The evaluation of p21 and p27 expression in HLA-DR negative AML patients

Hamideh Aghaee Nezhad (1)
Mohammad Hossein Mohammadi (2)
Mohammadreza Reza Khosravi (3)
Sina Salari (4)
Abbas Hajifathali (5)
Mehdi Allahbakhshian Farsani (6)

(1) MSc in Laboratory Hematology and blood Banking, Laboratory Hematology and blood Banking Department, School of Allied medical sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
(2) (PhD), Assistant Professor in Laboratory Hematology and blood Banking, Laboratory Hematology and Blood Bank Department, Faculty of Paramedical, Shahid Beheshti University of Medical Sciences; HSCT Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
(3) (MD), Assistant Professor of Medical Oncology and Hematology in adults, Cancer prevention research center, Isfahan University of Medical Science, Isfahan, Iran
(4) (MD), Assistant Professor Of Medical Oncology, Hematology and Bone Marrow Transplantation; Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran; HSCT Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
(5) Professor of Medical Hematology and Bone Marrow Transplantation; HSCT Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
(6) (PhD), Assistant Professor in Laboratory Hematology and blood Banking, Laboratory Hematology and Blood Bank Department, Faculty of Paramedical, Shahid Beheshti University of Medical Sciences; HSCT Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding author:
Mehdi Allahbakhshian Farsani,
HSCT Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Tel.: (+98) 21 2271 850 Fax: (+98) 21 2272 115
Email: Allahbakhshian@sbmu.ac.ir

Abstract

AML is a heterogeneous type of leukemia with a high variation in the biological features of the leukemic cells and disease outcomes. The biological features of the leukemic cells have a very close correlation with the disease outcomes. In this way, leukemic cells with higher levels of proliferation and lower levels of maturation undoubtedly would lead to poorer outcomes. Based on immunophenotyping, HLA-DR negative AMLs constitute an important category in AML classification. The majority of patients with this immunophenotype belong to APL subtype with PML-RARA fusion gene and the others are non-APL subtype without PML-RARA fusion gene. As disease outcome and cell biological features have a very close correlation, it is important to evaluate essential molecules involved in the biological processes of the leukemic cells which are helpful in the determination of disease outcome. Therefore in this study we evaluated the expression of p21 and p27 as two key molecules involved in the regulation of cell cycle, proliferation, maturation and apoptosis to determine whether there is any significant difference between these two subgroups of HLA-DR negative AMLs. We studied p21 and p27 mRNA levels by real-time RT-PCR in 41 primary HLA-DR negative AML samples, compared with normal bone marrow and peripheral blood cells. p21 expression was significantly higher in APL cases than non-APLs but there was no significant difference in p27 expression between APL and non-APL patients. According to our results, it seems that p21 can be considered as a critical gene involved in the determination of the levels of cell differentiation between these two subtypes.

Key words: Acute Myeloid Leukemia; HLA-DR negative; p21; p27

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Introduction

Acute myeloid leukemia (AML) is a cancer of hematopoietic system which results from a clonal proliferation of hematopoietic precursors that have lost their maturation capacity to various degrees (1, 2). Patients with AML constitute a highly heterogeneous class of neoplasms because of their variation in the biological features of the malignant cells and disease outcome. Disease outcome is strongly dependent on the biological features of the malignant cells. In this way, leukemic cells with higher levels of proliferation and lower levels of maturation undoubtedly would lead to poorer disease outcomes (3,4). The importance of disease outcome prediction in designing best treatment strategies led to various AML classifications including FAB, WHO 2001 and 2008 and finally the 2016 revision. These classification systems have been introduced mainly based on the leukemic cell features including morphology, immunophenotype and genetic abnormalities (5). Standing on these criteria, human leukocyte antigen-DR (HLA-DR) negative AMLs represent an important category with prognostic significance in most cases. Most patients with HLA-DR negative AML belong to acute promyelocytic leukemia (APL) cases which lack HLA-DR antigen on their surface. However infrequent cases of non-APL AMLs do not express HLA-DR as well. The APL cases have PML-RARA fusion gen as their underlying genetic defect and they represent good prognosis; however, other non-APL cases do not have a common known genetic defect and prognostic significance until now. Thus the biological characteristics of these non-APL HLA-DR negative cases and their outcomes have remained elusive. It has been shown that both subgroups are biologically distinct in some aspects (6). These two categories are also morphologically distinct, as APL cases show higher levels of maturation and differentiation than non-APL cases. Some key molecules involved in the cell cycle regulation, including the CIP/KIP family and the INK4 family, play a pivotal role in the determination of the extent of cell proliferation and differentiation. Their functional inhibition can induce cell proliferation and inhibits differentiation while their activation do vice versa (4). In other words, in leukemic transformation, they are at the center of cell transformation (7). The CIP/KIP family includes p21, p27 and p57 genes. They do their role by binding to and inactivating cyclin-CDK complexes; hence, they halt cell cycle in the G1 phase (8, 9). They directly regulate cell proliferation, maturation and apoptosis. In this way, they have opposite effects on the extent of proliferation and maturation (10,16). Since they induce a delicate and opposite balance between proliferation and differentiation, whenever cells are let go faster through the cell cycle, they proliferate but do not differentiate and thus have leukemic transformation without maturation. Defects in p21 and p27 have been demonstrated in a wide range of human malignancies and usually these defects have value in determination of patient prognosis (17-19). The purpose of this study was to evaluate the expression of p21 and p27, as two key molecules in cell cycle regulation, in HLA-DR negative AML patients. In this study the expression of p21 and p27 genes as two biological targets which are critically involved in the determination of the extent of cell proliferation and maturation was considered to determine whether there is any significant difference in the pattern of these key molecules in HLA-DR negative APL and non-APL cases. Any difference can help us to further sub-classify AML patients based on their leukemic cells biological features which have shown to greatly affect disease outcomes.

Material and Methods

Patients and normal controls:

Bone marrow and peripheral blood samples were obtained after obtaining informed consent from 41 patients who were subsequently diagnosed with HLA-DR negative AML between July 2013- and June 2015 (20 male and 21 female subjects, age median 48 years, range 18-80 years) and from 14 healthy donors that included bone marrow samples from five patients with early stage Hodgkin and non-Hodgkin Lymphoma and peripheral blood samples from eight normal donors. (6 males and 8 females, age median 44 years, range 3-81 years). The diagnosis was established by morphologic examination, cytochemistry staining, flow cytometric analysis and molecular testing. All of the patients were new cases and the mean proportion of leukemia cells (blasts, promyelocytes) was 60±2.0 (mean±S.E.M) in BM and PB samples, which were enriched by Ficoll density gradient centrifugation of sample.

Immunofluorescence staining and flow cytometry:

Flow cytometric analysis was performed through mixing between 30-50 Micro L of BM or PB sample mix with 5 Micro L of monoclonal anti bodies including HLA-DR, CD3, CD13, CD33, CD14, CD64, CD11b and CD45 Labeled with PE/FITC (all of them DAKO Ab, DAKO company, Denmark) and they were incubated for 20 minutes at 2-4ºC. The sample was prepared with RBC lysis buffer (DAKO-, Denmark) and analyzed by flow cytometery instrument (Beckman-coulter XL, USA). Threshold for confirmation of marker positive was more than or equal to 20% of the cells.

RNA isolation, c-DNA synthesis, real-time PCR

For the isolation of mononuclear cells from peripheral blood and bone marrow of both patient and control samples; Ficoll density centrifugation was used according to the manufacturer's instructions. Then for total RNA extraction QIAmp RNA blood extraction kit was used and total RNA was extracted using the manufacturer's instructions. We determined the quantity and purity of total RNA by Nano drop (Thermo fisher USA). All samples had an OD 260/280 nm ratio of 1.8-2.0, indicating their high purity. The integrity of RNA was confirmed by agarose gel electrophoresis.
One µg of RNA was transcribed into first-strand c-DNA using random hexamer primer and First Strand c-DNA Synthesis Kit (Thermo-scientific, USA.) in a 20 µl reaction, under standard conditions.

The expression level of p21, p27 and ABL as reference gene was determined with SYBR Green I real-time PCR. An aliquot of 1/10th of the resulting c-DNA was used for

quantitative PCR amplification. Real-time PCR amplification was performed with Rotor-gene 6000. The master mix was prepared in 15µl mix containing 1.5µl cDNA samples, 7.5µL of 2.5× SYBR Green mix (Ampliqon, Holland), 1.2 µl 10 pmol forward and reverse primer and 4.8 µl water. The thermal cycling conditions included 10 minutes at 95 ˚C followed by 40 cycles of denaturation for 15 s at 95˚C, annealing at 62˚C for 15 s and final extension at 72 ˚C for 20 s. To check the PCR mix for any kind of contamination, we took a negative control (2 ml water instead of c-DNA). Additionally, the specific amplification of the PCR products was analyzed by melting curve analysis and agarose gel electrophoresis.

The Standard curve was produced by four continuous 1:10 dilutions of a positive sample, for each PCR. Using the following calculation: the relative rate of mRNA in the samples was counted as the percentage of ABL gene.

Statistical analysis:
Skewness, kurtosis and Kolmogorov-Smirnov tests were applied for normality. The distribution of relative expression of p21 and p27 in control normal samples and HLA DR-negative AML samples was normal. Differential p21 and p27 expression between the two groups was evaluated using t-test. Differential distribution of p21 and p27 expression in HLA DR- negative AML according to FAB classification was assessed using the Kruskal-Wallis test. Data are presented as mean ± standard error of the mean (SEM). Spearman's rank correlation analysis was used to analyze the p21and p27 mRNA levels in different samples and evaluate correlation with age and blast count using the SPSS 11 statistical and GhraphPad Prism5 software. Differences were considered statistically significant at $p < 0.05$.

Results

Patient's characteristics
HLA-DR negative AML cases were divided into two subgroups, APL and non-APL based on immunophenotyping, cytochemistry, morphology and molecular testing for PML/RARα fusion gene. Thirty APL and eleven non-APL cases were diagnosed in this study. According to FAB classification, non-APL cases were M1 and M2.

p21 and p27 expression:
The expression of the p21 and p27 was evaluated in HLA-DR negative patients (n=41) in comparison with healthy control samples (n=14). In this regard, p21 expression was significantly lower in HLA-DR negative patients (mean p21in patients: 1.00±0.13) compared with normal controls (mean p21 in controls: 1.44± 0.14) ($p<0.05$, Figure 1).

But p27 expression levels did not show significant difference between patients (mean p27 in patients: 0.82±0.12) and control groups (mean p27 in controls: 0.63±0.10) ($p> 0.05$, Figure 2).

After that, we evaluated the expression levels of p21 and p27 between HLA-DR negative APL versus HLA-DR negative non-APL patients. P21 expression levels were significantly higher in APL cases compared with non APL cases (mean p21 in APL: 1.17±0.15 vs non-APL: 0.55±0.20, $p<0.05$, Figure: 3A). But it was not significant for p27 expression (mean p27 in APL: 0.82±0.15 vs non-APL: 0.82±0.20, $p>0.05$, Figure 3B).

p21 and p27 expression according to FAB classification:
The expression levels of p21 and P27 were not significantly related to sex and age in patients and control group. We did not see any difference between groups with different blast percentage. As we evaluated a variety of FAB subtypes including M1, M2 (known as non-APL) and M3 (known as APL) in HLA-DR negative AML subtypes, fluctuation in p21 and p27 expression levels was observed subjectively; but it was not statistically significant ($p>0.05$, Figure 4A and 4B - page 256).

In particular, although the p21 and p27 did not demonstrate significant difference between different subtypes of FAB (M1, M2, and M3) in our patients, we can see variable expression in p27 and trend to increase in p21 expression from M1 to M3, respectively.

Discussion

The classification of AML attempts to identify biologic entities of leukemic cells in the hope that future work will clarify molecular basis of the disease; this might help us to employ targeted therapies. Now evidence strongly suggests that AMLs consist of a group of relatively well-defined hematopoietic neoplasms affecting hematopoietic precursors and they should not be considered as a uniform kind of disease any more. Therefore these neoplasms should be divided into separated entities based on their unique biological characteristics. To date literature has classified AML patients as HLA-DR positive and negative subtypes based on their leukemic cells immunophenotype. Although the majority of HLA-DR negative cases have PML-RARA fusion gene (APL cases) as their underlying genetic defect, others do not have such a certain genetic lesion (non APL cases). Because of this, they are completely separated entities. As a general rule, APL cases enjoy a better prognosis due to their higher levels of cell maturation while non-APL cases mostly exhibit lower levels of cell maturation, so they have poorer prognosis and outcome. As we know, cell differentiation occurs in parallel with proliferation arrest which is mediated by up regulation of cell cycle regulators particularly cyclin-dependent kinase inhibitors. In the present study, the investigation of any significant difference in p21 and p27 expression (as two keys cyclin-dependent kinase inhibitor) was undertaken in a number of experimental steps in HLA-DR negative AMLs in comparison with normal controls. Firstly, we aimed to characterize the nature of p21 and p27 in HLA-DR negative AMLs and secondly we decided to determine the relationship between HLA-DR negative/APL and HLA-DR negative/non APL cases with p21 and p27 expression. Any significant difference in p21 and p27 expression can possibly somewhat justify different extent of cell maturation/differentiation in our evaluated patient subtypes. It helps us to more differentiate these

Figure 1: comparison between patients and control, p21 expression

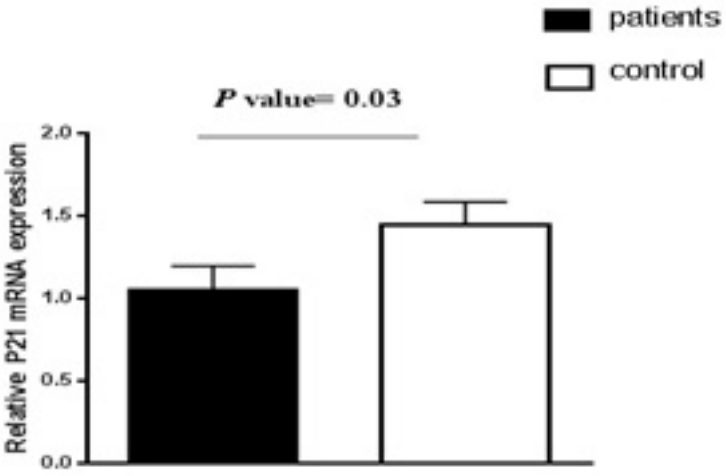

Figure 2: comparison between patients and control, p27 expression

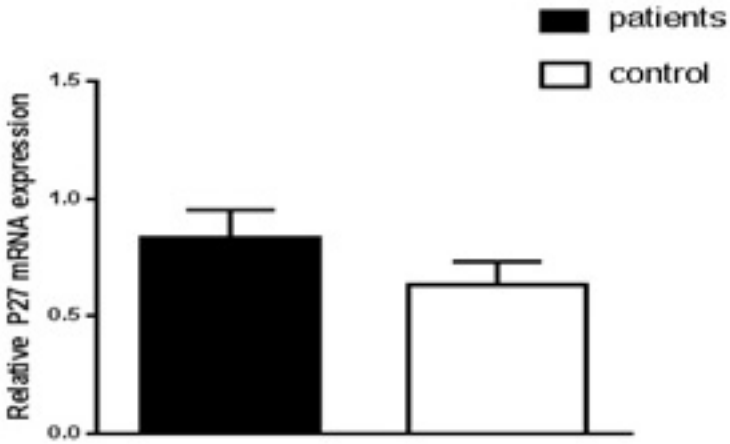

Figure 3: comparison between HLA-DR negative APL and HLA-DR negative non-APL, p21 (A) and p27 (B) expression

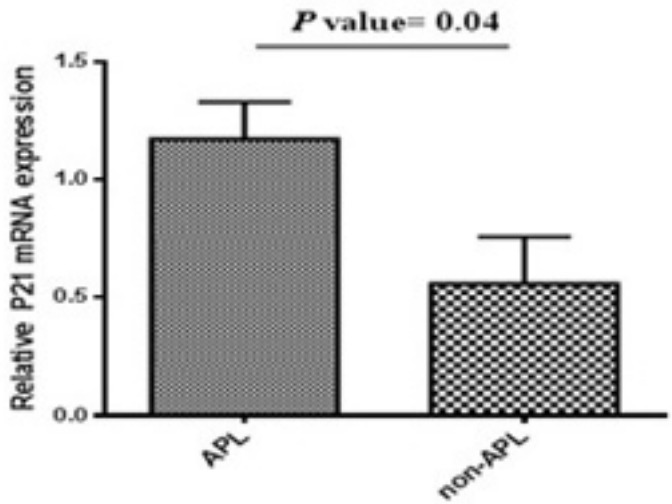

Figure 3A

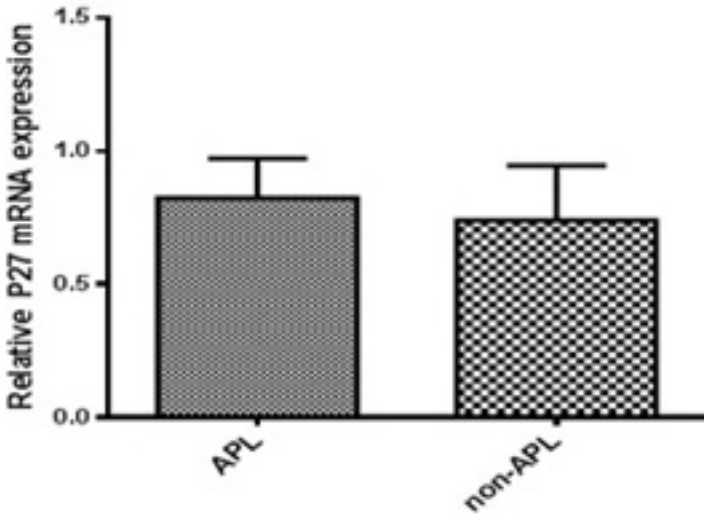

Figure 3B

Figure 4: gene expression in according to FAB classification, p21 expression (A), p27 expression (B)

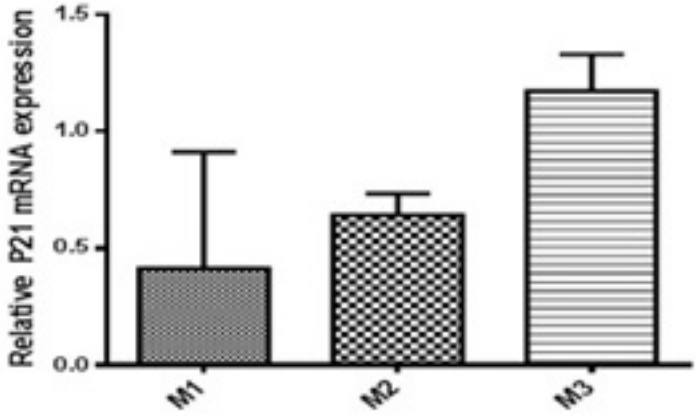

Figure 4A

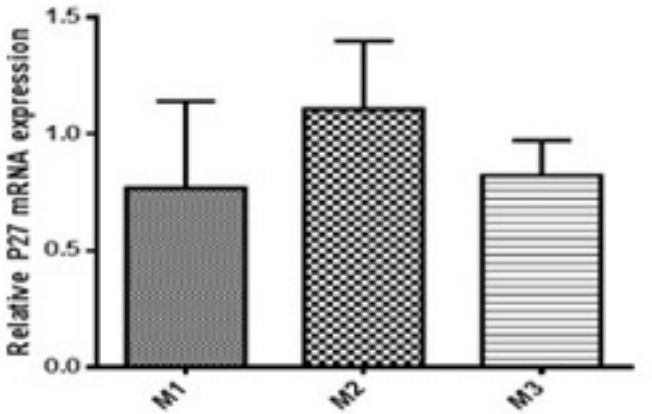

Figure 4B

subtypes based on their molecular features. P21 and p27 expression levels were measured in HLA-DR negative AML patients and compared with a normal control group. It was found that p21 but not p27 is markedly decreased in HLA-DR negative AML patients compared with normal controls (mean p21: 1.00±0.13 vs 1.44± 0.14, $p<0.05$) and it increased in HLA-DR negative/APL cases compared with HLA-DR negative non-APL cases (mean APL: 1.17±0.15 vs non-APL: 0.55±0.20, $p<0.05$). It strongly suggests that p21, but not p27, has a role in leukemic cells differentiation in AML. In contrast, p27 expression did not show any significant difference between HLA-DR negative AML patients (mean p27: 0.82±0.12) and normal controls (mean p27: 0.63±0.10, $p>0.05$). Also it was not expressed significantly different between HLA-DR negative/APL cases and HLA-DR negative/non APL cases (mean APL: 0.82±0.15 vs non-APL: 0.82±0.20, $p>0.05$. In this study we also detected a trend in p21 expression from a lower level of expression in M1 to a higher level of expression in M2 and M3 respectively, although it was not statistically significant. This pattern of increase in gene expression was compatible with the increase in the maturation levels through the FAB classification from M1 to M3 and indicates the possible role of p21 in myeloid differentiation.

In agreement with these findings, Taniguchi and colleagues in 1999 demonstrated that p21 mRNA expression increases in all myeloid colonies (granulocytes, macrophages, megakaryocytes, and erythroblasts) during differentiation of normal hematopoietic stem cells (HSCs), but they could not show increase in p27 mRNA expression except for erythroid bursts (20). In addition, Yaroslavskiy and colleagues in 1999 also studied normal CD34+ HSCs which were motivated to differentiate through the myeloid lineage. They found that the p27 but not p21 was expressed in freshly harvested resting CD34+ cells. During differentiation, p21 levels peak synchronized with cellular proliferation and then drop in expression when cells undergo terminal differentiation but p27 expression was steady (21). Moreover, Weng-Lang Yang and colleagues in 1999 in their study concluded that APL cells treated with retinoic acid (RA) show p21 transcription up regulation. They suggested that APL cells differentiation induction using RA may be due to P21 activation (22). Steinman and colleagues in 1998 have shown that P21 was elevated in myeloid maturation of CD34+ precursor cells and this increase was associated with decreased binding to a highly conserved 44-bp fragment within the p21 promoter (23). However other studies somewhat contradict the aforementioned observations as they observed both P21 and P27 are mainly involved in cellular differentiation. In one study conducted by Muto and colleagues in 1999, they reported that the induction of RA-resistant UF-1 cells by 1,25(OH)2D3 toward granulocytes differentiation was associated with the G1 arrest of the cell cycle and an increase in the expression of the both cdk inhibitors p21 and p27 (24). Another study by Jean Q. Tian and colleagues in 1999, observed that p21 and p27 are strongly overexpressed during intestinal epithelial cells differentiation but with different kinetics: p21 induction was rapid and transient while the p27 induction was delayed and sustained (25). Overall our results and these recent studies clearly indicate that p21 may be strongly involved in the myeloid differentiation of hematopoietic precursor cells. It also can be concluded that the levels of P21 expression may be an important molecular difference between HLA-DR negative/APL cases and DR negative/non APL cases. It might be concluded that P21 induction in HLA-DR negative AMLs leukemic cells can induce cell differentiation and malignancy resolution. Although we expected that like p21, p27 expression be lower in leukemic cells in comparison with normal controls, the level of p27 in HLA-DR negative AML cases did not show significant difference in comparison with normal controls. These findings demonstrate that more studies may be needed to be done to clarify the reason for this controversy about P27 and why p27 expression does not show any significant changes in HLA-DR negative AMLs. Although some studies demonstrated that P27 expression has a differentiation role, they were not in myeloid cell differentiation or they have not been performed on AML patients. These findings suggest that p21 and p27 may have distinct functions in controlling cell differentiation and maturation in myeloid cells and these two highly related proteins perform unique biological activities during myeloid differentiation and cannot simply substitute for each other to facilitate hematopoietic differentiation. Altogether, according to obtained results in this study it seems that p21 is a more critical factor in differentiation and maturation of DR-negative AMLs in comparison with p27 and it can be one of the discriminating factors between

these two closely related groups of DR-negative AMLs. However, there is controversy about the behavior of p21 in malignancies. Some observations have demonstrated that p21 have opposing functions in some kind of malignancies in a tissue-type dependent manner or localization menner (whether it has nuclear or cytoplasmic localization). For example, p21 can be both pro- and anti-apoptotic, and can both promote and inhibit differentiation and transcription (26). As we did not observe increase in p21 or p27 expression, we could not suppose oncogenic or tumor promoting role for p21 or p27 in our study.

Conclusion

The aim of this study was to evaluate the p21 and p27 expression in HLA-DR negative AMLs (APL and non-APL) to determine whether there is any difference in the pattern of expression of these two critical factors in myeloid differentiation. The main conclusion to be drawn from this study was that p21 expression in this category of AML was higher in APL cases with more maturation levels versus non-APL cases with lower levels of maturation. But there was no significant difference in p27 expression. It was supposed that p21 might be determinant factor that differentiates HLA-DR negative APL cases from non APL cases in comparison with p27. Considering the significant role of cell cycle regulatory proteins in differentiation and maturation of hematopoietic cells and according to the acquired results in this study, we suggest that further experimental investigations should be done to determine whether other critical cell cycle regulatory proteins such as p14, p16, p53 and c-myc have difference in their expression pattern between these two categories (27-29).

Conclusion

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