which affects leukaemogenesis in the CN-AML, it may predict the disease progression and overall survival during the follow-up of the patients.

Keywords CN-AML · PPP2R5C · B56 γ · PP2A · Real time-polymerase chain reaction

Introduction

Human cellular protein serine-threonine phosphatases (PSPs) comprise three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and aspartate-base protein phosphatases [1].

The protein phosphatase 2A (PP2A) holoenzyme is a crucial member of the PPPs family that controls many cellular processes, including the critical checkpoints of the cell cycle and its signalling pathways, such as the Wingless/Integrated (Wnt), mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK) and Hedgehog (HH) pathways [2–4].

The trimeric form of PP2A consists of a scaffold (A), catalytic (C) and variable regulatory (B) subunit; the regulatory subunit is mainly related to the functional diversity of PP2A [5–7]. The B subunit controls PP2A activity and directs its cellular localization and substrate specificity. There are four families of B subunit: B55, B56, PR72, and PR93; each family has many isoforms encoded by distinct genes [5–7]. The B56 family has five isoforms (α , β , γ , δ , ϵ), with B56 γ expressed in cardiac tissue and skeletal muscles. B56 γ has five splice variants which are encoded by the PPP2R5C gene [7–9].

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The B56 isoforms regulate various proliferative and apoptotic networks of the cell cycle [10]. After DNA damage, Ataxia telangiectasia mutated (ATM) protein kinase promotes the dephosphorylation and activation of P53 via B56 γ , thus inducing apoptosis [11–13]. It is likely that murine double minute 2 (mdm2) enhances B56 γ and P53 ubiquitination, which inhibits apoptosis in normal cell growth conditions [11]. Few studies have shown that B56 γ can regulate the B cell lymphoma gene 2 (BCL2) gene stability, it may dephosphorylate BCL2 and prevent its proteasome degradation to enhance proliferation [14] (Fig. 1).

B56 γ destabilizes B-catenin and dephosphorylates extracellular receptor kinases (ERKs); therefore, it acts as a negative regulator of the Wnt and MAPK signalling pathways, respectively [5, 9]. Alternatively, cellular exposure to any anti-apoptotic signals potentiates the expression of immediate early gene-X (IEX-1), which stimulates ERKs to phosphorylate B56 γ and reverse its inhibitory effect [9, 15] (Fig. 1).

The Nuclear accumulation of B56 γ monitors cell cycle progression during G1/S phase, exerts vital functions in G2 phase to maintain normal G0/G1 control and plays an essential role in mitosis [16–18].

There is a link between PPP2R5C (B56γ-encoded gene) and tumorigenesis, but there is controversy relating to its expression levels in different cancers [19–24]. Therefore, our study aimed to analyse the mRNA expression levels of PPP2R5C and to estimate the role of PPP2R5C in the progression of de novo acute myeloid leukaemia with normal cytogenetics in a cohort of adult Egyptian patients.

Fig. 1 The relation of the PP2A-B56 γ isomer with the proliferative and apoptotic molecules in the different signalling pathways of the cell cycle. Normally, B56 γ is a negative regulator of both Wnt and MAPK signalling pathways. After DNA damage, B56 γ interacts with P53 in a reaction mediated by ATM. ATM-B56 γ -P53 mediated reaction is inhibited by mdm2 in normal cell growth conditions. The IXE-1 expression stimulates ERK to inhibit B56 γ during the exposure to anti-apoptotic signals. B56 γ can dephosphorylate BCL2 to promote proliferation

Materials and Methods

Patient Selection

The patients comprised 50 adults with CN-AML, who were referred to the haematology/oncology clinic of National Cancer Institute, Cairo University and Beni-Suef University Hospital, Egypt. They were enrolled in our study at the time of diagnosis prior to chemotherapy.

The CN-AML patients comprised 32 males and 18 females, with an age range of 20–65 years; median: 41 years. They were compared to a control group of 20 healthy age- and sex-matched individuals with no medical history of cancer or any other serious illnesses. The group included 10 males and 10 females with an age range of 23–55 years; median: 38 years.

The study was performed in accordance with the revised Helsinki Declaration of Bioethics [25], and written informed consent was obtained from all participants.

The diagnosis of AML was based on the criteria of the 2016 WHO classification [26, 27] and the data extracted from the patients' files. The characteristics of the patients are summarized in Table 1. The median follow-up of the patients was 12.4 months (range: 0.53–25.5 months).

Patient Samples

Ethylene diamine tetra acetic acid (EDTA) and lithium tubes were used to collect peripheral blood (PB) and bone marrow (BM) samples, respectively. PB was used for routine laboratory analyses such as morphology and immunophenotyping, while BM was used for cytogenetic analysis.

Cytogenetic Analysis

Karyotypes were examined using conventional cytogenetic analysis. The patients were considered negative for any chromosomal abnormalities when at least 20 metaphase cells were examined and found to be normal [28].

Detection of FLT3-ITD and NPM1-A Mutations

All samples were routinely examined for Fms-like tyrosine kinase3-internal tandem duplication (FLT3-ITD) mutation as previously described [29]. Only 10 samples were examined for the nucleophosmin (NPM1) mutation because its assessment was previously researched [30], and the results were needed for standardization in our laboratory by the time this study was performed.

Table 1 Haematological laboratory data and clinical characteristics of the studied adult CN-AML patients

Characteristic	
PPP2R5C gene expression	10.78 ± 17.8 (0.2–100.2) ^a
Median	3.85
Gender: no (%)	
Male	32 (64%)
Female	18 (36%)
Age at diagnosis (years)	40.2 ± 11.4 (20–65)
Median	41
Lab findings	
Total leucocyte count × 10 ⁹ /L	51.2 ± 63.7 (2.5–327)
Median	42.1
Haemoglobin (gm/dl)	7.6 ± 1.8 (2.7–11.6)
Median	8
Platelets × 10 ⁹ /L	52.8 ± 49 (7–150)
Median	36
PB blasts	62 ± 25 (18–100)
Median	66
FAB subclassification: no (%)	
M0	8 (16%)
M1	12 (24%)
M2	17 (34%)
M4	7 (14%)
M5	6 (12%)
FLT3-ITD	
Wild	47 (94%)
Mutant	3 (6%)
Clinical response to induction chemotherapy: no (%)	
Favourable	29 (58%)
Unfavourable	21 (42%)

^aMean ± SD (range) FAB classification French American British classification

RNA Isolation and Analysis of PPP2R5C Expression by Real Time Quantitative-Polymerase Chain Reaction (RTQ-PCR)

Peripheral blood mononuclear cells (PBMNCs) were isolated from 2 mL of EDTA-treated blood samples collected at diagnosis by Ficoll density gradient centrifugation using Ficoll-Hypaque 1077 (Sigma).

Total RNA was extracted from MNCs using a QIA-amp RNA blood mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The cellular pellets were stored at − 80 °C until used for reverse transcription. Complementary DNA (cDNA) was synthesized from extracted RNA using a (dT) 15-mer primer by superscript 111 reverse transcriptase and stored at − 20 °C.

The Real-time PCR amplifications were performed in a reaction mixture comprising 1 µL of cDNA, 9 µL of 2.5 × SYBR Green Master mix kit (Qiagen, Hilden, Germany), and 10 µmol/L of each of the specific forward and reverse primers of the PPP2R5C and β2Microglobulin genes (β2MG) [both sets of primers synthesised by Applied Biosystems] to reach a final volume of 25 µL. β2MG was used as an internal control (reference gene) for normalization.

RTQ-PCR was performed using the ABI PRISM 7000 Light-Cycler system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions, and the reaction protocol involved initial denaturation at 95 °C for 2 min, followed by 44 amplification cycles at 95 °C for 15 s, and 81 °C for 1 min [23, 31].

The PCR products derived from the reactions were analysed by melt curve analysis, and their relative concentrations in the tested samples were calculated using Light-Cycler system software. The results were normalized to the measured levels of β2MG. All experiments were performed in triplicate, and the results were averaged for each sample.

The Sequence of the Primers used for PPP2R5C and β2-MG Gene Amplification [23]

PPP2R5C

Forward: 5'-GTAATAAAGCGGGCAGCAGG-3'

Reverse: 5'-CAAAGTCAAAGAGGACGCAACA-3'

β2MG

Forward: 5'-CAGCAAGGACTGGTCTTTCTAT-3'

Reverse: 5'-GCGGCATCTTCAAACCTC-3'

Protocol of Treatment

The patients were treated with a “three and seven regimen”, which consists of 3 days of anthracycline combined with 7 days of arabinosyl cytosine (Ara-C) daily as an induction therapy to be followed by intensified post-remission chemotherapy for patients who achieved complete remission [32, 33].

The haematological response of the patients was assessed by examining their BM aspirates between the 21st and 28th day of the induction therapy. Follow-up of the patients was performed periodically via clinical examination and complete blood counts.

Assessment of the clinical response was performed according to the response criteria of 2017 European Leukaemia Net (ELN) recommendations [34].

Definitions

Complete Remission (CR)

Complete haematological recovery with no extramedullary disease and < 5% blasts in the bone marrow. The absolute neutrophil count is $\geq 1000/\mu\text{L}$, platelet count is $\geq 100,000/\mu\text{L}$, and circulating blasts with or without Auer rods are absent [34].

Primary Refractory Disease

No CR or CR with incomplete haematologic recovery after 2 cycles of induction therapy, the patients who die due to aplasia or an intermediate cause must be excluded [34].

Statistical Methods

Statistical analysis was done using IBM® SPSS® Statistics version 22 (IBM® Corp., Armonk, NY, USA). Numerical data were expressed as the mean and standard deviation or the median and range as appropriate. The median value was used as a cut-off for defining high and low expression levels of PPP2R5C because the RQ values of PPP2R5C were considered to not have a homogenous distribution. Qualitative data were expressed as frequency and percentage. Chi square test or Fisher's exact test was used to examine the relation between qualitative variables. For non-normally distributed quantitative data, comparisons between two groups were performed using the Mann–Whitney test (non-parametric t-test). The Spearman-rho method was used to test the correlation between numerical variables. Survival analysis was performed using the Kaplan–Meier method, and comparisons between two survival curves were performed using the log-rank test. A Cox-proportional hazard regression model was used to determine the Hazard ratio (HR) with a 95% confidence interval (CI) for risk estimation. All tests were two-tailed. A P -value < 0.05 was considered significant.

Results

The expression levels of the PPP2R5C gene in the CN-AML samples ranged from 0.2 to 100.2 (mean 10.78 ± 17.8 , median 3.85), while its expression levels ranged in the control samples from 0.002 to 1.4 (mean 0.51 ± 0.4 , median 0.4). The levels of PPP2R5C mRNA were significantly higher in the CN-AML samples than in the control samples ($P < 0.001$).

The median PPP2R5C expression levels was taken as a cut-off value to compare the clinical and laboratory features of the patients with low and high expression levels

(Table 2). There was a statistically significant difference between the CN-AML patients with low PPP2R5C expression levels and those with high PPP2R5C expression levels with regard to age (Fig. 2).

Correlations

The FLT3-IDT mutation could not be correlated statistically with PPP2R5C expression levels due to the small number of patients who had this mutation (3/50). The PPP2R5C levels were negatively correlated with age ($r = -0.447$, P -value = 0.001) but were not correlated with the other patients' data.

Nucleophosmin mutations were detected in 2/10 cases, so it was not possible to statistically analyse the results due to the small number of cases.

Prognostic Relevance of PPP2R5C Expression

After the initial course of induction chemotherapy, 29 cases showed a favourable response (Complete Remission; CR) and 21 cases showed an unfavourable response (refractory disease/relapse): 4/21 died within the 1st 28 days of induction therapy, 7/21 showed CR with incomplete haematological recovery after 2 cycles of intensive induction treatment, 5/21 died after the 28 days of induction therapy, and 5/21 were experienced relapse.

The results revealed that patients with an unfavourable response had significantly higher PPP2R5C expression levels than those with a favourable response (P -value = 0.002) (Fig. 3).

There was a significant influence of PPP2R5C expression levels on the overall survival (OS) of the studied CN-AML patients. The predicted 1-year OS was 88% and an estimated survival of 25.5 months in cases with low PPP2R5C expression levels, while cases with high PPP2R5C expression had an OS of 63% and an estimated survival of 15.2 months (P -value = 0.031) (Fig. 4).

Based on the univariate Cox hazard regression, PPP2R5C was independently predictive of a high risk of death (HR 3.299; 95% CI 1.046–10.47) and a high risk of disease progression (HR 2.551; 95% CI 1.086–5.994) (Table 3; Fig. 4). Univariate analysis indicated that no other studied prognostic parameters could predict either overall survival or progression free survival (Table 3).

Discussion

AML is one of the known leukaemias that contributes to poor prognosis in adult patients. According to the revised 2008 and 2016 WHO classifications, the levels of specific molecular markers in blood have a great impact on

Table 2 Comparison between adult CN-AML patients with lower and higher PPP2R5C expression levels (median cut-off value = 3.85)

Characteristic	Low expression < 3.85	High expression $\geq$ 3.85	<i>P</i> value
Age (years): no (%)			
< 40	7 (29.2%)	17 (70.8%)	0.005
$\geq$ 40	18 (69.2%)	8 (30.8%)	
Gender: no (%)			
Male	15 (46.9%)	17 (46.9%)	0.55
Female	10 (55.6%)	8 (44.4%)	
Lab findings: no (%)			
Total leucocyte count $\times 10^9/L$			
< 50	17 (56.7%)	13 (43.3%)	0.24
$\geq$ 50	8 (40%)	12 (60%)	
Haemoglobin (gm/dl)			
< 9	19 (47.5%)	21 (52.5%)	0.48
$\geq$ 9	6 (60%)	4 (40%)	
Platelets $\times 10^9/L$			
< 50	14 (43.8%)	18 (56.3%)	0.24
$\geq$ 50	11 (61.1%)	7 (38.9%)	
PB blasts			
< 50	21 (51.2%)	20 (48.8%)	1
$\geq$ 50	4 (44.4%)	5 (55.6%)	
FAB classification			
M0, M1, M2	17 (45.9%)	20 (54.1%)	0.33
M4, M5	8 (61.5%)	5 (38.5%)	
FLT3-ITD: no (%)			^a
Wild	24 (51.1%)	23 (48.9%)	—
Mutant	1 (33.3%)	2 (66.7%)	
Response to induction therapy: no (%)			
Favourable	20 (69%)	9 (31%)	0.002
Unfavourable	5 (23.8%)	16 (76.2%)	

P-value < 0.05 is significant. The bold *P*-values are statistically significant

Low and high expression are defined as below and above the median cut-off, respectively

FAB classification French American British classification

^aNo *P*-value due to the small number of cases harbour FLT3-ITD mutation

Fig. 2 A comparison between the low and high expression levels of PPP2R5C mRNA in CN-AML patients with regard to age, being statistically higher in the younger ages (*P*-value = 0.005) **Fig. 3** A comparison between the low and high expression levels of PPP2R5C mRNA with regard to the clinical response of the patients, being statistically higher in the group with unfavourable response

prognosis of CN-AML, as most patients can achieve

Fig. 4 Survival times according to low and high expression levels of PPP2R5C: **a** overall survival. **b** Progression free survival

complete remission after induction chemotherapy [26, 35].

PP2A is a molecular marker that controls cell proliferation, differentiation, apoptosis, adhesion, migration, and metabolism in humans [6, 36]. It exerts opposing functions by acting on various cellular signalling pathways [5, 9]. In some studies, PP2A was considered a tumour suppressor gene that induces apoptosis of harmful cells, while it was found to be a positive regulator of abnormal cell growth and survival in other group of studies [4, 13, 37].

The current study showed higher mRNA levels of PPP2R5C in AML blasts than in normal cells, which was in agreement with Zheng et al. [23], the first group that described PPP2R5C overexpression in different leukaemia types. They reported that altered PPP2R5C expression

levels might affect the tumour suppressor activity of PP2A, thus resulting in abnormal cellular transformation and proliferation in AML.

Few studies have focused on the role of PPP2R5C in apoptosis because it dephosphorylates distinct pro-apoptotic molecules, such as P53, P21 and P27, and inactivates protein kinase B (AKT) [12, 20, 38]. Although PPP2R5C has pro-apoptotic activity, it can stimulate proliferation, as many kinases can control the phosphorylation of PPP2R5C [19].

Minimal levels of PP2A must be sustained for cellular survival, as PP2A controls G1-S transition of the cell cycle and B56 isoforms can be detected at the centromeres from prophase to metaphase during mitosis [5, 9]. This finding

Table 3 Univariate analysis for overall survival and Progression free survival

Characteristic	Overall survival			Progression free survival		
	Number of events (deaths)	Cumulative survival at 12 months (%)	<i>P</i> value	Number of events (deaths and relapses)	Cumulative survival at 12 months (%)	<i>P</i> value
PPP2R5C mRNA expression						
< 3.85	5	88	0.031	9	75.8	0.026
≥ 3.85	11	63.3		16	56	
Age (years)						
< 40	9	66.7	0.481	13	54.2	0.571
≥ 40	7	84.4		12	68.7	
Gender						
Male	10	70.9	0.629	17	65.6	0.222
Female	6	83.3		8	66.7	
<i>Lab findings</i>						
Total leucocyte count × 10 ⁹ /L						
< 50	8	83.3	0.194	15	60	0.712
≥ 50	8	62.5		10	52	
Haemoglobin (g/dL)						
< 9	12	76.9	0.944	21	72.5	0.214
≥ 9	4	70		4	70	
Platelets × 10 ⁹ /L						
< 50	9	80.8	0.318	14	71.9	0.201
≥ 50	7	65.8		11	48.9	
PB blasts						
< 50	3	87.4	0.185	7	75.6	0.904
≥ 50	13	69.4		18	55.6	
FAB classification						
M0, M1, M2	11	80.7	0.390	18	72.9	0.502
M4, M5	5	60.6		7	44.9	
FLT3-ITD						
Wild	13	78.2	—	22	58.5	—
Mutant	3	33.3		3	33.3	

P-value < 0.05 is significant. The bold *P*-values are statistically significant

FAB classification French American British classification

^aNo *P*-value due to the small number of cases harbour FLT3-ITD mutation

might explain the very low levels of PPP2R5C mRNA found in blood samples of healthy individuals.

Except for the negative correlation found with the age of the patients in our cohort, no correlations of PPP2R5C levels with the available clinical and laboratory data were detected, which was similar to the results of a previous study [39].

Similar to the results by Benton et al. [40], our results confirmed the significant association of high PPP2R5C levels with poor prognosis and shorter overall survival in AML. It was suggested that PPP2R5C might be an essential biomarker in estimating AML prognosis due to its crucial role in tumorigenesis.

Normally, B56γ dephosphorylates paxillin protein at the focal adhesions which stops cellular migration. Conversely, altered B56γ increases cell metastasis, invasion, and migration in tumours such as melanoma via paxillin phosphorylation [8, 10, 41–43]. Therefore, the role of deregulated B56γ in tumour invasiveness and genetic instability [44] might reflect the association of high PPP2R5C expression levels with low rates of CR, shorter overall survival and high probability of death/relapse in the studied CN-AML patients.

Recent studies [10, 45] discussed the ability of PP2A to enhance survival signalling pathways in the hypoxic microenvironments of certain solid tumours and

haematological malignancies that affect the response to chemotherapy. Hypoxia promotes PP2A activity by a hypoxia inducible factor1 (HIF1) α mediated mechanism, and PP2A can support leukaemia cells in the leukaemic niche by positively regulating certain pro-angiogenic cytokines, such as transforming growth factor β (TGF- β) [45].

Despite the elevated expression levels of PPP2R5C found in different investigations, Cristobal et al. [46] detected downregulated levels in AML. However, Chen et al. [47] and Shen et al. [48] provided convincing evidence that suppression of upregulated PPP2R5C in different leukaemia cell lines could induce apoptosis. Liu et al. [49] proposed that the inhibition of proliferation in chronic myeloid leukaemia cell lines (K562) via PPP2R5C knockdown was related to activation of the MAPK, Janus kinase/signal transducers and activators of transcription (JAK/STAT) and phosphatidylinositol 3-kinase (PI3K) pathways. This activation might occur via downregulation of certain genes, such as v-RAF murine sarcoma viral oncogene homologue B1 (BRAF). It was recommended to use agents that modulate PP2A subunits as potential therapeutic targets in leukaemia [19, 40, 47–49].

In haematological cancers, several genetic alterations antagonize the integration of PP2A subunits, which disrupt the function of the protein [50]. In addition, the cross talk among the different abnormal signalling pathways could direct PPP2R5C gene to acquire an oncogenic role in tumour cells [4].

In conclusion, we confirmed high expression levels of PPP2R5C previously detected in AML but also in patients with normal cytogenetics. We suggest the importance of the prognostic value of this gene. More studies will be needed to analyse its correlation with nucleophosmin and FLT3-ITD, to understand the signalling pathways affecting its expression and relationship with PP2A inhibitors and substrates in AML. Such studies may encourage therapeutic targeting in combination with conventional chemotherapies or other target therapies in various types of cancers.

Authors' contributions ME supervised and revised the study; RG shared in the practical work and in writing the article; RA provided the AML samples and share in the writing. All the authors approved the submitted version.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of

the NCI (Egypt) research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individuals participating in this research.

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