3 ISOFORMS OF PML RARα IN ACUTE PROMYELOCYTIC LEUKAEMIA

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ABSTRACT

Objective: To determine the frequencies of three isoforms of PML RARα fusion gene in APL.
Study Design: Descriptive cross sectional study.
Place and Duration of Study: Department of Hematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi Pakistan, from Apr 2015 to Oct 2015.
Material and Methods: This study involved 97 newly diagnosed cases of APL, aged between 15-70 years from both genders. Double nested PCR was carried out by Applied Biosystems 2720 Thermal Cycler in every patient to determine the isoform of PML RARα fusion gene. A written informed consent was obtained from every patient.
Results: The age of the patients ranged from 17 years to 69 years with a mean of 37.87 ± 12.89 years. There were 68 (70.1%) male and 29 (29.9%) female patients in the study group. The bcr1 isoform was found in 9 (9.3%) patients. There was no significant difference in the frequency of bcr1 isoform across age groups; 17-34 years vs. 35-52 years vs. 53-69 years (14.6% vs. 7.0% vs. 0.0%; p=0.223) and genders; male vs. female (7.4% vs. 13.8%; p=0.317). The bcr2 isoform was found in 26 (26.8%) patients. There was no significant difference in the frequency of bcr2 isoform across age groups; 17-34 years vs. 35-52 years vs. 53-69 years (19.5% vs. 27.9% vs. 46.2%; p=0.164) and genders; male vs. female (25.0% vs. 31.0%; p=0.539) while the bcr3 isoform was found in 62 (63.9%) patients. There was no significant difference in the frequency of bcr3 isoform across age groups; 17-34 years vs. 35-52 years vs. 53-69 years (65.9% vs. 65.1% vs. 53.8%; p=0.717) and genders; male vs. female (67.6% vs. 55.2%; p=0.242).
Conclusion: The most frequent isoform of PML-RARα fusion gene was bcr3 which was observed in 62 (63.9%) cases followed by bcr2 (26.8%) and bcr1 (9.3%). There was no significant difference in the frequency of isoforms with patient's age and gender.

Keywords: Acute Promyelocytic Leukemia, Isoforms, PML-RARα Fusion Gene.

INTRODUCTION

Acute Promyelocytic Leukemia (APL) classified as AML M3 according to French-American-British (FAB) classification system and APL with t(15;17) and PML RARα by WHO is a distinct subtype of Acute Myeloid Leukemia (AML)[1]. APL accounts for 5-8% of all cases of AML. The disease is uncommon in first decade of life with incidence increasing in second decade and decreasing after 60 years of age. The disease is relatively more commonly seen in females. Patients usually present with a severe hypercoagulable state with bleeding as the predominant clinical manifestation[2].

Characteristic features of APL were first reported by Douer *et al*[11]. APL has two morphological variants with hypergranular being the more common one[1,3]. Pancytopenia is a feature of hypergranular variant while micro-granular variant typically presents with a raised TLC[4]. Detection of PML RARα is the hallmark of diagnosis of APL[5]. It is found in more than 98% of patients of APL[3]. Absence of HLA DR on flow cytometry is the most sensitive finding of APL[5].

PML RARα has 3 possible isoforms. Breakpoint regions in chromosome 15 having PML gene include intron 3 (L-long isoform or bcr1 isoform), intron 6 (S-short isoform or bcr3 isoform) and exon 6 (V form or bcr2 isoform). Gonzales *et al*. has reported that S form has a

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short remission and overall survival[4]. Frequency of bcr1 isoform is 62%, bcr3 is 33% and bcr2 is 5%[5]. According to various studies in Europe and USA, bcr1 accounts for 50-55% of cases, bcr2 8-20% and bcr3 is found in 27-49% of cases[5]. All the 3 isoforms can be detected by real time quantitative PCR[6].

All trans retinoic acid (ATRA) is the mainstay of treatment of APL with a cure rate of >80%[7]. Different isoforms have different sensitivities to ATRA. Bcr3 isoform is found to have a lack of sensitivity to ATRA and a longer time to remission[8]. The rationale of my study is to determine the frequency of 3 isoforms of PML RARα which helps to determine treatment protocols and prognosis. While bcr1 is more sensitive to ATRA, bcr 3 not only lacks sensitivity but is also associated with a poorer outcome; high TLC and CNS relapse in a number of cases. This will be the first study carried out in our population.

The objective of this study was to determine the frequencies of three isoforms of PML RARα fusion gene in APL.

Operational Definition

Acute Promyelocytic leukemia (APL): Patients with all of the following features were diagnosed as having APL:

- Peripheral blood or bone marrow showing myeloid blasts and abnormal promyelocytes.
- Abnormal promyelocyte was characterized by having a densely packed cytoplasm with coarse red granules, absence of golgi zone, nucleocytoplasmic asynchrony, bi-lobed or folded nuclei and occasional bundles of Auer rods or faggots.
- Characteristic immunophenotype of low expression of HLA DR and CD34, bright expression of CD33 and heterogeneous expression of CD13.
- Strongly positive for SBB.
- Positive for PML RARα fusion gene on double nested conventional PCR.

PML RARα Fusion Gene: Retinoic acid receptor alpha (RARα) gene on chromosome 17 fuses with a nuclear regulatory factor gene on chromosome 15 (Promyelocytic leukemia or PML gene) giving rise to a PML RARα fusion gene product. The PML RARα fusion gene was detected by conventional double nested PCR.

BCR 1 Isoform (Long Isoform): BCR 1 Isoform was detected by conventional double nested PCR. Fusion of PML gene involving intron 3 of breakpoint cluster region (bcr) on chromosome 15 with intron 2 of RARα gene on chromosome 17 leads to formation of bcr1 (long isoform). It was detected by conventional double nested PCR.

BCR 2 Isoform (Variable isoform): Fusion of PML gene involving exon 6 of breakpoint cluster region (bcr) on chromosome 15 with intron 2 of RARα gene on chromosome 17 leads to formation of bcr2 (variable isoform) which was detected by conventional double nested PCR.

BCR 3 Isoform (Short isoform): Fusion of PML gene involving intron 6 of breakpoint cluster region (bcr) on chromosome 15 with intron 2 of RARα gene on chromosome 17 leads to formation of bcr3 (short isoform) which was detected by conventional double nested PCR.

MATERIAL AND METHODS

This was a descriptive cross sectional study conducted at department of Hematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi. A total of 97 newly diagnosed cases of APL, aged 15-70 years and both genders were included in the study. Patient presenting with other leukemias like Acute Lymphoid leukaemia, secondary leukaemia evolving from Myeloproliferative neoplasia & Myelodysplastic syndrome and taking treatment for APL were exclude form the study.

Data Collection

The study was conducted after approval by the Ethical Committee. All subjects fulfilling the inclusion criteria were elaborately apprised about the study to obtain their informed consent.

Patient particulars were endorsed in a proforma. 2.5 ml venous blood in ethylene diaminete traacetic acid (EDTA) was collected and blood complete count was performed by Sysmex KX-21 automated hematology analyzer. Blood smear was examined for DLC and blast morphology. Bone marrow examination (aspiration) and cytochemical staining (sudan black) was done. Flow cytometry was done on peripheral blood/ bone marrow aspirate of the patients suspected to have Acute Promyelocytic Leukaemia. 2-4 ml of venous blood was collected in EDTA. RNA was extracted from EDTA collected sample. cDNA was synthesized from extracted RNA. cDNA was constructed from 1 µg of total RNA with the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Tokyo, Japan). cDNA was amplified by using two step nested PCR in a 2 mmol/L $MgCl_2$, 200 mmol/L dNTP, 10x PCR Gold Buffer containing 15 mmol/L Tris-HCL (pH 8.0) and 50 mmol/L KCL and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). 0.2µmol/L of following primers was used.

- P1 5′-ATGCTGT-GCTGCAGCGCAT-3′
- R7 5′-CCATAGTGGTA-GCCTGAGGAC-3′

After 10 min of incubation at 95°C, PCR was performed at 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Five microliter of 50µL each from the first step PCR product was amplified with the nested primers

- P2 5′-CAGAGGATGAAGTGCTA-CG-3′
- R9 5′-GTCCTGACAGACAAAGCAAG-3′

Final PCR products were run on a 2% agarose gel electrophoresis.

Data Analysis

All the collected data was entered and analyzed through SPSS version 20. Numerical variables i.e. age was presented by mean ± SD. Categorical variables i.e. gender and positive fusion oncogenes on PCR was presented as frequency and percentage. Data was stratified for age and gender to deal with effect modifiers. Post-stratification chi-square test has been applied taking $p \leq 0.05$ as significant.

Table-I: Frequencies of three isoforms of PML RARα fusion gene in APL.

	bcr1 Isoform	bcr2 Isoform	bcr3 Isoform
	Number (%)	Number (%)	Number (%)
Positive	9 (9.28%)	26 (26.80%)	62 (63.92%)
Negative	88 (90.72%)	71 (73.20%)	35 (36.08%)
Total	97	97	97

PML: Promyelocytic leukemia protein, RAR: Retinoic acid receptor APL: Acute Promyelocytic Leukemia

Table-II: Frequencies of three isoforms of PML RARα fusion gene in APL in relation to age of patients.

	bcr1 Isoform		bcr2 Isoform		bcr3 Isoform	
	Number (%)		Number (%)		Number (%)	
	Positive	Negative	Positive	Negative	Positive	Negative
N	9	88	26	71	62	35
17-34	6 (66.67%)	35 (39.77%)	8 (30.77%)	33 (46.48%)	27 (43.55%)	14 (40%)
35-52	3 (33.33%)	40 (45.45%)	12 (46.15%)	31 (43.66%)	28 (45.16%)	15 (42.86%)
53-69	0 (0%)	13 (14.77%)	6 (23.08%)	7 (9.86%)	7 (11.29%)	6 (17.14%)
Chi-Square Test	2.997		3.619		0.666	
p-value	0.223		0.164		0.717	

n: Number, PML: Promyelocytic leukemia protein, RAR: Retinoic acid receptor APL: Acute Promyelocytic Leukemia

RESULTS

The age of the patients ranged from 17 years to 69 years with a mean of 37.87 ± 12.89 years. There were 68 (70.1%) male and 29 (29.9%) female patients. The bcr1 isoform was found in 9 (9.3%)

patients. There was no significant difference in the frequency of bcr1 isoform across age groups; 17-34 years vs. 35-52 years vs. 53-69 years (14.6% vs. 7.0% vs. .0%; *p*=0.223) and genders; male vs. female (7.4% vs. 13.8%; *p*=0.317).

The bcr2 isoform was found in 26 (26.8%) patients. There was no significant difference in the frequency of bcr2 isoform across age groups; 17-34 years vs. 35-52 years vs. 53-69 years (19.5% vs. 27.9% vs. 46.2%; *p*=0.164) and genders; male vs. female (25.0% vs. 31.0%; *p*=0.539). The bcr3 isoform was found in 62 (63.9%) patients. There was no significant difference in the frequency of bcr3 isoform across age groups; 17-34 years vs. 35-52 years vs. 53-69 years (65.9% vs. 65.1% vs. 53.8%; *p*=0.717) and genders; male vs. female (67.6% vs. 55.2%; *p*=0.242).

Table-III: Frequencies of three isoforms of PML RARα fusion gene in APL in relation to gender of patients.

	bcr1 Isoform		bcr2 Isoform		bcr3 Isoform	
	Number (%)		Number (%)		Number (%)	
	Positive	Negative	Positive	Negative	Positive	Negative
N	9	88	26	71	62	35
Male	5 (55.56%)	63 (71.59%)	17 (65.38%)	51 (71.83%)	46 (74.19%)	22 (62.86%)
Female	4 (44.44%)	25 (28.41%)	9 (34.62%)	20 (28.17%)	16 (25.81%)	13 (37.14%)
Chi-Square Test	1.002		0.377		1.372	
p-value	0.319		0.539		0.242	

n: Number, PML: Promyelocytic leukemia protein, RAR: Retinoic acid receptor APL: Acute Promyelocytic Leukemia

Table-IV: Frequent isoform in populations.

Author	Population	Year	bcr1	bcr2	bcr3
Chatterjee *et al*.[4]	India	2014	42.85%	14.28%	38.09%
Sazawal *et al*.[10]	India	2009	30%	-	70%
Ambayya *et al*.[5]	Malaysia	2014	62%	33%	5%
Susanne Schnittger *et al*[13]	Germany	2011	60.5%	35.4%	4.1%
Nikhil Rabade *et al*[14]	India	2018	55%	-	40%
Present Study	Pakistan	2016	9.3%	26.8%	63.9%

DISCUSSION

Acute Promyelocytic Leukemia (APL) is a distinct subtype of Acute Myeloid Leukemia (AML) and accounts for 5-8% of all cases[2]. Detection of PML RARα is the hallmark of diagnosis of APL[5]. It is found in more than 98% of patients of APL[3]. All trans retinoic acid (ATRA) is the mainstay of treatment of APL with a cure rate of >80%[7]. PML RARα has 3 possible isoforms. Breakpoint regions in chromosome15 having PML gene include intron 3 (L-long isoform or bcr1 isoform), intron 6 (S-short isoform or bcr3 isoform) and exon 6 (V form or bcr2 isoform). Different isoforms have different sensitivities to ATRA. bcr3 isoform is found to have a lack of sensitivity to ATRA and is also associated with a poorer outcome; high TLC and CNS relapse in a number of cases[4].

The frequency of bcr3 varied in the existing literature depending upon population under study while there was no such local published material. The objective of this study was to determine the frequencies of three isoforms of PML RARα fusion gene in APL. It was a descriptive cross sectional study conducted at Department of Hematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi 6 months after the approval of synopsis from 06 April 2015 to 05 October 2015. This study involved 97 newly diagnosed cases of APL, aged between 15-70 years from both genders. Double nested PCR was carried out by Applied

Biosystems 2720 Thermal Cycler in every patient to determine the isoform of PML RARα fusion gene. A written informed consent was obtained from every patient.

The age of the patients ranged from 17 years to 69 years with a mean of 37.87 ± 12.89 years. A similar mean age of 31.8 ± 1.68 years was observed in AML patients in another local study by Sultan *et al*. in 2015[9]. There were 68 (70.1%) male and 29 (29.9%) female patients in the study group. A similar male predominance has also been reported by Chatterjee *et al*. in 2014 (1.5:1) and Sazawal *et al*. in 2009 (61.76% vs. 38.24%) in Indian population[4,10]. Sultan *et al.* however observed a female predominance (38.5% vs. 61.5%)[9]. Similar female predominance has also been exported by Douer *et al.* (46.43% vs. 53.57%)[11].

The most frequent isoform of PML-RARα fusion gene was bcr3 which was observed in 62 (63.9%) cases followed by bcr2 (26.8%) and bcr1 (9.3%). As evident from the Table-IV, bcr1 is the most frequent isoform in populations other than Indian and Pakistani populations. However, Chatterjee *et al.* in 2014 (38.09%) and Dutta *et al.* in 2008 (72.7%) reported bcr3 isoform to be the most frequent in Indian population[4,12]. The results of these study match with those of our study where we also found bcr3 being the most common isoform. There was no significant difference in the frequency of isoforms across age groups and genders. Similar insignificant difference in the frequency of isoforms across age (*p*-value=0.99) and gender (*p*-value=0.25) groups has also been reported by Douer *et al*.[11].

Thus the most frequent isoform of PML-RARα fusion gene was bcr3 which was observed in 62 (63.9%) cases. This is the isoform which lacks sensitivity to ATRA and is also associated with a poorer outcome; high TLC and CNS relapse in a number of cases[4]. Keeping in view the higher frequency of bcr3 isoform in local population, it is advocated that patients in future practice should be screened for PML-RARα isoform and those with bcr3 isoform should be offered alternative treatments other than ATRA to decrease the risk of relapse.

The present study is first of its kind in local population and provides baseline statistical data about the isoforms of PML-RARα fusion gene. A very important limitation in the present study is that we didn't compare the outcome of patients in relation to the isoform of PML-RARα fusion gene which is a matter of future research.

CONCLUSION

The most frequent isoform of PML-RARα fusion gene was bcr3 which was observed in 62 (63.9%) cases followed by bcr2 (26.8%) and bcr1 (9.3%). There was no significant difference in the frequency of isoforms with patient's age and gender.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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