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Expression levels of the runt-related transcription factor 1 and 3 genes in the development of acute myeloid leukemia

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Abstract. The aim of the present study was to evaluate the mRNA expression level of the runt-related transcription factor 1 (*RUNX1)* and runt-related transcription factor 3 (*RUNX3)* genes in patients with acute myeloid leukemia (AML). The etiology of AML is not yet fully known, but certain genetic factors may contribute to its manifestation. The *RUNX1* and *RUNX3* genes have been demonstrated to serve a role in the transcription process. The group investigated in the present study included 43 patients diagnosed with AML, and the relative *RUNX1* and *RUNX3* expression levels were determined using reverse transcription-quantitative polymerase chain reaction. The results indicated that *RUNX1* and *RUNX3* expression was associated with clinicopathological features, including sex and mortality risk. Expression levels of the *RUNX1* gene were higher and more variable among females (P=0.044), and mortality was more frequent among patients with a higher *RUNX3* expression level (P=0.036). The data obtained from the present study suggested that *RUNX3* expression may have potential value as a prognostic factor; furthermore, sex is potentially a factor that may affect the difference in *RUNX1* gene expression level among females and males. Further analyses in this field will aid in the identification and elucidation of the molecular basis of leukemia.

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Abbreviations: AML, acute myeloid leukemia; *RUNX1*, runt-related transcription factor 1; *RUNX3*, runt-related transcription factor 3; qPCR, quantitative polymerase chain reaction; dNTP, deoxynucleotides; CN-AML, cytogenetically normal acute myeloid leukemia; OS, overall survival; EFS, event-free survival

Key words: acute myeloid leukemia, expression level, runt-related transcription factor 1, runt-related transcription factor 3, reverse transcription-quantitative polymerase chain reaction

Introduction

Acute myeloid leukemia (AML) is a cancer of the white blood cells characterized by the clonal proliferation of myeloid progenitor cells in the bone marrow and peripheral blood (1). AML accounts for ~80% of all acute leukemia cases in adults, and the incidence of this disease has been revealed to increase with age (2,3). At present, AML is curable in 35-40% of patients <60 years old; however, among patients >60 years of age, cases of full recovery are less common (5-15%) (1,4). Due to the fact that survival rates remain relatively low (overall 5-year survival is <5% in older patients), novel therapeutics and treatment strategies are required (3).

The etiology of AML is not yet fully known, but there are a number of genetic factors that may predispose patients to this disease, including chromosomal translocations (Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1), and mutations in FMs-Related Tyrosine Kinase 3 (*FLT3*), Tumor Protein 53 and Additional Sex Combs Like 1, Transcriptional Regulator genes (5). Among the genetic factors that are involved in the development of AML, are the runt-related transcription factor 1 (*RUNX1)* and runt-related transcription factor 3 (*RUNX3)* genes. These genes belong to the runt domain transcription factor family, which is responsible for encoding the DNA-binding α-subunits of the RUNT domain transcription factor and serve an important role in the regulation of transcription (6,7). However, they may be dysregulated in human cancer cells (as a result of mutations, translocations or inactivation), and therefore potentially serve a role in the pathogenesis of cancer (6,8). The *RUNX1* gene, which is located on chromosome 21 (locus q21.22), serves an important role in hematopoiesis during embryonic development (9). Furthermore, it is responsible for the formation of hematopoietic stem cells and progenitor cells due to its expression in all hematopoietic sites (10). Previous studies have demonstrated that the chromosomal translocations and mutations in the *RUNX1* gene may be associated with several types of leukemia, including AML (11,12).

The *RUNX3* gene also encodes transcription factors and is responsible for the regulation of a number of other genes, including Transforming growth factor-β and Notch 1 pathways, Core-Binding Factor β subunit, ETS proto-oncogene 1 and ETS proto-oncogene 2 transcription factor genes (11). The *RUNX3* gene is located on chromosome 1 (locus p36) (11,13)

and is highly expressed in all hematopoietic stem cells (14). Furthermore, the *RUNX3* gene is hypothesized to act as a tumor suppressor. There is evidence to suggest that the inactivation of this gene is associated with the development of various types of cancer, including breast cancer (15). Additionally, deletion of this gene is associated with hyperplasia of the gastric mucosa and gastric cancer development (16,17). Continuous research regarding the potential functions of *RUNX* family genes in tumor development and the influence of these genes on the expression of other genes may assist in the early detection of cancer, and the development of more effective treatment modalities and novel therapeutics for patients with cancer (8). The role of the *RUNX1* and *RUNX3* genes in AML have, thus far, not been completely elucidated. Therefore, the aim of the present study was to evaluate the mRNA expression level of the *RUNX1* and *RUNX3* genes in patients with AML.

Materials and methods

Sample collection and ethics statement. The investigated group comprised of 43 (22 female and 21 male) patients who had been diagnosed with AML at the Hematology Clinic, Medical University of Lodz (Lodz, Poland). The mean age at the time of diagnosis was 57.9 years (58.6 for females and 57.4 for males; age range 17-80 years). Patients were divided into subgroups according to the French-American-British (FAB) classification of AML (18). Peripheral blood (March 2016-May 2017) was used for research and the samples obtained were residual material remaining following routine blood tests. The Ethics Committee of the Medical University of Lodz approved the present study (RNN/88/16/KE). Written informed consent was obtained from the patients for participation in the study.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the blood cells of participants using the Total RNA Mini kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's protocol. The purity of obtained RNA was determined by the A260/280 ratio (DNA/RNA absorbance to protein absorbance). Absorbance at 260 nm was used to determine the amount of RNA required for reverse transcription. Isolated RNA samples were stored at -76˚C until further analysis. RNA samples were reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The thermocycling parameters were as follows: 25˚C for 10 min, then 37˚C for 120 min and 85˚C for 5 min. The final concentration of RNA in the reaction mixture in all samples was 0.02 μg/μl. The presence of cDNA was verified through PCR amplification normalized to *GAPDH* (19). PCR was performed for qualitative analysis of mRNA expression *RUNX1* and *RUNX3*. Amplification was performed according to the manufacturer's protocol for the AccuTaq™ LA DNA Polymerase kit (Sigma Aldrich; Merck KGaA, Darmstadt, Germany). The reaction mixture consisted of 1 μl cDNA template, 0.7 μl of 10 μM each primer, 3.5 μl of 1.5 mM 10x PCR buffer without $MgCl_2$ (Sigma Aldrich; Merck KGaA), 0.7 μl of 25 mM $MgCl_2$ reagent, 0.4 μl of 0.2 mM dNTP (deoxynucleotides) mix, 0.2 μl of 0.5 U AccuTaq LA DNA Polymerase and distilled water to the final volume of 21 μl. Primers used in the present study are listed in Table I. A negative control, without cDNA template, was included in every experiment. Amplification was performed using an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, denaturation at 92˚C for 1 min, primer annealing at 58˚C for *RUNX1* and 56˚C for *RUNX3* for 30 sec, elongation at 72˚C for 45 sec and final elongation at 72˚C for 7 min. Electrophoresis on a 2% agarose gel was used to assess the products of PCR amplification. The sizes of the reaction products were as follows: *RUNX1,* 96 bp and *RUNX3,* 120 bp. qPCR was used for quantitative assessment of *RUNX1, RUNX3* and *GAPDH* mRNA expression, and reactions were performed in a Rotor-Gene™ 6000 thermocycler (Corbett Life Science; Qiagen GmbH, Hilden, Germany). *GAPDH* is a housekeeping gene, the expression of which is often used to normalize mRNA levels between samples (19). The reaction mixture consisted of 5 μl RT HS-PCR Mix Sybr® B (A&A Biotechnology), 0.7 μl of 10 μM each primer, 1 μl cDNA template and nuclease-free water to a final volume of 10 μl. Experiments for investigated and reference genes were performed in triplicate and reactions were performed in separate tubes. A negative control, without cDNA template, in triplicate was also included in every experiment. The reaction parameters were as follows: Initial denaturation at 95˚C for 10 min, denaturation at 95˚C for 10 sec, primer annealing at 55˚C for *RUNX1* and 58˚C for *RUNX3* for 15 sec, elongation at 72˚C for 20 sec. In order to assess the specification of products, analysis of melting curves was performed following amplification. The $2^{-\Delta\Delta Cq}$ method was used to estimate relative changes in gene expression determined by RT-qPCR analysis (20). The mean C_q values of *GAPDH*, *RUNX1* and *RUNX3* genes were used in subsequent calculations.

Statistical analysis. Statistical analyses were performed using STATISTICA 12.5 (StatSoft Inc., Tulsa, OK, USA). A comparative statistical analysis was performed using the non-parametric U Mann-Whitney test in the absence of normality of relative levels of *RUNX1* and *RUNX3* gene expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Relative RUNX1 and RUNX3 gene expression level with sex and age of diagnosis. All 43 samples exhibited *GAPDH* expression. The presence of *RUNX1* and *RUNX3* gene expression was also identified in all selected samples. Quantitative analyses revealed that the transcript level of *RUNX1* and *RUNX3* genes varied among selected cases. It ranged between 0.13 and 18.37, with a median value 0.73 for the *RUNX1* gene and between 0.04 and 8.54 with a median value of 1.28 for the *RUNX3* gene. The investigated group comprised 22 females and 21 males. Statistically significant differences between patient sex and relative *RUNX1* expression were identified ($P=0.044$). Levels were higher and varied more among females (Fig. 1). However, no significant differences were identified between sex and relative *RUNX3* gene expression ($P=0.130$; data not shown). Another compared parameter was age at the time of AML diagnosis. The mean age was 57.9, 58.6 years

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Forward primer	Reverse primer
GAPDH	5'-TGGTATCGTGGAAGGACTCATGAC-3'	5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'
RUNX1	5'-AGTGGAAGAGGGAAAAGC-3'	5'-ATCCACTGTGATTTTGATGG-3'
RUNX3	5'-ATGACGAGAACTACTCCG-3'	5'-TCAGGGTGAAACTCTTCC-3'

RUNX1, runt-related transcription factor 1; *RUNX3*, runt-related transcription factor 3.

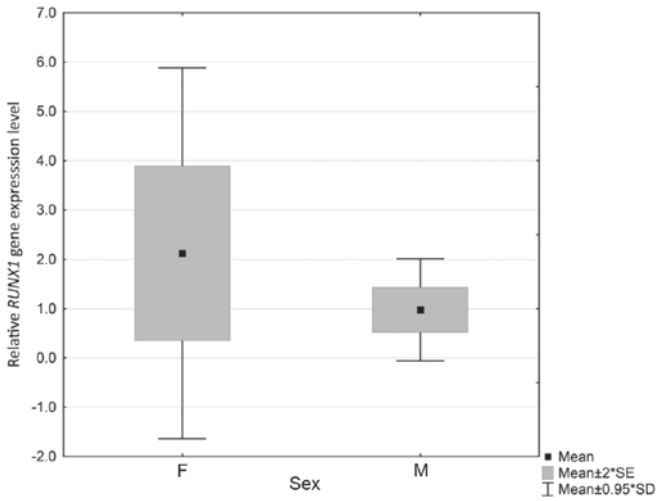

Figure 1. Associations between relative *RUNX1* gene expression level and patient sex. Statistically significant differences were observed between female and male patients (P=0.044). *RUNX1*, runt-related transcription factor 1; F, female; M, male; SE, standard error; SD, standard deviation.

for females and 57.4 years for males; however, no statistically significant associations were identified between age at the time of diagnosis and mRNA expression of *RUNX1* (P=0.970) or *RUNX3* (P=0.469).

Relative RUNX1 and RUNX3 gene expression level with FAB classification and mortality. Patients were also divided into subgroups according to the FAB classification of AML (18). Full details are presented in Table II. Statistical analysis revealed no significant associations between FAB classification subgroups and relative *RUNX1* (P=0.746) and *RUNX3* (P=0.771) expression. Relative expression was also compared with mortality among the enrolled patients. The results indicated that there is a statistically significant association between the relative expression of *RUNX3* and mortality among patients (P=0.036). Mortality was more frequent among patients with higher *RUNX3* expression levels (Fig. 2); however, no such association was observed between mortality and *RUNX1* expression (P=0.445, data not shown).

Discussion

Due to the presence of various mutations in the *RUNX1* and *RUNX3* genes in patients with AML, we hypothesized that these genes may influence mRNA formation and may contribute to the different levels of expression among the investigated cases. To the best of our knowledge, the present study is the first to present the *RUNX1* and *RUNX3* gene expression levels in patients with AML determined by RT-qPCR analysis in a Polish population, as previous studies have only been conducted in Chinese populations thus far.

The *RUNX1* gene serves an important role in hematopoiesis (9). Its abnormal expression is present in various malignancies, including ovarian cancer, cytogenetically normal AML (CN-AML) and breast cancer (21-24). However, the significance of the *RUNX1* gene in cancer development is not fully known. Previous studies have suggested that the *RUNX1* gene functions as a tumor suppressor in AML (25), and that loss of the *RUNX1* gene may lead to weak differentiation and leukemia development (26). One previous study has reported that a normal expression level of *RUNX1* gene inhibits cell proliferation and promotes differentiation of hematopoietic progenitor cells (21). By contrast, deactivating the *RUNX1* gene may cause amplification of myeloid progenitors and the subsequent development of AML. A previous study undertaken by Silva *et al* (25) suggested that the *RUNX1* gene acts as a classical tumor suppressor gene; however, other studies have suggested that *RUNX1* functions as an oncogene and that it may cause AML development due to its pro-survival role in leukemia cell proliferation (27-30). The results of these studies also suggested that the prognostic impact in CN-AML depends on the *RUNX1* expression level. A study undertaken by Goyama *et al* (28) reported that overexpression of the *RUNX1* gene inhibited the growth of regular cord blood cells by inducing myeloid differentiation. It was suggested that the *RUNX1* gene may be a valuable novel marker for risk stratification in patients with AML and that it is an excellent candidate for anticancer-targeted therapy due to the modulation of its post-translational modifications (29).

It is now hypothesized that, due to its expression level, the *RUNX1* gene may serve a role as a tumor promoter or tumor suppressor in different types of cancer and hematological malignancies including AML (21). A study undertaken by Fu *et al* (21) estimated *RUNX1* expression using microarrays and revealed that a high level of *RUNX1* mRNA expression in CN-AML was associated with a poorer overall survival (OS) and event-free survival (EFS) than low *RUNX1* mRNA expression. The median OS and EFS times in patients with a higher *RUNX1* expression level were poorer than that of the low *RUNX1* expression group (P=0.009 and P=0.011, respectively). Among 157 patients with CN-AML with a higher *RUNX1* gene expression, significantly more patients exhibited the FAB M2 subtype than in the group with lower *RUNX1*

Table II. French-American-British classification of investigated patients.

Diagnosis	Number of cases	*RUNX1* P-value	*RUNX3* P-value
AML undefined	21 (10F, 11M)		
AML 0	1F		
AML1	3 (2F, 1M)		
AML2	8 (5F, 3M)	0.746	0.771
AML3	2 (1F, 1M)		
AML4	4 (2F, 2M)		
AML5	3 (1F, 2M)		
AML6	1M		

RUNX1, runt-related transcription factor 1; *RUNX3*, runt-related transcription factor 3; AML, acute myeloid leukemia; F, female; M, male.

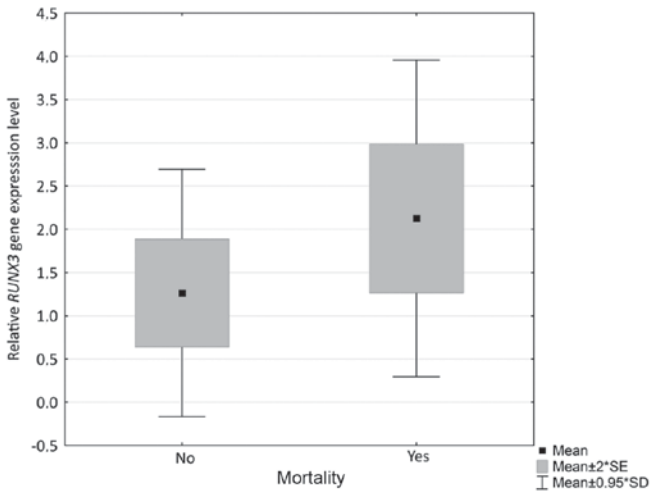

Figure 2. Associations between relative *RUNX3* gene expression level and mortality. Statistically significant differences were observed among subgroups (P=0.036). *RUNX3*, runt-related transcription factor 3; SE, standard error; SD, standard deviation.

gene expression. Furthermore, the *RUNX1* high expression group included significantly more patients with the FAB M1 subtype than the *RUNX1* low expression group (P=0.0014), suggesting that the leukemia cells from patients with a high expression of *RUNX1* are derive from relatively less mature cells. According to this aforementioned study, *RUNX1* gene expression may have prognostic significance in AML and it may be a biomarker of an unfavorable outcome in CN-AML, where overexpression of the *RUNX1* gene is widespread among patients (high expression of *RUNX1* is associated with poorer disease outcomes) (21).

These results differed from those obtained in the present study, where there were no associations among mortality, FAB classification of AML and the expression level of *RUNX1*. Furthermore, the present study revealed statistically significant differences in *RUNX1* gene expression levels between females and males; as females tended to exhibit a higher and more variable expression level. This suggested that sex may affect *RUNX1* expression, thereby influencing the process of leukemia development.

The *RUNX3* gene is involved in neurogenesis and thymopoiesis, and serves a role as a tumor suppressor in gastric cancer (7,31-33). A study undertaken by Jiang *et al* reported that the *RUNX3* expression level is associated with breast cancer development and that it is decreased in this type of cancer. The principal cause for this inactivation mechanism may be hypermethylation in the promoter region (34). A study undertaken by Cheng *et al* (7) demonstrated that *RUNX3* gene expression was an independent prognostic factor in childhood AML, and that a higher *RUNX3* gene expression level was associated with a shorter EFS and OS time (7). The results of a study undertaken by Lacayo *et al* (35) also demonstrated that a higher level of *RUNX3* gene expression was associated with a shortened EFS rate in childhood AML. However, this study was conducted on patients belonging to an *FLT3* mutant group, which may have also affected EFS (35). Based on these aforementioned studies, it is possible that the *RUNX3* gene expression level is associated with a shorter survival time in childhood AML (7,35). Furthermore, according to the study undertaken by Cheng *et al* (7), the *RUNX3* gene expression level was not associated with age or sex. However, in a group of patients with a lower *RUNX3* gene expression level, this was significantly associated with the presence of t(8;21) or inv(16) translocations (7). Lower *RUNX3* gene expression levels were frequently identified in patients with FAB M2 and M4 AML subtypes. Furthermore, *RUNX3* was significantly underexpressed in the prognostically favorable subgroup of AML with the t(8;21) and inv(16) translocations (7).

The *RUNX3* expression level differed among the patients enrolled in the present study, and the study undertaken by Cheng *et al* (7) obtained similar levels of *RUNX3* expression in patients with childhood AML, although the results of the present study were more varied and slightly higher. This may be due to differences in age between the investigated groups. Cheng *et al* (7) identified no statistically significant associations between clinicopathological features (sex, age or FAB classification) and relative *RUNX3* expression level. The results obtained in the present study are comparable, as no associations between sex or age at the time of diagnosis or FAB classification and *RUNX3* expression were identified. Statistically significant differences were identified between the expression level and the incidence of mortality among patients, as mortality occurred more frequently in the group with a higher *RUNX3* expression level. These observations are also similar to those reported by Cheng *et al* (7) which leads to the conclusion that *RUNX3* may serve as a potential prognostic factor in AML.

The lack of an association between the selected clinicopathological features and relative *RUNX1* and *RUNX3* expression may be a limitation of the present study, particularly due to the relatively small group of investigated patients. Future studies would benefit from an increased number of patients and the collection of more detailed clinical information, including the results of peripheral blood morphology, previously applied treatment, percent of blasts in bone marrow.

The results of the present study suggested that sex may be associated with the expression level of the *RUNX1* gene and may influence the difference in the process of AML development between females and males. Based on the results of

earlier studies (7,35) and those of the present study, *RUNX3* may serve as a potential novel prognostic factor. Patients with a higher *RUNX3* expression level generally have poorer outcomes. However, the obtained results must be confirmed in a larger cohort.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Author's contributions

AK and DS planned and conducted experiments, and assisted in the preparation of the manuscript for publication. MŻ and AJ conducted experiments. EB planned and supervised experiments, and assisted in the preparation of the manuscript for publication.

Ethics approval and consent to participate

The Ethics Committee of the Medical University of Lodz approved the present study (RNN/88/16/KE). Written informed consent was obtained from the patients for participation in the study.

Consent for publication

Written informed consent was obtained from the patients for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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