Impact of Simultaneous Presence of Multiple PML-RARA Isoforms on Phenotype in Patients with Acute Promyelocytic Leukaemia

Emine Goktas[1], Ayse Gul Zamani[1], Mahmut Selman Yildirim[1], Sinan Demircioglu[2], Atakan Tekinalp[2] and Ozcan Ceneli[2]

[1]*Department of Medical Genetics, Faculty of Medicine, Necmettin Erbakan University, Konya, Turkiye*
[2]*Department of Internal Medicine, Division of Haematology, Faculty of Medicine, Necmettin Erbakan University, Konya, Turkiye*

ABSTRACT

Objective: To determine the coexistence of multiple PML-RARA transcripts in adult APL (acute promyelotic leukaemia) patients, and its impact on the patients' laboratory parameters, treatment responses, and prognoses.

Study Design: Cross-sectional study.

Place and Duration of the Study: Department of Medical Genetics, Medical Faculty of Necmettin Erbakan University, Konya, Turkiye, from January 2015 to March 2023.

Methodology: The study group consisted of individuals diagnosed with APL. RNA isolation was performed by taking blood or bone marrow samples and the presence of breakpoints in PML-RARA bcr1, bcr2, and bcr3 was detected using the real-time PCR. However, the quantification of PML-RARA fusion transcripts cannot be provided using the utilised kit.

Results: Twelve women and eight men were examined with a mean age of 38 years (range: 19-80), and 46.5 years (range: 22-60) were examined, respectively. When evaluating patients based on isoforms, it was found that 40% had multiple isoforms. Nineteen (95%) patients achieved haematologic remission after the treatment. Only one patient who had three different isoforms did not achieve remission. The estimated median survival for patients with a single isoform and those with multiple isoforms was 78.1 months (95% CI: 37.8-117.6) and 71.7 months (46.2-97.2), respectively. Two of the patients with multiple isoforms were lost in the early stage, whereas no early-stage mortality was recorded among patients with a single isoform.

Conclusion: Identifying PML-RARA isoform subtypes is important for predicting prognosis and informing clinical follow-up.

Key Words: *Acute promyelocytic leukaemia, Breakpoint cluster region, Isoform, PML-RARA.*

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INTRODUCTION

Acute promyelocytic leukaemia (APL) is a subtype of acute myeloid leukaemia (AML) that is aggressive and has a high mortality rate. The global incidence of APL is estimated to be 0.42 per 100,000 people.[1] Before the 1970s, patients diagnosed with APL had an average life expectancy of only one week.[2] However, with the introduction of all trans-retinoic acid (ATRA) and arsenic trioxide (ATO) agents into treatment, 5-year disease-free survival rates have now reached up to 96%.

Correspondence to: Dr. Emine Goktas, Department of Medical Genetics, Faculty of Medicine, Necmettin Erbakan University, Akyokus/Meram, 42080, Konya, Turkiye
E-mail: emineaktas88@hotmail.com
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In most cases, there is a presence of t(15;17)(q24:q21) translocation, resulting in the fusion of the *PML* gene on chromosome 15 with the *RARA* gene on chromosome 17, forming the PML-RARA chimeric fusion of oncoprotein that obstructs the differentiation of myeloid precursors. This results in the build-up of abnormal promyelocytes in the bone marrow.[3,4]

The *RARA* gene has a single breakpoint cluster region (bcr) in intron 2. In contrast, the *PML* gene has three different bcr-localised areas in intron 3, intron 6, and exon 6. The breakpoint in the sixth intron of the *PML* gene results in the bcr 3 (short, S) isoform, while the breakpoint in the third intron yields the bcr 1(long, L) isoform, and the breakpoint in the sixth exon produces the bcr 2 (variable, V) isoform. The BCR 1 isoform is present in approximately 55% of cases, B-cell receptor (BCR) 3 in 35%, and BCR 2 in around 8%.[5] The L and S isoforms involve the excision of the chimeric intron structure and the joining of the reading frames of both genes. In contrast, the V isoform is more complex, resulting from splicing between the remaining part of the exon 6 of the *PML* gene and exon 2 of the *RARA* gene.[6] Several studies have identified associations between

PML-RARA transcript types and patient characteristics or outcomes, but the literature lacks studies on the clinical impact of the presence of multiple isoforms.[2]

The objective of this study was to investigate the coexistence of multiple PML-RARA transcripts in adult APL patients and to determine its correlation with the patients' laboratory parameters, treatment responses, and prognosis. To the best of the authors' knowledge, this is the first study to discuss the clinical characteristics of adult patients simultaneously carrying more than one PML-RARA transcript.

METHODOLOGY

The clinical data of the patients was collected retrospectively by evaluating the hospital database and patient files which were filled for the diagnosis and treatment of APL subjects. This study was conducted from January 2015 to March 2023 in the Department of Medical Genetics of the Medical Faculty at Necmettin Erbakan University in Konya, Turkiye. Non-probability sampling method was utilised as the sampling technique, and the study included twenty patients with at least one positive transcript in the PML-RARA fusion analysis. Patients who were initially diagnosed with APL but tested negative for the PML-RARA fusion analysis were excluded from the research. Informed consent forms were obtained from patients who met the inclusion criteria.

Total RNA isolation was performed from peripheral blood (4 ml) or bone marrow (500-1000 µl) samples using the Hybrid-RTM Blood RNA kit. The RNA was then extracted from the upper phase using organic extraction and precipitated with ethanol. Subsequently, the GeneMAPTM PML-RARA t(15;17) bcr1, bcr2, and bcr3. Detection kit was used to detect the presence of breakpoints in PML-RARA bcr1, bcr2, and bcr3 through real-time PCR method. However, the quantification of PML-RARA fusion transcripts cannot be provided using the utilised kit. Additionally, fluorescence *in situ* hybridisation (FISH) analysis was performed on all patients using locus-specific probes, specifically the PML/RARA Dual-Color, Dual-Fusion Translocation Probe (Cat. no. 17-043 - primeFISH® PML-RARA t(15;17) DF DC Probe Kit), according to the manufacturer's instructions.

The statistical analysis for this study was conducted using IBM SPSS version 22.0. Descriptive characteristics were presented as median (minimum-maximum). To evaluate central tendency, the median was preferred after normality tests were performed. Skewness-kurtosis range and the Shapiro-Wilk's test were used to test normality,[7] as the sample size was 20.

Group comparisons were performed using the Mann-Whitney U test.[8] Categorical variables were expressed as frequency and percentages. The Kaplan-Meier method[9] was used for survival analysis, and survival rates were compared using the log-rank test. Results were accepted as statistically significant if the p-value was less than or equal to 0.05.

RESULTS

Twelve (60%) patients were female, and 8 (40%) were male. The median age was 38 years (range 19-80) for women and 46.5 years (range 22-62) for men. When evaluating patients based on the number of isoforms, 60% had a single isoform and 40% had multiple isoforms. Simultaneously, FISH was performed on all patients to detect t(15;17), and all patients were found to have translocation at varying rates. Three (15%) patients had additional cytogenetic abnormalities: One patient with the bcr3 isoform had trisomy 8, and one patient each with bcr1 and bcr3 isoforms had p53 deletion. Patients with multiple isoforms did not have any additional cytogenetic abnormalities.

All patients with the microgranular variant had multiple isoforms (2 patients with BCR1 + BCR2, one patient with BCR1 + BCR2 + BCR3). In contrast, only 5 (24.9%) patients with the hypergranular variant had multiple isoforms. Clinical symptoms such as petechiae, ecchymosis, nosebleeds, and gum bleeding were observed in 75% of patients with a single isoform and in 87% of patients with multiple isoforms. However, in one patient with multiple isoforms, gastrointestinal system bleeding was observed. Additionally, disseminated intravascular coagulation (DIC) was observed in half of the patients with a single isoform, whereas this rate was 75% in the group with multiple isoforms. Regarding remission induction therapy, 18 patients (90%) received ATRA-idarubicin, and one (5%) patient each received ATRA-daunorubicin + ARA-c, and ATRA alone.

Table I: Comparison of patients according to isoform type.

	Single Isoform	Multiple Isoforms
Total Number	12	8
Age (years)		
Mean	33.67	50.75*
Median	32	52
Range	19-48	24-80
Gender		
Female, n (%)	7 (58.7)	5 (62.5)
Male, n (%)	5 (41.3)	3 (37.5)
Haemoglobin (g/dL)		
Median	7.8	8.1
Range	2.7-14.5	7.1-11.4
Platelet Count (10^3xµL)		
Median	26.6	15.5
Range	12-84	5-94
Neutrophil Count (10^3xµL)		
Median	1.1	2.1
Range	0.1-15.4	0.2-3.8
Leukocyte Count (10^3xµL)		
Median	5.8	7.0
Range	0.6-13.2	0.8-1.7
Morphology, n (%)		
Hypergranular, n (%)	12 (100)	5 (67.5)
Microgranular, n (%)	-	3 (37.5)
Bleeding, n (%)		
Positive	9 (75)	7 (87.5)
Negative	3 (25)	1 (12.5)
Site of bleeding, n (%)		
Petechiae, ecchymosis	8 (66.7)	4 (50)
Nose bleeding, bleeding gums	1 (8.3)	2 (25)
GIS bleeding	-	1(12.5)
DIC, n (%)		
Positive	6 (50)	6 (75)
Negative	6 (50)	2 (25)
Survival (months)		
Median	78.1	71.7
Range	37.8-117.6	46.2-97.2
Breakpoint cluster region, n (%)		
BCR1, n (%)	2 (16.7)	-
BCR2, n (%)	1 (8.3)	-
BCR3, n (%)	9 (75)	-
BCR1+ BCR2, n (%)	-	3 (37.5)
BCR1+BCR2+BCR3, n (%)	-	5 (62.5)

Table II: Group comparisons according to breakpoint numbers.

	Single Breakpoint (n = 12) Median (Minimum-Maximum)	Multiple Breakpoint (n = 8) Median (Minimum-Maximum)	p-value
Age	32 (19-48)	52 (24-80)	0.025*
Total leucocyte counts (10^3xμL)	5.8 (0.6-13.2)	7.0 (0.8-13.7)	0.840
Neutrophil counts (10^3xμL)	1.1 (0.1-15.4)	2.1 (0.2-3.8)	0.840
Haemoglobin (g/dL)	7.8 (2.7-14.5)	8.1 (7.1-11.4)	0.778
Platelet (10^3xμL)	26.6 (12-84)	15.5 (5-94)	0.109
Myeloperoxidase (%)	90 (26-99)	87 (50-95)	0.902
CD34 (%)	3.5 (0-52)	2 (1-67)	0.227
HLA-DR (%)	12.5 (5-89)	6.5 (0-18)	0.592
CD117 (%)	62.5 (22-92)	72.5 (44-88)	0.768
CD33 (%)	92.5 (84-97)	86.5 (45-97)	0.494
CD13 (%)	77 (27-92)	62 (53-86)	0.536
t(15;17) (%)	87.5 (21-95)	77 (60-80)	0.596
LDH (IU)	544 (181-1845)	331 (224-605)	0.238
C-reactive protein (mg/L)	18.1 (2.5-24)	29 (1-46)	0.696
Creatinine (mg/dL)	0.73 (0.57-1.0)	0.59 (0.48-0.69)	0.026*

**p <0.05, calculated by Mann-Whitney U test.*

The patients had a median follow-up time of 62.7 months (range: 0.7-173.2), and the estimated median overall survival (OS) time could not be calculated. The one-year OS rate was found to be 70%. Nineteen patients (95%) achieved haematologic and molecular remission after remission-induction treatment. Only one patient did not achieve remission and had three different isoforms (bcr1+bcr2+bcr3). The estimated median survival for patients with a single isoform (12 patients, 60%) and those with multiple isoforms (8 patients, 40%) was 78.1 months (95% CI: 37.8-117.6) and 71.7 months (46.2-97.2), respectively (p = 0.577). Among patients with multiple isoforms, 2 (25%) were lost in the early stage. No early-stage mortality was recorded among patients with a single isoform. Table I presents the patients' clinical features, laboratory values, and isoform subtypes.

In addition, age and certain laboratory findings were compared to elucidate if any significant differences were present (Table II). Patients who have multiple breakpoints were older and the age difference was statistically significant (p = 0.025). Whereas creatinine was the only laboratory marker that was significantly higher in the patients with single breakpoint, other laboratory values such as leukocyte count, neutrophil count, etc. showed no significant change between the two groups.

DISCUSSION

The localisation of the breakpoint in the *PML* gene leads to the formation of different PML-RARA isoforms in individuals with acute promyelocytic laeukemia.[6] In this study, it was found that 60% had a single isoform, while 40% had multiple isoforms. Among the patients with a single isoform, 75% had the S variant, 17% had the L variant, and 8% had the V variant. These findings are inconsistent with previous studies, which have consistently reported a higher prevalence of bcr1 isoforms compared with bcr3. However, the prevalence of the bcr2 isoform was extremely low, consistent with previous studies.[5,10] Moreover, some researchers have reported the S isoform as the predominant isoform. It has been emphasised that the frequency of the PML-RARA sub-isoform may vary according to geographical location and ethnic origin.[5,11] To the authors' knowledge, the literature on the coexistence of more than one PML/RARA transcript in APL patients is limited to a single case report. In the case presented by Chu *et al*.,[12] the coexistence of S and L isoforms was mentioned.

In studies with numerous samples conducted on APL, patient groups were created based on isoform subtypes.[13,14] Comparisons were made concerning age and gender variables, but no significant differences were found.[15,16] A comparison of the two patient groups in terms of age and gender revealed no significant differences in the gender distribution between the groups. However, the average age of patients with multiple isoforms was significantly higher (p = 0.025).

Research has shown that the isoform types of APL patients can affect their clinical characteristics, prognosis, response to treatment, and relapse durations. A large-scale study of 167 newly diagnosed APL patients found that those with S and V isoforms had significantly higher leukocyte counts than those with the L isoform. Furthermore, patients with the S isoform displayed increased microgranular features and CD34 expression levels. Although there were no significant differences in terms of overall remission rates among the three groups, individuals with the V variant exhibited lower disease-free survival rates than the other groups over three years.[16]

Following the findings of other published studies, a correlation was identified between the S-form type and an elevated white blood cell (WBC) count. Additionally, higher mean leukocyte count and microgranular morphology were observed in patients with multiple isoforms compared to those with a single isoform. Except for one patient with multiple isoforms, all patients achieved haematological remission. The shorter estimated median survival time for patients with more than one isoform in this study suggested

that having multiple isoforms (p = 0.577), along with the V and/or S isoform highlighted in previous studies, may also be linked to a shorter survival time.[15,17]

Siahbani *et al.*[5] and Chu *et al.*[12] emphasised that low initial platelet count is associated with higher mortality rates. The observation that platelet levels are lower and DIC clinic occurrences are more frequent in cases with multiple isoforms indicates that the platelet count, which is an effective prognostic indicator for the disease, may be influenced by the isoform count.

Approximately 45% of APL patients exhibit additional chromosomal anomalies besides t(15;17). The most commonly observed alterations include trisomy 8, 7q deletion, 9q deletion, and 17p deletion. Trisomy 8 contributes to APL pathogenesis through MYC deregulation in APL cells.[17,18] In one of the cases, trisomy eight was observed alongside the PML-RARA fusion, while two had deletions in the *TP53* gene located in the 17p region.

The t(15;17) translocation which is present in 90% of APL cases and underlies the molecular basis of the disease, can be detected using techniques including RT-PCR.[18] The PCR method is frequently used in gene expression analysis and allows the identification of specific isoforms, facilitates early diagnosis for patients and enables prognosis prediction through isoform typing.[19,20]

Limitations of this study include the small number of patients, the short follow-up period, and the inability to obtain an estimated median survival time. However, the fact that APL is a rare disease and that there is no research in the literature on the effect of the presence of multiple isoforms suggests that this study may lead to multicentric studies with larger numbers of patients.

CONCLUSION

Despite extensive research on APL patients worldwide, there remains a lack of agreement on prognostic factors and the potential links between bcr isoforms and various prognostic and demographic variables. These findings indicate the need for further studies to identify more consistent prognostic markers and to gain a clearer understanding of how bcr isoforms may influence APL outcomes.

ETHICAL APPROVAL:
This study was approved by the University's Faculty of Medicine's Drugs and Non-Medical Devices Research Ethics Committee with approval number 2023/4281.

PATIENTS' CONSENT:
Since it was a retrospective study, the data were collected from the hospital's archive, following the approval of the Ethics Committee. Informed consent was obtained from all the patients before the procedure.

COMPETING INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
EG, SD, AT: Study conception, design, literature search and drafting and editing of the manuscript.
EG, AT, SD, OC: Data collection.
EG, AGZ, MSY: Analysis and interpretation of the results.
All authors approved the final version of the manuscript to be published.

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