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EVALUATION OF P14ARF, P27KIP1 AND P21CIP1, CELL CYCLE REGULATORY GENES, EXPRESSION IN ACUTE MYELOID LEUKEMIA PATIENTS

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Abstract – *Objective:* *Acute myeloid leukemia (AML) is an excessive proliferation of immature malignant myeloid progenitors essentially affected by cell cycle abnormalities. Generally, cell cycle is tightly regulated by cyclin, cyclin-dependant kinases (CDKs) and CDK inhibitors (CDKIs). Their defects can result in abnormal proliferation of leukemic cells. Since P14ARF, P27kip1, P21Cip1, and CDKIs, traditionally have a crucial role in cell cycle regulation and they have been reported to be frequently involved in human tumor initiation and progression, we aimed to evaluate the expression of these genes in patients with* de novo *AML.*

Patients and Methods: *In this case-control study, quantitative Real-time PCR was used to rate P14, P27 and P21 expression levels in bone marrow and peripheral blood samples of 93 newly diagnosed AML patients (39 male and 54 female) and 13 healthy people (8 male and 5 female) as the control group. Data were analyzed via SPSS16 software and p<0.05 was designated as the significant level.*

Results: *Our results revealed that P14 and P27 are overexpressed in patients with AML compared to the control group (p<0.05). However, there was no significant change in P21 expression in our patients in comparison with control group. In addition, a significant and positive correlation was observed between expression of these genes in AML patients (p<0.0001, r=0.359, for P14 and P27, p<0.0001, r=0.674 for P14 and P21, p<0.0001, r=0.501, for P27 and P21).*

Conclusions: *Our study indicated P14 and P27 overexpression in AML patients. Although CDKIs down regulation clearly indicates their inability to guard cells against cancer, it is completely difficult to justify their overexpression as it can be a part of normal reaction of cells against malignant process or can indicate their functional switch in favor of malignant process. However, recent studies support the second hypothesis strongly.*

KEYWORDS: *Acute myelogenous leukemia, Cell cycle regulatory genes, P27, P21, P14.*

LIST OF ABBREVIATIONS: *AML: Acute myelogenous leukemia, CDK: Cyclin-Dependant Kinase, CDKI: Cyclin-Dependent Kinase Inhibitor, KIP: Kinase Inhibitor Protein.*

INTRODUCTION

Acute myelogenous leukemia (AML) is a significantly heterogeneous disorder in the cytogenetic and molecular genetic characteristics. This malignancy is identified with abnormal proliferation and differentiation of myeloid stem cells arisen from cell cycle aberrancies[1-7]. Cyclin, cyclin-dependant kinases (CDKs) and CDK inhibitors are molecules that tightly control the cell cycle and govern cell cycle balance[8]. Activation and inactivation of CDK proteins regulate the G1/S and

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G2/mitosis entry[9-13]. CDK inhibitors contribute to cell cycle regulation by cyclin-CDKs inhibition or limitation. Two classes of CDK inhibitors include inhibitors of CDK4 (INK4) and kinase inhibitor proteins (KIPs)[14]. Noticeably, aberrancy existence in P21Cip/WAF1 and P27Kip1 as kinase inhibitor proteins has a crucial role in human tumor origination and progression[15]. One of the most important cyclin-CDK inhibitors is P21 which can affect all cyclin-CDK complexes[15,16]. It has been demonstrated that P21 overexpression inhibits proliferation in mammalian cells[16]. Also, the correlation between P21 and p53 could indicate the P21 importance in human cancers[17]. Additionally, lack of P21Cip1 has been shown to increase the frequency of spontaneous tumors in various tissues in mice. However, increased P21 expression in some gliomas has been shown[18]. P27 has a remarkable homology with P21 and implicates in plenty of malignancies particularly leukemia and its function is context-relevant[19]. P27 expression is a prognostic factor in non-Hodgkin lymphomas and some solid tumors[20,21]. It has been reported that poor outcome in AML and other malignancies arise from low levels of P27 protein[22]. In addition P14ARF, a pivotal CDK inhibitor, plays a role in cell cycle arrest in both G1 and G2/M through stabilization both P53 and MDM2 by directly binding to MDM2[23,24]. In fact, the direct interaction between P14 and mdm2 inhibits degradation of p53 by mdm2 leading to the activation of p53 and its tumor suppressor activities. P14 expression alteration has been demonstrated in some leukemia[25]. Therefore, P21, P27 and P14 are three essential cell cycle regulators with altered expression in different kinds of malignancies such as AML. Therefore, the present study aimed to investigate these essential cell cycle regulators (P21, P27, and P14) in bone marrow aspiration and peripheral blood obtained from newly diagnosed acute myeloid leukemia patients.

PATIENTS AND METHODS

Patients

In the present study, gene expression evaluation results were obtained from 93 bone marrow (BM) and peripheral blood (PB) samples of newly diagnosed AML patients and 13 BM and PB samples of healthy people. Our research received the Ethics Committee approval by our hospital (Code No. IR.SBMU.RETECH.REC.1396.800). Patients were diagnosed at the Taleghani Hospital, Shahid Beheshti University of Medical Sciences (Tehran, Iran) and acute myeloid leukemia was confirmed according to the FAB classification system by morphological assessment and specific immunophenotyping.

RNA Isolation and cDNA Synthesis

The extraction of total cellular RNA from PB and BM samples was performed using RNase Kit (Qiagen, Germany). Subsequently, the Nano Drop (Thermo Scientific, Waltham, MA, USA) was applied to measure RNA integrity. All samples investigated in the present study indicated high purity (OD 260/280 nm ratio >1.8). A final volume of 20 μl cDNA was synthesized from 1 μg of RNA using a cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). An equal amount of patients and normal controls cDNA (50 ng of cDNA) was used in Q-PCR reactions.

Quantitative Real-Time PCR (Q-PCR)

The primer sequences were designed for Real-time PCR via Oligo 7.56 software (Table 1) and their specification was checked through NCBI Blast database. ABL was considered as a housekeeping gene in this study. Utilization of these primers allowed P21, P14, P27, and ABL cDNA to be specifically amplified. Consequently, P21, P14, P27, and ABL mRNA expression in patient and healthy volunteer samples were analyzed by Q-PCR (Rotor-Gene 6000, Bosch, Qiagen, Germany). The components in the Q-PCR reaction for each target consisted of 1 μl of template target cDNA, 1 μl of forward and reverse primers, 7 μl of Real Plus 2x Master Mix Green- Low ROX (Ampliqon, Denmark), and 6 μl water for a total reaction volume of 15 μl. 1:10 serial dilutions of an appropriate cDNA (1, 0.1, 0.01, and 0.001) were provided for standard curve in each Q-PCR reaction. The thermal cycling conditions for each reaction included an initial hold at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Fold change of mRNA expression of patients and normal control samples was obtained according to the Livak method[26].

Statistical analysis

Analysis of the data was implemented using the SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 6.07 software (La Jolla, CA, USA). In SPSS, normal distribution of data was

TABLE 1. Real-time PCR oligonucleotide primers.

Gene		Sequence	TM
P14	P14 F	CCCTCGTGCTGATGCTACTG	60.53
	P14 R	CATCATGACCTGGTCTTCTA	53.86
P21	P21 F	ACTAGGCGGTTGAATGAGAG	57.03
	P21 R	GAGAGGAAAAGGAGAACACG	55.81
P27	P27 F	GGCTTTCAGATTCCCAACTT	55.91
	P27 R	AGCCTCCCCACTCTCGTCT	61.93
ABL	ABL F	AGTCTCAGGATGCAGGTGCT	59.1
	ABL R	TAGGCTGGGGCTTTTTGTAA	60.0

TABLE 2. Specifications of newly diagnosed AML patients.

Study population (N=93)	
Age, y (median, y)	47 (8-85)
Sex (Male/Female)	39 (41.94%)/54(58.06%)
Sample type (Peripheral blood/ Bone marrow)	19 (20.43%)/74(79.57%)
Blast percent, median (range)	69 (20-95)
Immunological classification APL	45 (48.39%)
Non-M3 AML	48 (51.61%)

achieved through log10 computation. The Shapiro-Wilk test was applied to determine data distribution normality. According to Shapiro-Wilk test, we used one-way ANOVA or Kruskal-Wallis for multistate variables and *t*-test or Mann-Whitney U test for two state variables. Pearson test was performed to show correlation. Two tailed $p<0.05$ was considered as significant level.

RESULTS

In this study, peripheral blood and bone marrow specimens were collected from 93 newly diagnosed AML patients that belonged to various gender, age and distinct malignancy morphological features (Table 2). Prevalence of patients in various subtypes according to FAB category was 45 (48.39%) APL and 48 (51.61%) non-M3 AML (including 38 (40.63%) AML M0-M2 and 13 (13.98%) AML M4 and M5).

P14, P21 AND P27 EXPRESSION IN AML PATIENTS AND HEALTHY CONTROLS

P14, P21 and P27 expression levels were analyzed in leukemic patients and control group samples using quantitative Real-time PCR. ABL gene was considered as internal reference gene. In control group, the mean Ct values (±SD) of ABL, P14, P21, and P27 were 30.49±1.64, 28.91±1.96, 27.89±2.07, and 29.30±1.43, respectively. The mean Ct values (±SD) of ABL, P14, P21 and P27 were 28.78±3.44, 26.05±3.62, 25.65±2.40, and 25.88±3.36 respectively, in AML patients. Subsequently, the Ct values obtained from P14, P21, and P27 were normalized against the ABL gene, for both AML patients and control group samples. Statistical analysis revealed a significant difference in P14 and P27 mRNA expression between AML patients and control group ($p = 0.005$ and $p = 0.004$, respectively). The P21 mRNA expression showed no significant alteration ($p = 0.799$). The mean expression level ±SD measured for AML patients and control group was 13.25±0.1.2 and 5.45±1.01 for P14, 12.25±3.1 and 10.87±2.04 for P27 12.28±1.37 and 3.22±1.14 for P21, respectively. Analysis revealed that 17 (18.28%), 29 (31.18%) and 29 (31.18%) of AML pa-

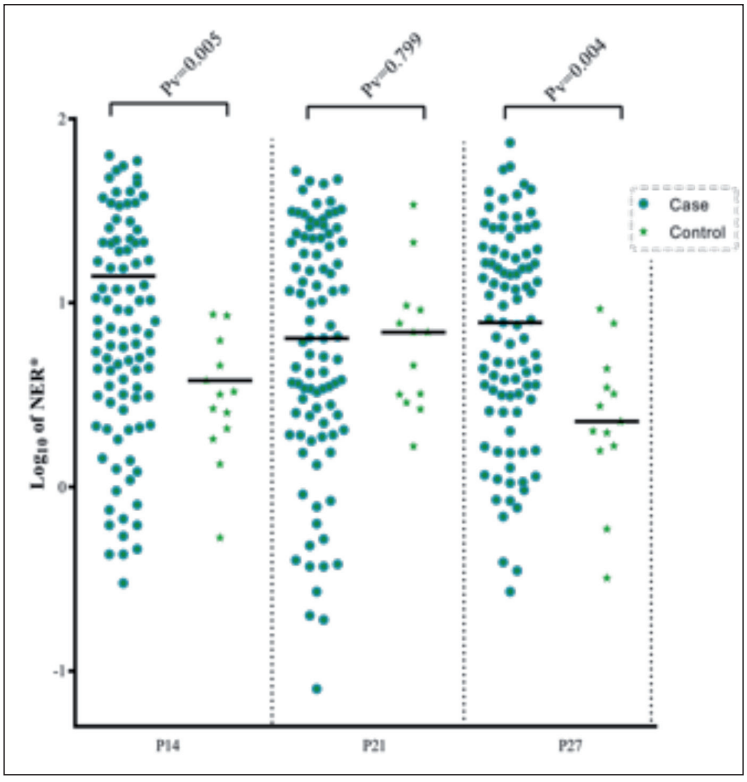

Fig. 1. Quantitative PCR analysis of p14, p21, and p27. Normalized gene expression of p14, p21, and p27 in AML patients and normal subjects has been shown in distinct plots.

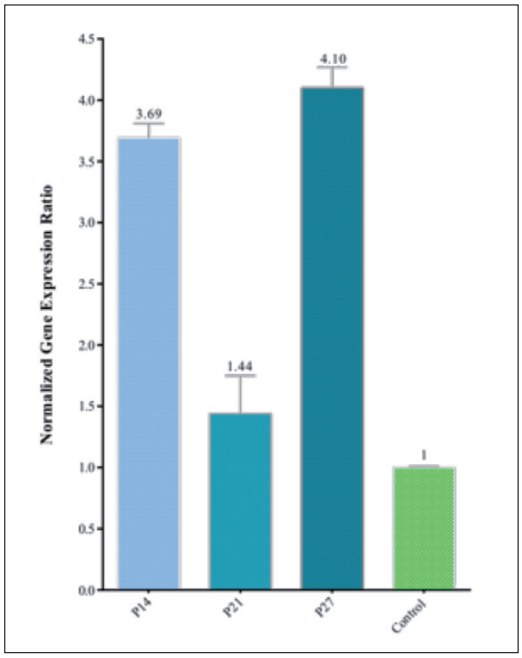

Fig. 2. Normalized gene expression ratio of P14, P21, and P27. Comparison of gene expression in control group with AML patients.

tients carried intermediate P14, P27, and P21 expression, respectively. Low expression levels of P14, P27 and P21 were observed in 22 (23.66), 30 (32.26%) and 17 (18.28%) of AML patients, respectively. In addition, 54 (58.06%), 34 (36.56%), and 47 (50.54%) of AML patients showed high expression levels of P14, P27, and P21, respectively.

Correlation between P14, P27 and P21 expression levels

Pearson test was applied for the analysis of correlation between P14, P27, and P21 as data distribution were normal. The significant positive correlation was revealed between P14, P27 and P21 in AML patients with $p<0.0001$, r=359, for P14 and P27, $p<0.0001$, r=0.674 for P14 and P21, $p<0.0001$, r = 0.501, for P27 and P21. The significant positive correlation was shown between P14 and P27 ($p<0.0001$, r=0.830) and P21 ($p< 0.007$, r=0.709) in control group. However, there was no significant correlation between P27 and P21 gene expression (p=112, r=0.462) in control group (Figure 3)

DISCUSSION

Self-renewal is the only cell fate decision for malignant cells as they have nonstop cell division and resistance to apoptosis and they have also lost their differentiation capacity[27-29]. In hematologic cancers, malignant cells self-renewal will result in their accumulation in bone marrow; subsequently these cells spread throughout the human body [30,31]. This nonstop self-renewal arises from the cell cycle abnormalities[32]. Cell cycle regulators have shown considerable aberrations in their expression patterns in different kinds of cancers including solid tumors and hematologic malignancies[33]. CDKIs (P14, P21, and P27) tightly regulate CDK activation, which ensures the correct timing of CDK activation in different phases of the cell cycle. In normal situation,

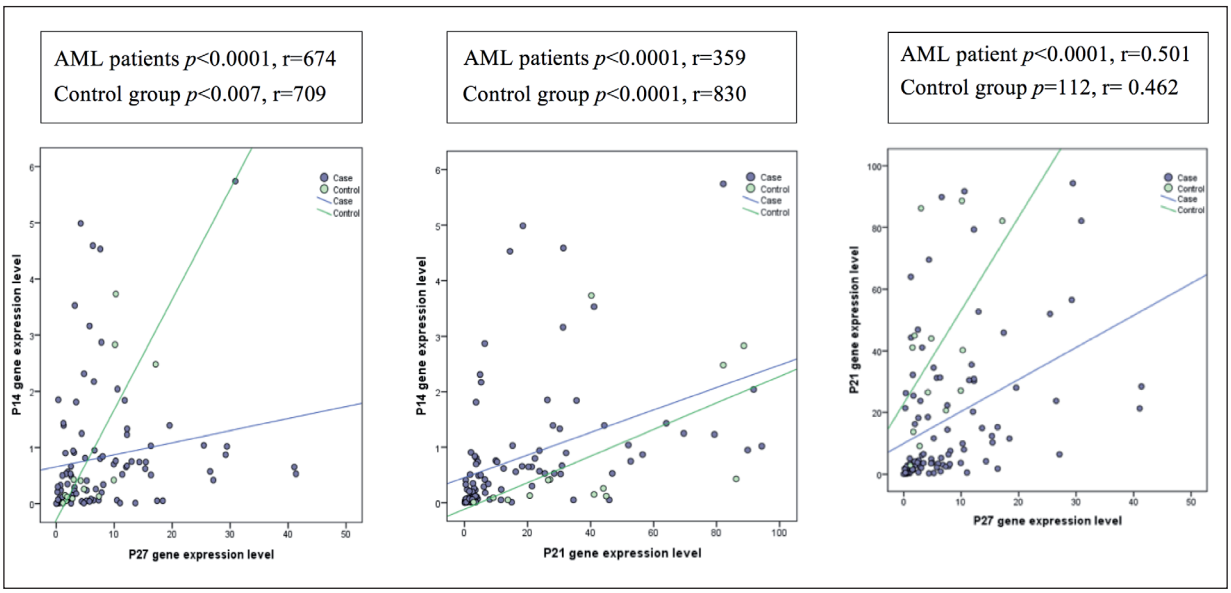

Fig. 3. Significant positive correlation was revealed between p14, p27, and p21 in AML patients. Significant positive correlation was shown between p14 and p27 and p21 in control group. However, there was no significant correlation between p27 and p21 gene expression in control group.

timely cell cycle regulation is tightly dependent on the presence of enough amounts of CDKIs and also their proper localization and function in the nucleus[34,35]. Various studies have clearly shown that CDKIs defects have a fundamental role in cancer and they are early events during tumorigenesis[36]. DNA sequence analysis of CDKIs (P27, P21, and P14) indicated that these proteins are unconventional tumor suppressors, as mutations of these genes have been detected at an extremely low frequency in different kinds of tumors. However, recent studies have indicated deranged function of these genes as CDKIs in those tumors. It has been shown that gene mutation is not the only way of gene function disruption in these cases[37]. In this condition, the main cause for this wide spectrum of gene defects is possibly gene expression deregulation. Since there are insufficient studies to evaluate changes in CDKIs genes expression in acute myeloid leukemia, as a disease with serious and poor outcomes, we examined P14, P21 and P27 expression levels in AML patients. The present study demonstrated significant overexpression of P14 and P27 (3.6 and 4.10 fold change respectively) in AML patients in comparison with normal control group. P14 and P27 overexpression was observed in 66.7% and 76.3% of AML patients, respectively; however, there were no significant changes in P21 expression in these patients. In agreement with our results, Zolota et al[27] used IHC technique to show high levels of P14 and P27 proteins in bone marrow biopsy samples obtained from AML patients. Also, P21 protein was detected in only small parts of their samples. However, this study was not performed at RNA level. In Müller-Tidow et al[38] study on 18 cell cycle regulatory genes, including P14ARF and Radosevic et al[39] study on P21Cip1 and P27Kip1, the P14 and P27 overexpression and the absence of P21 were reported, respectively. Congruent with our study, high expression of P14 and low expression of P21 were demonstrated by Pare et al[40] in invasive ductal carcinomas. Garcia-Rendueles et al[41] in 2017 showed P27 up-regulation in papillary thyroid cancer. In Fanoodi et al[42] study, P21 had no significant expression changes in esophageal cancers. It seems that these CDKIs have a similar role in some malignancies and these tumors induce similar expression patterns of these genes. Brakensiek et al[15] represented hypomethylated promoter of P21 and P27 genes with their overexpression; however, we did not find P21 overexpression. Cabral et al[43] also contradictorily revealed absence of P14 expression in malignant ovarian tumors in comparison with borderline and benign types. Lee et al[44] determined P21 and P27 overexpression in malignant ovarian tissues. These studies have shown expression dysregulation of CDKIs in different tumors with different origination and contexts. According to our results and similar studies, it is possible that P14 and P27 overexpression display tumor suppressor activity through cancer progression and their overexpression is a normal response to cancer development. However, more recent investigations suggest a contradictory role for traditional tumor suppressors. In these situations, these tumor suppressors have altered function and perform tumor promoter activities. They showed that overexpressed form of tumor suppressors such as P14 and P27 have cytoplasmic localization resulting in their inactivation. P27 and P14 after re-localization from nucleus to the cytoplasm may have gained an ability to induce tumor development. Another possible explanation for the CDKIs overexpression in our study is that tumor cells have overexpressed genes that lead to generation of a short form of proteins. It seems that tumor cells remove functional parts of CDKIs which are essential for their function against tumor using alternative splicing. As a tumor suppressor, P21 participates in the cell cycle regulation, gene transcription, DNA repair, and apoptosis. It has been shown that P27 exerts a negative effect on P21 expression[45]. This may be involved in our observation of no significant changes in P21 expression. Although there was no significant alteration in P21 expression in our study, others demonstrated its functions as a tumor suppressor[46,47] or tumor promoter protein[48,49].

CONCLUSIONS

Defining the expression patterns of cell cycle regulators in hematologic malignancies may lead to enhance our understanding about molecular basis of these malignancies. Even though cell cycle regulators have preserved their function as tumor suppressor in some tumors, they have probably gained an ability to promote tumor progression in hematologic malignancies as well[50,51]. Future studies should investigate the epigenetic, post-translational modifications and their relevance in such malignancies.

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Author contribution:
All authors contributed to the design of the research. MAF, PK, FM,NES,MRK and MHM collected the Data. MAF, PK, FM,NES,MRK ,MHM and ZKH conducted analysis and interpretation of data. All authors drafted The first version. MAF, PK, FM,NES,MRK and MHM edited the first draft. All authors reviewed, Commented and approved the final draft.

Conflict of Interest:
The authors declare no conflict of interest.

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