Changes of AML 1 and P53 tumor suppressor gene expression in patients de novo acute myeloid leukemia

Fatemeh Salarpour[1], Kourosh Goudarzipour[2], Ahmad Ahmadzadeh[3], Mohammad Hossein Mohammadi[4], Mehdi Allahbakhshian Farsani[5,*]

[1] Laboratory Hematology and Blood Banking Department, School of Allied Medical Sciences, Shahid Beheshti University of Medical Science, Tehran, Iran.
[2] Pediatric Congenital hematologic Disorders Research Center, Shahid Beheshti University of Medical Science, Tehran, Iran.
[3] Health Research Institute, Research Center of Thalassemia & Hemoglobinopathy, Ahvaz jundishapur University of Medical Science, Ahvaz. Iran.
[4] Laboratory Hematology and Blood Banking Department, School of Allied medical Sciences, Shahid Beheshti University of Medical science, Tehran, Iran.
[5] Laboratory Hematology and Blood Banking Department, School of Allied medical Sciences, Shahid Beheshti University of Medical Science, Tehran, Iran.

*Corresponding Author: email address: allahbakhshian@sbmu.ac.ir (M. Allahbakhshian)

ABSTRACT

P53 and AML1are two important tumor suppressor genes in regulation of hematopoiesis with a critical role in keeping balance between proliferation and differentiation. Alternations in the expression of these genes can be resulted in malignancy. The present study investigated the expression levels of P53 and AML1 genes in 82 de novo AML patient specimens against 12 normal control group using Real-Time-PCR. The results presented in this study revealed that AML1 gene expression was significantly higher and P53 gene expression levels was significantly lower in patients with AML in comparison with the normal control group (P = 0.016 and P = 0.002). Furthermore, the correlation between P53 and AML1 was significant and positive (P= 0.037 and r= 0.231). The lower levels of P53 expression were expected and in line with the normal role of this gene as a tumor suppressor gene, however AML1 over expression was in contrast with of its well-known role in myeloid maturation. However, this findings suggest that despite the current established role this genes in myeloid cell differentiation, oncogenic form of AML1 (AML1a) has possibly increased and high expression of this isoform may act as an inhibitor for other normal AML1 isoforms and P53 as well.

Key words: Acute myeloid leukemia (AML); P53; AML1; oncogene; malignancy

INTRUDOCTION

Acute myeloid leukemia (AML) forms due to genetic abnormalities within the normal hematopoietic cells. These genetic abnormalities affect the expression pattern of various key genes involved in the regulation of cell behavior. These affected genes are belonged to transcription factors, cell signaling molecules or cell cycle regulation proteins. They generally act as tumor suppressor or proto-oncogene [1, 2]. The tumor suppressors mainly perform their role by participation in cell differentiation and repair. Among tumor suppressor genes, P53 is one of the most important. P53 induces different molecules involved in cell cycle check points, apoptosis, autophagy, differentiation and Senescence thereby it finally regulates DNA damage response. Various P53 gene abnormalities including deletion, insertion, point mutations and epigenetic changes happens in the vast spectrum of human cancers with high frequency [3-7]. This high rate of abnormalities in cancer is possibly due to central role of P53 in association with lots of other cell regulatory proteins.

Acute Myeloid Leukemia 1/Runt Related Transcription Factor 1(AML1/RUNX1) is an essential gene in prevention of myeloid malignancies by induction of genes involved in myeloid differentiation [8-10]. AML1 is belonging to the Runt-related transcription factor

(RUNX) family of transcription factors. AML1 is involved in normal hematopiesis and it has a vital role in the regulation of hematopoietic cell differentiation. AML1 disruption has been reported with high frequency in AML patients [11]. AML1 gene produces at least three isoforms, AML1a, AML1b and AML1c. AML1a isoform, in contrast with AML1b and c isoform, lacks C terminal part which has transactivation activity. Pervious studied indicated overexpression of AML1a isoform in mouse can be resulted in the development AML. This isoform lacks normal function of AML1 and interferes with the function of AML1b/1c; therefore its high level leads to hematopoietic abnormalities. AML1 isoforms transcription occured using two different promoters. AML1c transcription, as the longest isoform, occurs from distal promoter while other isoforms are expressed from proximal promoter. In leukemia, this transcription factor is disturbed repeatedly [12-14].

Various studies indicated the functional link between AML1 and P53. P53 inhibits AML1 expression through sitting in the promoter of AML1, AML1 overexpression has been observed in cell under stress situation or when P53 has low levels in some conditions such as lymphomas **[**3, 4, 15, 16].

In present study, we supposed that AML is originated due to a lack of balance between proliferation and differentiation. Several cellular changes should occur to be resulted in malignancy no cellular change alone sufficient for formation of malignancy [17, 18]. In the present study, we showed that AML1 had significantly higher levels of expression in patients with AML compared to healthy group, which was unrespectable, while P53 had lower levels of expression which was expectable. Our results suggest that in AML patients the lower levels of P53 probably lacks its inhibitory effects on AML expression. This can explain why AML1 has unexpectedly overexressed in AML patients.

MATERIAL AND METHODS

Patients and healthy volunteers

Twelve bone marrow (BM) and peripheral blood (PB) samples of healthy volunteers and 82 BM and PB samples of de novo AML patients(the new case patients without any treatment) were obtained between 2012-2014. The sample size was determined based on previous literature review. The patients were referred to Emam Khomeini and Mofid hospitals, Tehran, Iran and they received informed consent. Our patients were 45 male and 37 female and they were in the range of 2 to 87 years, with mean age of 44.6 years. in this study, The prevalence of various morphological subtypes of FAB/WHO was: 9 cases of M0, 18 cases of M1, 12 cases of M2, 27 cases of M3, 10 cases of M4 and 6 cases of M5 (there were no cases of M7and M6 in this study).

RNA isolation, cDNA synthesis, real-time PCR

RNA was extracted using RNasey Kit (Qiagen,Germany) from BM and PB was extract. The samples had High purity (OD 260/280 nm ratio >1.8), as it was evaluated by Nanodrop (Thermo Scientific, USA). Consequently, cDNA was synthesized from 2µL (0.5mg) of RNA in a final volume of 20µL using a Thermo Scientific kit (USA). An aliquot of 1/10th (1µL of cDNA / 9 of water) was used as substrate for qRT-PCR amplification (Rotor Gene 6000, Qiagene. In our study, primers were designed using Oligo 7.56 software and NCBI-Blast database. Primers sequences of AML1, P53 and ABL (housekeeping gene for normalization) genes were demonstrated in Table 1.

Table 1. Nucleotide sequences of primers used for ABL,AML1 and P53 qRT-PCR reactions

Gene	Forward	Reverse
ABL	AGTCTCAGGATGCAGGTGCT	TAGGCTGGGGCTTTTTGTAA
AML1	ATGGCACTCTGGTCACTG	TTAAATCTTGCAACCTGG
P53	ACCGGCGCACAGAGGAAGAGAA	TGGGGAGAGGAGCTGGTGTTGT

The total reaction (volume of 15µl) components in qRT-PCR reaction consisted of, 1µL forward and reverse primer, 6µL water, 7µL of RealQ Plus 2x Master Mix Green- Low ROX (Ampliqon, Denmark) and 1µL of template cDNA. a standard curve was produced For each

qRT-PCR reaction with using five consecutive 1:10 dilutions of a positive sample (1, 0.1, 0.01 and 0.001). The thermal cycling conditions for each reaction (AML1, P53 and ABL) were evaluated (Table 2).

Table 2. The Program time and temperature of Real time PCR for target genes and reference gene

Primers	initial hold	denaturation	annealing/extension	final extension
ABL	95°C for 10 minutes	95°C for 10 seconds	62°C for 15 seconds	72°Cfor10minutes
AML1		95°C for 15 seconds	59°C for 15 seconds	
P53			62°C for 15 seconds	
	1 cycles	40 cycles		

The assays were performed in duplicate and negative controls were included. the Livak method ($2^{-\Delta\Delta ct}$) used for calculation of the relative quantification of mRNA expression for each sample (fold change=FQ)(19, 20).

Statistical analysis

The SPSS Statistics 16.0 and GraphPad Prism 6.07 software were used for data analysis. For evaluation of normal distribution of data, both the Shapiro-Wilk and the Kolmogorov-Smirnov tests were used. The Mann-Whitney U and ANOVA tests determined whether there is a significant difference between patients and normal control groups for AML1 and P53 gene expression. These tests were also used to evaluate differential expression of genes based on patient/disease characteristics. The Spearman's chi-squared test was used to measure the linear correlation between P53 and AML1 expression. A P value of 0.05 or less was considered as a significant difference (the results are expressed as mean ± standard error of the mean (SEM).

RESULTS

AML1 and P53 expression in AML and healthy patients

The expression level of P53 and AML1 was analyzed in AML samples using real-time PCR. The amplification efficiency of reference and target genes was obtained; it was approximately equal indicating the validity of the DDCt method. The mean expression levels detected for P53 and AML1 were 1.17 ±0.38 and 40.08 ±9.63 in AML patients and the normal control respectively. The normal expression levels of p53 and AML1 was 0.44-3.74 fold change and 0.72-3.65 fold change, respectively which was defined as 95% confidence interval range in healthy normal control population. According to this reference level, AML patients whose P53 and AML1 expression was within the normal range were considered as intermediate expression (18.3 % and 35.4%, respectively) while those with levels above the threshold of the intermediate were defined as high expression levels (3.775-423.631 for AML1 and 4.191-22.588 for P53 that was included 50% and 7.3% of AML patients respectively). Patients with expression levels under the reference range of normal controls were defined as LOW expression cases (0.0039-0.437 for P53 and 0.161-0.681(including 74.4% and 14.6% of AML patients, respectively). Statistical analysis revealed a significant difference ($P < 0.0001$) between both P53 and AML1 mRNA expression levels between normal healthy controls and AML patients (Figure 1, A and B). The majority of patients had high expression levels for AML1 and low expression levels for P53.

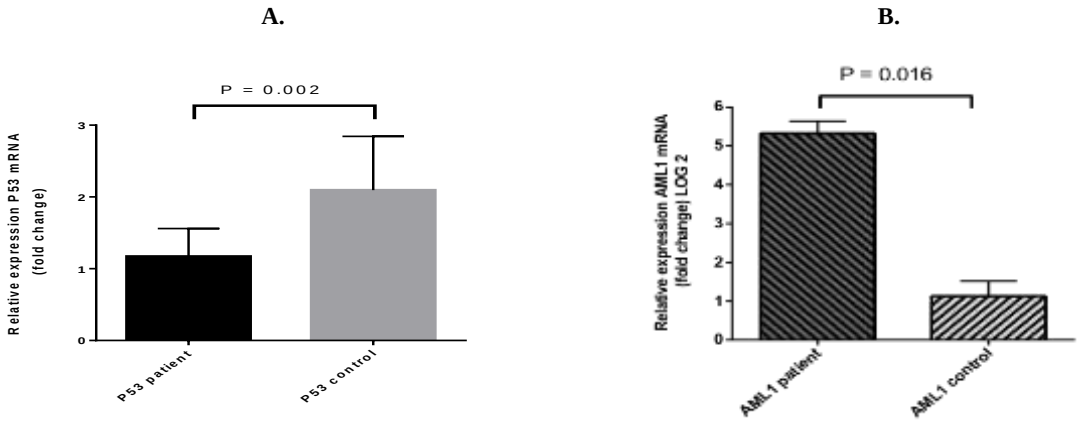

Figure 1. Relative expression of P53 and AML1 in 82 AML patients and 12 healthy patients. **A)** A significant difference (P= 0.002) between P53 expression in AML patients and healthy patients was identified. A relative P53 expression level of 1.17 ±0.38 (SD) was measured in AML patients in comparison to 2.09 ± 0.74 (SD) in the normal control group. **B)** A significant difference (P = 0.016) between AML1 expression in AML patients and healthy patients was also identified. A relative AML1 expression level of 40.08 ±9.63 (SD) was measured in AML patients in comparison to 2.19 ± 0.66 (SD) in the normal control group. AML1 expression levels exhibited a higher fold change in comparison to P53 (15.26 versus 5.46).

Correlation between P53 and AML1 expression levels

Spearman analysis was applied to identify any correlation between P53 and AML1 expression in AML patients. Our analysis demonstrated that these two genes have positive and significant correlation with each other (P= 0.037 and r= 0.231) in AML patients which suggest correlation between their expression. In comparison, correlation analysis between these genes in healthy control samples showed that there is no correlation between P53 and AML1(R: 0.098, P: 0.762) in the normal population (Figure 2, A and B).

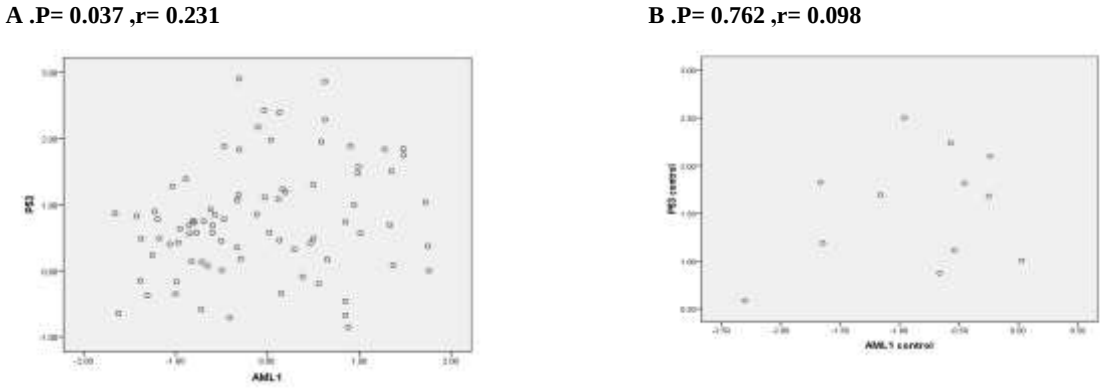

Figure 2. Statistical analysis by means of Spearman's chi-squared test reveals dependence and relation between the expression of P53 and AML1. **A)** Correlation between P53 and AML1 in 82 AML patients was determined to be positive and significant (P= 0.037, r= 0.231). **B)** Correlation between P53 and AML1 in 12 healthy patients was not determined (P= 0.762, r=0.098).

Differential expression of P53 and AML1 in AML FAB subgroup

ANOVA test was used for evaluation of P53 and AML1 differential expression between different FAB subtypes of AML patients. Statistically, there wasn't significant difference between various FAB subtypes for P53 and AML1 mRNA expression. Both of the lowest expression(0.58 ± 0.20) and highest expression (2.31 ± 1.10), levels of P53 (± SEM) was seen in M0/M1/M2 . In this regards, AML1 had lowest expression in M4/M5 (32.10 ± 10.80) and highest expression in M0/M1/M2 (47.52 ± 16.49) subtypes. However, in comparison to the normal control group, these subgroups showed significantly higher expression in AML patients than normal groups (in M0/M1/M2: P = 0.039, M3: P = 0.024 andM4/M5: P=0.023) in AML1 and lower expression in AML than control group (in M0/M1/M2: P = 0.011 and M4/M5 : P=0.020) and higher expression in AML than control group (in M3: P = 0.027) in P53 (Figure 3, A and B).

A. **B.**

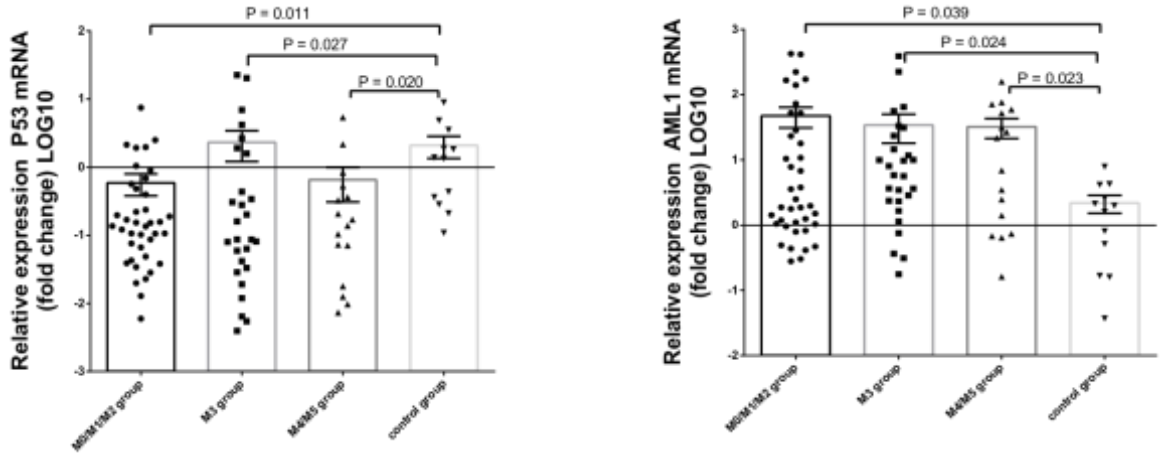

Figure 3. The relative expression of P53 and AML1 measured in 82 AML patients in their FAB subgroup and analysis by means of the ANOVA test determines that there is no significant difference in expression between these subgroups. **A)** The lowest relative expression of P53 was evaluated at 0.58 ± 0.20 (SEM) in the M0/M1/M2 subgroup and the highest at 2.31 ± 1.10 (SEM) in the M3 subgroup. The M0/M1/M2 , M4/M5 subgroups have significant low expression and M3 subgroup has high expression in comparison to the normal control group (P = 0.011, P=0.020 and P = 0.027,respectively) but there is no significant difference in P53 expression between these subgroups. **B)** The lowest relative expression of AML1 was measured at 32.10 ± 10.80 (SEM) in the M4/M5 subgroup and the highest at 47.52 ± 16.49 (SEM) in the M0/M1/M2 subgroup. The M0/M1/M2 , M3 and M4/M5 subgroups have significant over expression in comparison to the normal control group ((P = 0.039, P = 0.024 and P=0.023) but similarly to P53 expression there is no observed correlation between these subgroups.

DISCUSSION

Inactivation of tumor suppressors plays a major role in the molecular pathogenesis of leukemia; P53 and AML1 are two important tumor suppressor genes in the regulation of hematopoiesis. Various studies indicated the functional link between P53 and AML1. Different studies showed functional link between P53 and AML1 genes. AML1 is involved in increased acetylation of P53 by P300, it increases transcriptional activity of P53 and inhibits the proliferation and increases apoptosis [3, 4, 16]. In this study, we found that AML1 and P53 as two important tumor suppressor genes, which are involved in the proliferation prevention and differentiation induction. They have impaired pattern of expression in AML patients. We showed a significant decrease in the expression of P53 gene and significant and unexpected increased in the expression of AML1 gene in AML patients in comparison with healthy normal controls. The observed increase in the level of AML1 expression was in the controversy with its well-known role in the differentiation induction. Previous reports have shown such unexpected increase in other genes involved in the tumor formation prevention such as WT1[21]. A positive and intermediate correlation was

observed between these two genes in our studied AML patients.

These studies showed that this unexpected high expression level of AML1 may be part of malignant process in AML patients; however the underlying mechanism is unknown. One possible explanation can be related to different isoforms of AML1 including AML1a, b and c. Isoform a lacks the trans activating domain in its c-terminal part which is essential in its differentiation induction function but the other isoforms have this functional domain. Different studies have shown that high expression levels of AML1a isoform can be involved in the development of malignancy in human and mice. AML1a contributes in leukomogenesis process in several children leukemia, mix lineage leukemia and T-cell lymphomas as an oncogene. AML1a acts as a negative regulator for other isoforms including b and c. Since the c terminal domain is essential for the normal function of AML1, high levels of AML1a expression without its functional domain may guide the normal hematopoiesis to malignant form. On the other hand, in some situations isoform a can also increase the number of early hematopoietic cells and may block primitive cells differentiation, by interference with the AML1b / c isoforms. This oncogenic isoform inhibit normal isoforms function by binding to their target genes promoters. These studies are in agreement with our observation of high levels of AML1 expression and its possible role as oncogenic protein [10, 12, 13, 22]. According to these evidences, this pattern of AML1 expression in AML patients can be justified with the expression of isoform a in malignant process. One of the possible explanations of underlying mechanism of AML1 overexpression can be related to lower levels of P53 in AML patients as we observed this finding in our patients. In the other hand, because of the oncogenic isoform without function domain, is able to connect to the target genes of AML1, it can be act as blocker. So, in situation with increased of this isoform, we expect to decreased in acetylation and activation of P53 in AML patients. Since the studied indicated that P53acts as a critical tumor suppressor gene trough several mechanisms including cell cycle arrest and increased apoptosis in genetically damaged cell, it is not unrespectable that reduced P53 expression due to mutations or epigenetic modifications can be involved in malignancy process [7, 23, 24]. In agreement with our findings, previous studies have shown that P53 gene expression reduced due to mutations in patients with myelodysplastic syndrome and acute myeloid leukemia, which was in agreement with our results in AML patients [25].

CONCLUSION

Together with these findings, probably oncogenic form of AML1 has increased. High expression of this isoform act as an inhibitor for other normal isoforms. It is suggested that future studies to investigate changes in the expression of these isoforms of AML1 along with P53 protein levels in AML patients for investigate the relationship between this oncogenic isoforms with reducing P53.

ACKNOWLEDGMENTS

This work was supported in part by Shahid Beheshti University of Medical Science, Tehran, Iran and Department of Medical Research.

"The authors declare no conflict of interest"

REFERENCES

1.Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, et al. Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). Blood. 2002 Oct 15;100(8):2717-23. PubMed PMID: 12351377. Epub 2002/09/28. eng.

2.D'Alo F, Di Ruscio A, Guidi F, Fabiani E, Greco M, Rumi C, et al. PU.1 and CEBPA expression in acute myeloid leukemia. Leukemia research. 2008 Sep;32(9):1448-53. PubMed PMID: 18308386. Epub 2008/03/01. eng.

3.Wu D, Ozaki T, Yoshihara Y, Kubo N, Nakagawara A. Runt-related transcription factor 1 (RUNX1) stimulates tumor suppressor p53 protein in response to DNA damage through complex formation and acetylation. Journal of Biological Chemistry. 2013;288(2):1353-64.

4.Ito Y, Bae S-C, Chuang LSH. The RUNX family: developmental regulators in cancer. Nature Reviews Cancer. 2015;15(2):81-95.

5.de Stanchina E, Querido E, Narita M, Davuluri

RV, Pandolfi PP, Ferbeyre G, et al. PML is a direct p53 target that modulates p53 effector functions. Molecular cell. 2004;13(4):523-35.
6.Zhao Z, Zuber J, Diaz-Flores E, Lintault L, Kogan SC, Shannon K, et al. p53 loss promotes acute myeloid leukemia by enabling aberrant self-renewal. Genes & Development. 2010;24(13):1389-402.
7.Hou H, Chou W, Kuo Y, Liu C, Lin L, Tseng M, et al. TP53 mutations in de novo acute myeloid leukemia patients: longitudinal follow-ups show the mutation is stable during disease evolution. Blood cancer journal. 2015;5(7):e331.
8.Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene. 2002;21(21):3475-95.
9.Silva FP, Morolli B, Storlazzi CT, Anelli L, Wessels H, Bezrookove V, et al. Identification of RUNX1/AML1 as a classical tumor suppressor gene. Oncogene. 2003;22(4):538-47.
10.Goyama S, Schibler J, Cunningham L, Zhang Y, Rao Y, Nishimoto N, et al. Transcription factor RUNX1 promotes survival of acute myeloid leukemia cells. The Journal of clinical investigation. 2013;123(9):3876-88.
11.Zheng X, Gao Y, Zhang Q, Liu Y, Peng Y, Fu M, et al. Identification of transcription factor AML-1 binding site upstream of human cytomegalovirus UL111A gene. PLoS One. 2015;10(2):e0117773. PubMed PMID: 25658598. Pubmed Central PMCID: 4320089. Epub 2015/02/07. eng.
12.Lam K, Zhang D-E. RUNX1 and RUNX1-ETO: roles in hematopoiesis and leukemogenesis. Frontiers in bioscience: a journal and virtual library. 2012;17:1120.
13.Real PJ, Navarro-Montero O, Ramos-Mejía V, Ayllón V, Bueno C, Menéndez P. The role of RUNX1 isoforms in hematopoietic commitment of human pluripotent stem cells. Blood. 2013;121(26):5250-2.
14.Tang JL, Hou HA, Chen CY, Liu CY, Chou WC, Tseng MH, et al. AML1/RUNX1 mutations in 470 adult patients with de novo acute myeloid leukemia: prognostic implication and interaction with other gene alterations. Blood. 2009 Dec 17;114(26):5352-61. PubMed PMID: 19808697. Epub 2009/10/08. eng.
15.Shimizu K, Yamagata K, Kurokawa M, Mizutani S, Tsunematsu Y, Kitabayashi I. Roles of AML1/RUNX1 in T-cell malignancy induced by loss of p53. Cancer science. 2013;104(8):1033-8.
16.Kitabayashi I, Yokoyama A, Shimizu K, Ohki M. Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. The EMBO Journal. 1998;17(11):2994-3004.
17.Jan M, Majeti R. Clonal evolution of acute leukemia genomes. Oncogene. 2013;32(2):135-40.
18.Hatlen MA, Arora K, Vacic V, Grabowska EA, Liao W, Riley-Gillis B, et al. Integrative genetic analysis of mouse and human AML identifies cooperating disease alleles. The Journal of experimental medicine. 2016;213(1):25-34.
19.Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25(4):402-8.
20. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008;3(6):1101-8.
21.Yang L, Han Y, Saiz FS, Minden M. A tumor suppressor and oncogene: the WT1 story. Leukemia. 2007;21(5):868-76.
22.Rodriguez-Perales S, Torres-Ruiz R, Suela J, Acquadro F, Martin M, Yebra E, et al. Truncated RUNX1 protein generated by a novel t (1; 21)(p32; q22) chromosomal translocation impairs the proliferation and differentiation of human hematopoietic progenitors. Oncogene. 2015.
23.Melo M, Ahmad NN, Lima C, Pagnano K, Bordin S, Lorand-Metze I, et al. Mutations in the p53 gene in acute myeloid leukemia patients correlate with poor prognosis. Hematology (Amsterdam, Netherlands). 2002;7(1):13-9.
24.Abramowitz J, Neuman T, Perlman R, Ben-Yehuda D. The P53 Pathway Is Inactive in Acute Myeloid Leukemia. Blood. 2012;120(21):5122-.
25.Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. Journal of Clinical Oncology. 2001;19(5):1405-13.