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Original article

Fatal intracranial haemorrhage in acute promyelocytic leukemia patients with short isoform of PML-RARα: Review of molecular and radiological data

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ABSTRACT

Three major *PML-RARα* fusion gene transcripts (long [bcr1], variant [bcr2], and short [bcr3]) are currently used in clinical laboratories for the diagnosis and treatment monitoring of APL patients. Despite highly improved outcome, relapse and intracranial haemorrhage that may lead to early death is still an unsolved complication in APL. We reviewed APL patients confirmed by qPCR for the presence of PML-RARα transcripts (n = 27) and studied their outcome in relation to the isoform expression at diagnosis and follow-up in King Fahad Medical City. Eight in twenty-seven patients showed bcr3 and nineteen patients with bcr1 as major isoforms at diagnosis. Half of the bcr3 patients (n = 4/8) showed early mortality, prolonged qPCR positivity, 4-fold higher neutrophil/lymphocyte ratio, higher creatinine levels, and significantly reduced relapse free and overall survival time compared with bcr1 patients. Radiological findings in bcr3 patients revealed CNS involvement in the form of intracranial haemorrhage and periventricular microangiopathy and no CNS involvement in bcr1 patients. In conclusion, *PML-RARα* isoform expression at diagnosis in selective patients influences disease course over time and may even lead to early mortality due to haemorrhage. Thus, timely reporting of the specific PML-RARα isoform by clinical laboratories and CNS assessment by radiology can prevent complications leading to death in some APL patients.

1. Introduction

Acute promyelocytic leukemia (APL), accounting for about 10–15% of acute myeloid leukemia (AML) globally (Dinmohamed and Visser, 2019; Tallman and Altman, 2008) is characterized by the presence of specific cytogenetic and molecular abnormality in the form of the most common *PML-RARα* fusion transcripts [t(15;17)(q22;q12) in 95% of patients] culminating in the differentiation block at the promyelocytic stage. The development of targeted therapies with the introduction of all-*trans*-retinoic acid (ATRA) and arsenic trioxide (ATO), advances in supportive care in addition to the technological improvements in molecular diagnostics and monitoring have been instrumental in improved outcomes in APL patients. The detection of *PML-RARα* using a highly sensitive quantitative polymerase chain reaction (qPCR) for diagnosis and minimal residual disease (MRD) monitoring after targeted therapy has been a gold standard in the workup of patients with APL (Huang et al., 1993; Lo-Coco et al., 2003).

Depending upon the breakpoint cluster region (bcr) involved, two major types of *PML-RARα* transcripts have been reported: long (bcr1, intron 6 between exon 6–7 in *PML* gene) and short (bcr3, intron 3 between exons 3–4 in *PML* gene) isoforms with only one breakpoint in intron 2 in the *RARα* gene (Liquori et al., 2020; De Thé et al., 1990; Pandolfi et al., 1992). The variant (bcr2, in exon 6 of *PML* gene) isoform is also known to occur rarely from more complex splicing (Pandolfi et al., 1992). The clinical relevance of major *PML-RARα* isoforms remains inconclusive. Previous studies

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revealed poor prognosis, shorter disease-free and over-all survival in APL patients with bcr3 than bcr1 isoform (Huang et al., 1993; Park et al., 2009; Jurcic et al., 2001). Others have reported association of *PML-RARα* isoforms with pre-treatment hematological parameters but not treatment outcome in APL (Gallagher et al., 1997). Studies in APL patients with *FLT3* mutation (*FLT3-ITD* or *FLT3-TKD*) and Central Nervous System (CNS) involvement have also revealed the presence of the bcr3 isoform (Esnault et al., 2019; Lu et al., 2014). However, a limited number of patient data that are available showed the role of *PML-RARα* isoform expression in disease progression and CNS involvement.

Herein we review and present our molecular genetics, clinical laboratory and radiological findings at diagnosis and follow-up post ATRA-ATO treatment of a series of risk-stratified APL patients from a single Tertiary-care Medical City in Saudi Arabia. To our knowledge, this is the first study form Saudi Arabia that describes patients specifically having short isoform of *PML-RARα* (bcr3) expression at diagnosis with CNS involvement, reduced molecular relapse-free/overall survival and early mortality.

2. Methods

Collection of blood specimens and processing: Patients visited the tertiary care and teaching hospital, King Fahad Medical City, Riyadh for APL workup. Blood specimens were collected in the EDTA vacutainer and stored at 4 °C until processing within 24 to 48 h. The clinical team collected bone marrow (BM) at day 0 (presentation) and day 28 post-treatment (for confirmed APL cases) as per clinical workup guidelines and sent to KFMC molecular laboratory for qPCR analysis. Peripheral blood (PB) was collected afterwards from patients with haematological remission for *PML-RARα* fusion transcript monitoring by qPCR or FISH as required. Ethical approval was obtained from the institutional ethical committee. Specimens were processed for RNA Extraction, cDNA Synthesis, and qPCR on Qiagen Rotor-Gene Q.

APL workup (diagnostic and follow-up): This included bone marrow morphology assessment at day 0, day 28 and follow-up when required, immunophenotypic analysis for specific flow-cytometry markers, hematological analysis (WBC, platelets etc.), biochemical analysis (creatinine etc.), cytogenetic and molecular genetic analyses for *PML-RARα* fusion transcripts at diagnosis and follow-up intervals. The data were collected at diagnosis (day 0), post-first induction (day 28), and every month till complete molecular remission and at 3-month follow-up intervals. Consolidation and maintenance therapy is given as per established clinical protocols. Risk stratification was done based on standard white blood and platelet count. The immunophenotypic markers used in bone marrow assessment at diagnosis and follow up were CD2, CD11b, CD11c, CD13,CD14, CD15, CD16, CD19, CD33, CD34,CD38, CD45, CD56, CD58, CD64, CD67, CD71, CD117, MPO, HLA-DR in addition to B/T-cell markers such as CD3, CD4, CD5, CD19 and CD20.

Radiological analysis: Chest X-ray and CT-Scan brain/chest was done and available for eight patients with bcr3 isoform of *PML-RARα* according to the standard hospital protocol. To rule out intracranial haemorrhage, radiological assessment through CT brain was clinically indicated in bcr3 patients who presented with severe headache, abnormal promyelocytes in PB and some bleeding complications, whereas no clear indication for radiological assessment in bcr1, was observed.

PML-RARα detection by FISH and qPCR: Our laboratory performs fluorescence in-situ hybridization (FISH) and qPCR based molecular testing using commercially available probes and kits (Abbot and IPSOGEN, Qiagen, Germany) for each case at diagnosis and MRD monitoring (data not shown). Both the assays that have been validated in-house are part of the College of American Pathologist proficiency testing (CAP-PT) for *PML-RARα*. Blood specimens were processed for RNA Extraction, cDNA synthesis, and qPCR on Rotor-Gene Q in our Molecular Pathology (Genetics) laboratory using established protocols in accordance with the manufacturer's instructions. RNA extraction is performed using QIAamp RNA Blood Mini Kit Qiagen (Hilden, Germany) and the quality and quantity measured by the Nanodrop spectrophotometer. cDNA synthesis is performed using Qiagen's ipsogen RT kit (Germany) followed by the detection and quantification of *PML-RARα* by using the Ipsogen PML-RARa kit (ipsogen® FusionQuant®) on Rotor-Gene Q 5plex HRM instrument with a 72-tube rotor (Qiagen, Hilden, Germany). The primer and probe mix are unique to the isoforms bcr1 and bcr3 tested separately with Abl1 gene used as internal control and known copy number standards used for copy number calculation and rule out any artefacts due to RNA degradation. The quantitative results are reported based on normalized copy number (NCN%) using fusion gene (bcr1 or bcr3) and control gene (Abl1) standards and extrapolating the patient specimen data by predetermined formula.

Statistical analysis: All categorical variables such as gender frequencies are expressed as percentages. Continuous variables such as age, lymphocytes, hemoglobin, APTT, fibrinogen etc. expressed as Mean ± SD while OS time, laboratory parameters such as Neutrophil-to-lymphocyte ratio (NLR), WBC, etc. presented as Median [IQR]. The Shapiro–Wilk test was used to confirm the assumption of normal distribution. If the data was biased, a non-parametric test was used. Pearson chi-square / Fisher's exact test was used to determine significant associations between categorical variables, depending on whether the cell was expected to have an expected frequency of<5. Independent sample *t*-test / Mann Whitney *U* test performed to compare the differences between bcr1 and bcr3 with factors and also outcome of the patients was compared with study factors. Kaplan–Meier survival curves were used to estimate the relapse free survival (RFS) rate and overall survival (OS) rate of the patients with respect to bcr1 and bcr3. A two-sided p-value less than 0.05 was considered statistically significant. All data was entered and analysed using the SPSS 25 Statistics Package (SPSS Inc., Chicago, Illinois, USA) and MEDCALC version 18.11.6 (Acacialaan 22 8400 Ostend Belgium). We have also taken effect size into consideration, which means that we can be very confident that there is some difference or association but it is probably small with a small sample size.

3. Results

3.1. APL patients present with variable patient demography (descriptive statistics)

Patients who came to the Emergency Department at KFMC with a suspicion of APL were sent for molecular confirmation of PML-RARα by qPCR (n = 106). The presence of PML-RARα transcripts was observed in 27 patients thereby confirming the diagnosis of APL by qPCR, with 8 patients showing bcr3 isoform and 19 showing bcr1 isoform of PML-RARα at diagnosis (Tables 1 and 2). The median age at the time of diagnosis for all APL patients was 44 years (19–74 years) and a male to female ratio of 1.1: 1 (14 males and 13 females). Most of the bcr3 patients were younger adults less than 45 years of age with a male predominance (6 male and 2 female in 8 patients, Tables 1, 2). Patients with bcr3 isoform but with early mortality had a significantly higher median age than those bcr3 patients who survived (46 ± 3.2 vs 21 ± 2.8, Tables 1, 2). The median overall survival (OS) was also significantly lower in bcr3 patients than bcr1 patients (0.84 vs 4.88, Tables 2).

Table 1
Risk stratification of APL patients based on Sanz score and *PML-RARa* isoform expression.

PML-RARα isoform at diagnosis	Case#	Age	Gender	WBC (4.3–11.3 × 10e9/L)		Platelets (155–435 × 10e9/L)	
				Day 0	Day 28 post treatment	Day 0	Day 28 post treatment
bcr3	1	23	m	**15.5*****	1.19	70	695
	2^{X}	48	m	**123.1*****	0.06	10	28
	3	43	m	1.21	1.85	7	346
	4 ^{X}	71	f	1.69	3.48	11	88
	5	19	f	2.06	6.33	5	121
	6 ^{X}	44	m	**156.2*****	NA	38	NA
	7	19	m	**21.9*****	1.24	7	316
	8 ^{X}	43	m	**26.7*****	NA	8	NA
bcr1	9	56	f	**115*****	11.2	14.2	31.8
	10	74	m	**16.6*****	2.2	27.6	1096
	11	57	f	**23.8*****	2.43	32	269
	12	35	f	**128.9*****	0.84	12	257
	13	53	f	**69.54*****	0.54	31	80
	14	23	f	**77.7*****	2.8	12	230
	15	50	f	**23.8*****	1.31	14	41
	16	24	f	3.46	2.24	7	66
	17	46	f	1.86	2.43	25	382
	18	37	f	1.01	1.22	11	179
	19	48	m	0.78	1.28	26	171
	20	43	f	1.5	3.38	29	77
	21	29	m	1.06	2.22	4	53
	22	17	m	3.71	4.2	27	65
	23	32	m	0.515	7.02	10.9	68
	24	49	m	1.17	2.17	27	70
	25	44	m	0.66	2.35	41	390
	26	5	m	5.27	1.4	37	92
	27	36	f	0.79	3.3	9	179

Note: *** indicates high risk based on Sanz score; NA, not available; ^{X} indicates patients who died;
m is male and f is female.

Table 2A
Comparative laboratory biomarkers of bcr1 and bcr3 APL patients at presentation and of bcr3 patients who survived vs dead.

Variables	Description	bcr1 (n = 19)	bcr3 (n = 8)	bcr3 survived	bcr3 died	Reference Range /Unit
Gender	Male	8	6	3	3	
	Female	11	2	1	1	
Age (years)	Median ± SD	46 ± 14.8	43 ± 17.7	21 ± 11.48	46 ± 13.17	Years
OS Time (p<*0.002)	Median [IQR]	4.88[6.43–2.8]	0.84[2.43–0.02]	4.1[5.77–2.36]	0.03[0.16–0]	Years
Hematology/Biochemistry laboratory parameters: baseline at presentation						
WBC (p<*0.06)	Median [IQR]	1.86[23.75–1.01]	18.69[99–1.78]	2.06[21.88–1.06]	74.9[147.94–7.94]	(4.3–11.3 × 10e9/L)
Platelets	Median [IQR]	26[29 – 11]	9[31.25–7]	25[29 – 7]	10.5[31.25–8.5]	(155–435 × 10e9/L)
NLR	Median [IQR]	0.25[0.42–0.18]	1.05[1.97–0.19]	0.28[0.51–0.18]	1.94[9.67–0.51]	Ratio
LDH (p < 0.09)	Median [IQR]	293[512 – 230]	416[1352 – 283]	293[512 – 230]	1352[0–326]	(125–220 U/L)
Creatinine (p < 0.03)	Median [IQR]	65[76 – 60]	102[198.5–60.5]	65[80 – 60]	188[246.5–128]	(49–90 μmol/L)
Hemoglobin Hb	Mean ± SD	8.63 ± 1.34	9.05 ± 2.94	8.64 ± 1.96	9.43 ± 2.25	(11–15 g/dl)
DDimer	Median [IQR]	20[34.98–11.4]	15.09[36.09–5.8]	20[34.98–11.2]	14.85[35.65–5.8]	(0–0.5 ug/ml)
Fibrinogen	Mean ± SD	1.66 ± 0.63	1.73 ± 0.7	1.7 ± 0.59	1.6 ± 0.96	(1.61–4.39 g/L)
ALT	Median [IQR]	20[28 – 12]	27[45.75–16.25]	20[32 – 13]	34[55.75–13.75]	(0–55 U/L)
AST	Median [IQR]	24[32 – 17]	24.5[48.5–17.25]	23[32 – 17]	32[86–18.5]	(5–34 U/L)

Note: Categorical data presented as frequency (%) while continuous data expressed as Mean ± SD & Median [IQR: Interquartile Range, Q3 – Q1]; * shows that P-value is significant at P < 0.05; WBC, White blood cells; NLR, Neutrophil to Lymphocyte ratio; LDH, Lactate dehydrogenase.

Table 2B
Comparative laboratory biomarkers of bcr1 and bcr3 APL patients at day 28 and of bcr3 patients who survived vs dead.

Variables	Description	bcr1 (n = 19)	bcr3 (n = 8)	bcr1/bcr3 survived (n = 23)	bcr1/bcr3 died (n = 4)	Reference Range /Unit
Hematology/Biochemistry laboratory parameters: at day 28 post first induction treatment						
WBC	Median [IQR]	50.8[72.5–42.1]	33.85[76.9–22.1]	2.22[3.33–1.24]	1.77[0–0.06]	(4.3–11.3 × 10e9/L)
Platelets	Median [IQR]	32.71 ± 19.5	23.95 ± 19.32	171[346 – 68]	58[0–28]	(155–435 × 10e9/L)
NLR	Median [IQR]	1.55[1.99–0.18]	1.41[1.97–0.19]	1.5[1.97–0.19]	35.9[38.4–10.5]	Ratio
LDH	Median [IQR]	9.59 ± 1.21	10.23 ± 1.52	272[323.5–226]	265[265–265]	(125–220 U/L)
Creatinine	Median [IQR]	3.5[11.4–1.5]	1.44[3.19–0.7]	52[64 – 38]	225[0–142]	(49–90 μmol/L)
Hemoglobin Hb	Mean ± SD	9.59 ± 1.21	10.23 ± 1.52	9.78 ± 1.23	9.75 ± 2.62	(11–15 g/dl)
DDimer	Median [IQR]	293.5[348 – 236]	240[261 – 204]	2.1[6.9–1.4]	4.15[0–0.8]	(0–0.5 ug/ml)
Fibrinogen	Mean ± SD	4.41 ± 1.97	3.31 ± 1.73	4.09 ± 1.89	4.13 ± 3.21	(1.61–4.39 g/L)
ALT	Median [IQR]	35[68 – 20]	32.5[41.75–20.5]	35[64 – 23]	28.5[0–13]	(0–55 U/L)
AST	Median [IQR]	33[63 – 18]	21[52.5–19.5]	33[63 – 19]	21 (Gameiro et al., 2001)	(5–34 U/L)

Note: Categorical data presented as frequency (%) while continuous data expressed as Mean ± SD & Median [IQR: Interquartile Range, Q3 – Q1]; WBC, White blood cells; NLR, Neutrophil to Lymphocyte ratio; LDH, Lactate dehydrogenase.

3.2. Risk stratification of patients

The patients were classified into three risk group categories according to Sanz score based on WBC and platelets count (Sanz et al., 2000). There were 10 patients at high-risk (***), 16 patients at intermediate-risk (*) and 1 patient at low-risk. 5 in 8-patients with bcr3 isoform and 7 in nineteen- patients with bcr1 isoform at diagnosis were in the high-risk group (Tables 1 and 2).

3.3. PML-RARα isoform characteristics

While FISH detects the *PML-RARα* fusion transcripts without the ability to distinguish the isoforms, qPCR however, was used to detect two main *PML-RARα* isoforms: the long (bcr1/L-) and the short (bcr3/S-) isoform (Fig. 1A, 1B). The low positive and high positive standards serve as controls (LP* and HP*** in Fig. 1B) in each run. Based on *PML-RARα* transcript isoform expression at diagnosis, APL patients were grouped into two: bcr3 and bcr1 patients. The representative Karyotype, FISH and qPCR data for *PML-RARα* are shown (Fig. 1B-panels a, b, c, d). Molecular genetic analysis by qPCR revealed bcr1 and bcr3 as major isoforms at diagnosis in nineteen and eight of twenty-seven patients, respectively (Table 1). APL patients who died early had bcr3 isoform at diagnosis (Table 1 and Supplementary Figure S1A, panels #2, #4, #6, #8).

3.4. Clinical laboratory characteristics, therapy response and clinical outcome in APL patients

Comparative routine clinical laboratory analyses of bcr3 and bcr1 APL patients at presentation and post treatment at day 28 are summarized (Tables 1 and 2). The data revealed high median [IQR] WBC in bcr3 vs bcr1 patients (18.69[99–1.78] vs 1.86 [23.75–1.01], Table 1) indicating a 10-fold difference, very low median [IQR] platelet counts in bcr3 vs bcr1 patients (9[31.25–7] vs 26[29–11], Table 1) indicating 2.8-fold difference and high median [IQR] NLR in bcr3 vs bcr1 patients (1.05[1.97–0.19] vs 0.25 [0.42–0.18], Table 1) indicating 4-fold difference at presentation (Fig. 2a). APL bcr3 patients also showed higher median [IQR] LDH, creatinine and ALT at presentation vs bcr1 patients (Table 2, Supplementary S3B). All the APL patients (n = 23/27, 85%) except those with bcr3 who died early achieved hematological remission post treatment at day 28 (Tables 1, 2 and Fig. 2b).

3.5. Prolonged qPCR positivity, early reappearance of PML-RARα post treatment, reduced relapse free and overall survival in bcr3 patients

Since qPCR is a gold standard in monitoring treatment response of APL patients, we next analysed *PML-RARα* transcript expression at day 28 post-treatment. FISH and/or qPCR showed detectable levels of bcr3 not only at day 28 but also at day 60 post treatment in some (Supplementary Table S1, representative case shown in Fig. 3A). This prolonged qPCR positivity was not observed in bcr1 patients, who went into molecular remission with undetectable levels of the transcript at day 28 (Supplementary Table S1, representative case shown in Fig. 3B). Monitoring of *PML-RARα* transcript expression over an extended period revealed that bcr3 APL patients exhibit earlier reappearance of the isoform than bcr1 APL patients (Median 2 ± 0.2 years for bcr3 vs 5 ± 0.2 years for bcr1, Fig. 3C and Supplementary Figure S1B). Furthermore, Kaplan–Meier survival curve estimation revealed significantly shorter RFS and OS in bcr3 compared with bcr1 patients (Fig. 4A, 4B and Supplementary S3A).

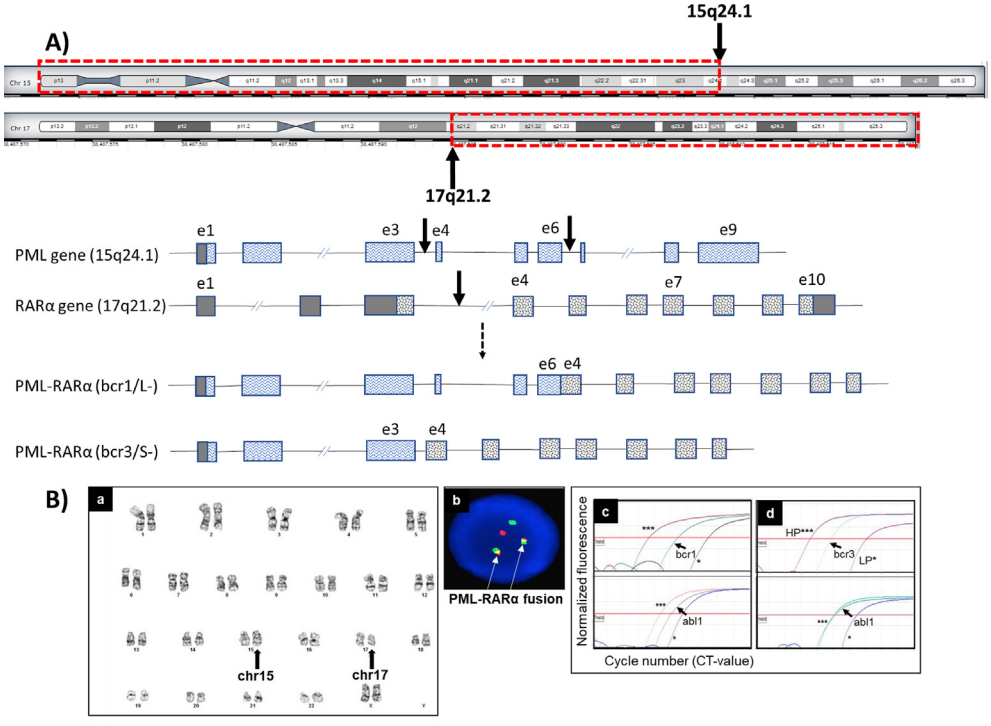

Fig. 1. Genomic browser image with exon map showing different PML-RARα isoforms and their detection by karyotyping, FISH and qPCR at diagnosis: A) upper panel-chromosome 15 and 17 map showing the breakpoints (arrow) involved in translocation t(15;17) with the regions shown (dashed red box), lower panel-detailed exon map of the *PML* and *RARα* genes on respective chromosomes with coding exons (e1, e2, e3 etc., texture boxes) and non-coding exons (solid boxes). The arrows represent the breakpoints at respective locations to generate the two isoforms of *PML-RARα* bcr1 (long L- isoform) and bcr3 (short S- isoform), **B)** representative APL case showing detection of *PML-RARα* at diagnosis by Karyotype (a), FISH (b) and qPCR for bcr1 (c) and bcr3 (d) on RotorGene instrument using commercially available kits (Ipsogen Qiagen, Germany). The graph shows a high positive HP (***), low positive LP (*) and abl1 gene as internal control used in each qPCR run.

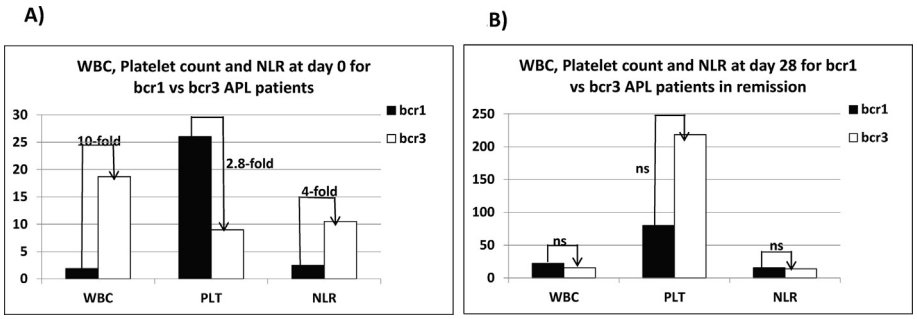

Fig. 2. Clinical laboratory markers WBC, PLT and NLR at presentation and at day 28 post induction treatment for bcr1 vs bcr3 APL patients: A) WBC, platelet (PLT) counts and NLR median values at presentation (day 0) for bcr3 vs bcr1 APL patients, **B)** WBC, platelet (PLT) counts and NLR median values post induction treatment (day 28) for bcr3 vs bcr1 APL patients. The fold changes are shown, ns means not significant changes since APL patients go into remission post induction.

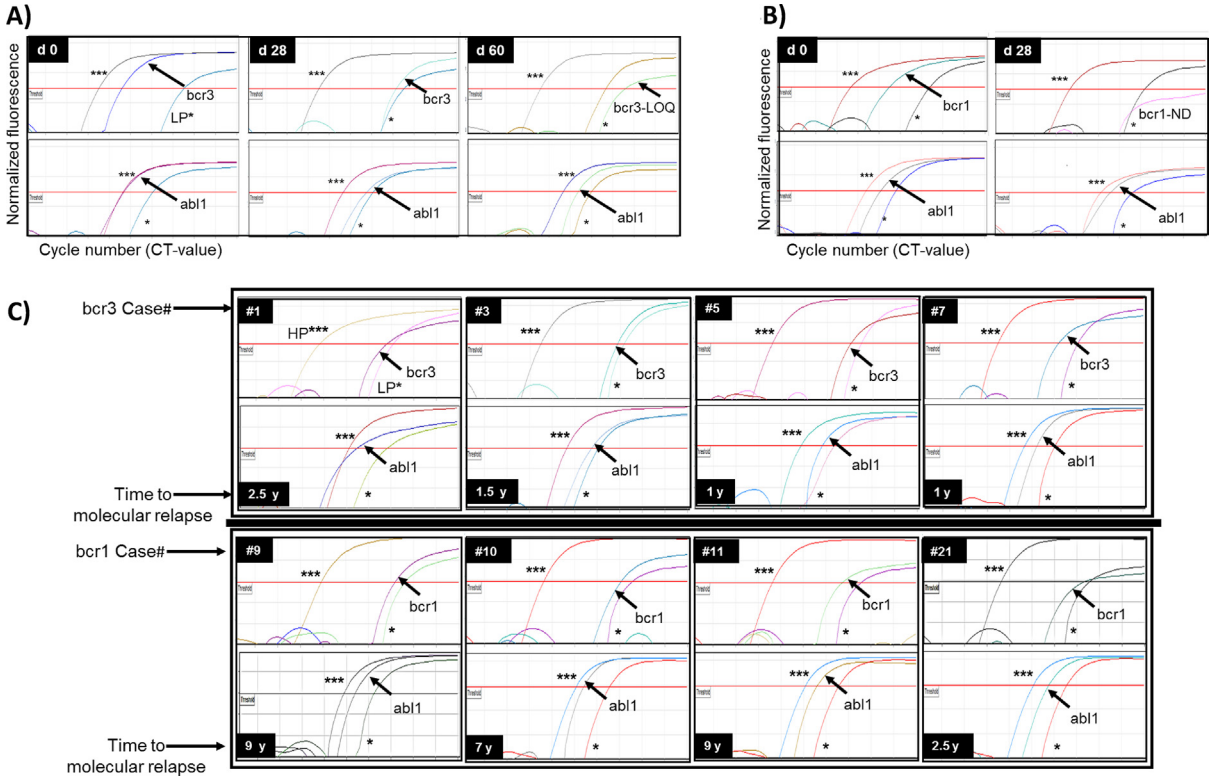

Fig. 3. Prolonged qPCR positivity post induction and early reappearance (time to molecular relapse) of PML-RARα in bcr3 vs bcr1 patients: A) representative APL case showing qPCR data for bcr3 at diagnosis (day 0), and post treatment monitoring at day 28, and at day 60 showing isoform detected but below level of quantification (LOQ, positivity), **B)** representative APL case showing qPCR data for bcr1 at diagnosis (d 0), and post treatment monitoring at d 28 showing isoform not detected (ND), Arrows show bcr3/abl1 and/or bcr1/abl1 detection, **C)** qPCR monitoring data over extended period of time for bcr3 vs bcr1 APL patients. # on the upper left side of the figures represent representative case numbers and numbers on the lower left side represents time for the reappearance of bcr3 and/or bcr1 isoform (time to molecular relapse). The graphs also show a high positive HP (***), low positive LP (*) and abl1 gene as internal control used in each qPCR run. An arrow also represents amplification of bcr3 and/or bcr1.

3.6. Abnormal imaging findings in APL patients with bcr3 isoform of PML-RARα at presentation

We next analysed radiological images of the APL patients at presentation. We found that CT-scans were mainly requested for bcr3 patients due to their CNS involvement at presentation. The findings in bcr3 patients revealed various abnormalities such as right cerebellar hematoma pulmonary edema, periventricular microangiopathic changes, pulmonary opacities, bilateral pleural and pericardial effusion, intracranial haemorrhage in right occipital and right medulla, left intraventricular and bilateral orbital haemorrhage, left cerebellar hypodensity, hepatomegaly and nodular opacities (Fig. 5A and Supplementary Figure S4A, S4B, S4C).

Collectively, we present and interpret the clinical, molecular and radiological findings of the APL patients with different *PML-RARα* isoforms at the time of diagnosis and during monitoring of the treatment outcomes. These interpretations suggest that selective *PML-RARα* transcript expression is not a coincidence but seems to impact disease course in APL patients over time.

4. Discussion

Here, we present comprehensive morphological, clinico-pathological, imaging and molecular real-time data, without any potential study bias of APL patients who visited King Fahad Medical City for clinical work-up and management. We

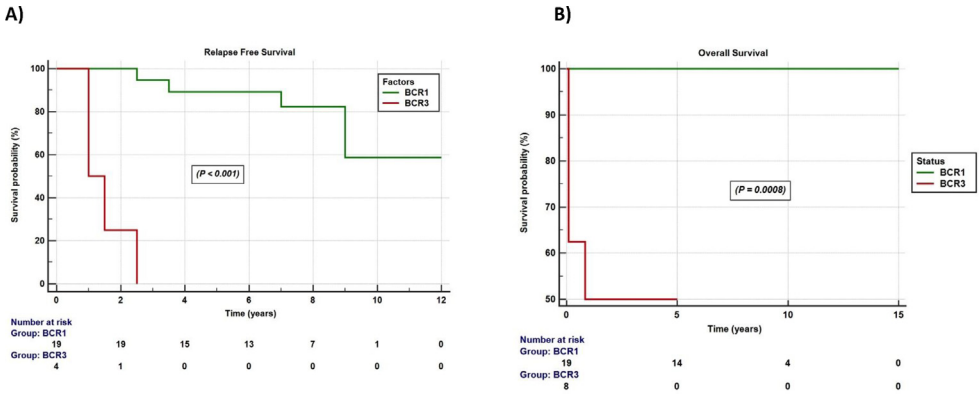

Fig. 4. Reduced relapse free survival (RFS) and overall survival (OS) in bcr3 vs bcr1 APL patients: Kaplan Meier-based estimation of RFS (4A) and OS probability (4B) in bcr3 vs bcr1 patients ($p < 0.05$). Red lines represent bcr3 patients with reduced survival compared with green lines representing bcr1 patients. Numbers at risk for bcr1 and bcr3 groups are shown at the bottom. Note that four patients that showed early mortality were excluded in the RFS analyses.

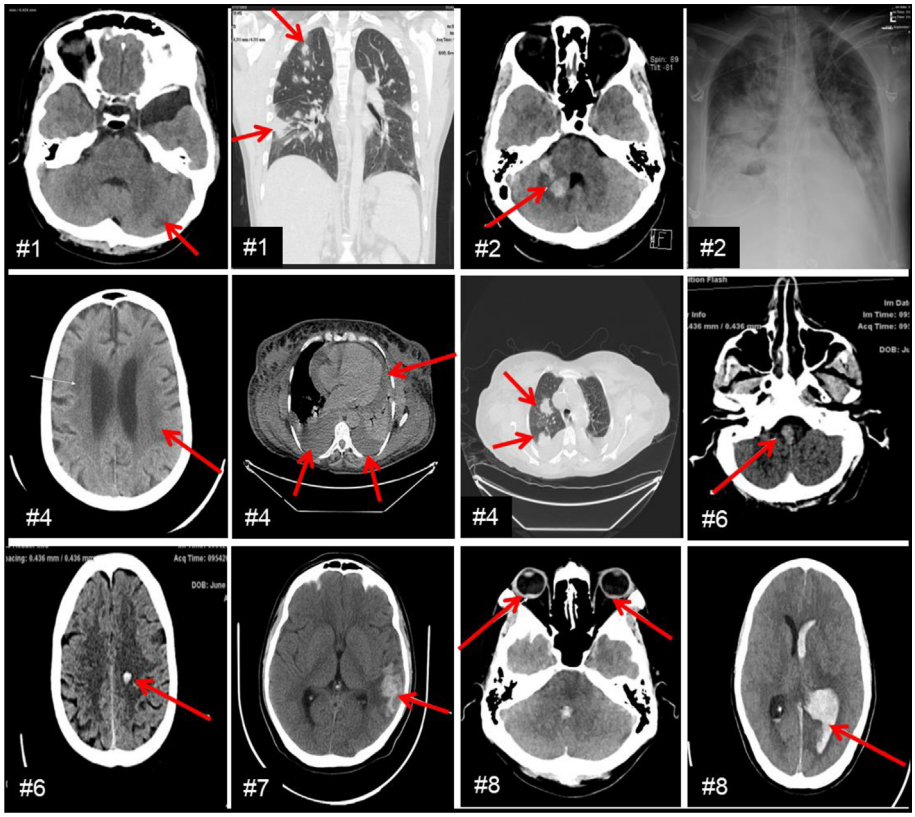

Fig. 5. Abnormal imaging findings in APL patients with bcr3 isoform of PML-RARα at presentation: A) Brain, chest, abdomen computerized tomography (CT) scans, and chest X-rays in bcr3 patients showing various abnormalities such as left cerebellar hypodensity, hepatomegaly and nodular opacities (arrows #1), right cerebellar hematoma and pulmonary edema (arrows #2) periventricular microangiopathic changes, pulmonary opacities, bilateral pleural and pericardial effusion (arrows #4), intracranial haemorrhage in right occipital and right medulla, pulmonary edema (arrows #6), left cerebral hematoma (arrows #7), left intraventricular and bilateral orbital haemorrhage (arrows #8).

observed that APL patients displayed different disease course and outcome that in part, depends on *PML-RARα* transcript expression at diagnosis. The two key points evident from the data presented here are first, APL patients with the short isoform of *PML-RARα* at the time of diagnosis exhibit high early mortality with abnormal imaging in the form of CNS complications, prolonged PCR-positivity, molecular relapse and reduced overall survival; second, the data raise the possibility of incorporating *PML-RARα* isoform expression at diagnosis into APL risk-stratification system and the use of CNS prophylaxis is implicated.

APL patients are typically risk-stratified based on WBC and platelet count into three groups according to the Sanz score (Sanz et al., 2000): a) low risk group if WBC count is $\leq 10 \times 10^9$/L, platelet count $> 40 \times 10^9$/L; b) intermediate risk group if WBC count is $\leq 10 \times 10^9$/L, platelet count $\leq 40 \times 10^9$/L; and c) high risk group if WBC count is above 10×10^9/L. In this risk categorization (Fig. 2) we observed significant fold changes in baseline WBC, platelet counts and NLR when individual patient data were analysed between bcr3 vs bcr1 APL patients.

The overall survival rate of about 86% after 5 years with high probability of relapse in high-risk APL undergoing treatment with

a combination of ATRA/ATO and cytotoxic chemotherapy has been reported (Abaza et al., 2017; Iland et al., 2015; Lancet et al., 2016). Approximately 10–20% of APL patients relapse irrespective of their risk status (Tallman et al., 2002). Very few studies have been published that have correlated APL prognosis with *PML-RARα* isoform expression (Liquori et al., 2020). Despite discrepant results of these studies, the most plausible theory that correlates with the presentation and outcome in our patients is the one of poor prognosis and an aggressive disease course in bcr3 patients (Huang et al., 1993; Park et al., 2009; Jurcic et al., 2001). The support for this theory is not just speculative but comes directly from the way our APL patients behaved at the time of diagnosis, post-induction treatment and monitoring over years with a combination of clinical, morphological and molecular genetic analyses.

First, bcr3 patients presented with high WBC, very low platelet count, high NLR, high LDH, high creatinine and ALT (Table 1). Similar data for WBC were presented by PETHEMA group and others although they had included bcr2 isoforms of *PML-RARα* in their study (Gonzalez et al., 2001; Gupta et al., 2004). The unique finding in our study is high median [IQR] NLR in bcr3 vs bcr1 patients.

Second, bone marrow morphology in bcr3 patients (Supplementary Table S2), which is slightly distinct from the study by PETHEMA (29% microgranular morphology reported) group (Gonzalez et al., 2001) revealed hypergranular promyelocytes with bilobed nuclei and auer rods in all the cases (100%), and morphology in bcr1 patients displayed both hypogranular/hypergranular promyelocytes. Furthermore, CD2 marker was not found in any bcr1 patient but in three bcr3 patients, two of whom passed-away (Table S2). CD34 was not found in bcr3 patients but in many bcr1 patients where it is rare (Gonzalez et al., 2001).

Third, bcr3 patients exhibited prolonged qPCR positivity post-treatment and early reappearance of the *PML-RARα* isoform after first molecular remission. In accordance with other published studies revealing 90% complete molecular remission (CMR) after induction therapy (Gonzalez et al., 2001; Gupta et al., 2004; Daver et al., 2015; Gameiro et al., 2001), we obtained similar results with the difference that bcr3 patients went into CMR only after 28-day post induction in contrast to bcr1 patients (10 in thirteen $\sim$ 76%) who achieved CMR within first month after induction treatment. There is no formal data to conclude that this prolonged qPCR positivity in bcr3 will impact treatment decision, although the most recent recommendations do not indicate change in standard APL therapy based on the *PML-RARα* isoform (Sanz et al., 2019). With the accumulation of more cases showing poor prognosis of bcr3 patients, updates in these recommendations may be warranted. Prolonged qPCR positivity of bcr3 patients followed by shorter RFS certainly points to predict increased risk of relapse and poor prognosis as suggested by other studies (Pandolfi et al., 1992).

Fourth, bcr3 patients exhibited very high early mortality (n = 4/8) compared with bcr1 patients who all survived (Supplementary Table S1). APL in general, is characterized by an abnormally high early mortality with bleeding complications and differentiation syndrome in patients undergoing APL treatment (Park et al., 2011). Studies from Sweden and Brazil have reported high early death rate of about 29%, which is higher than in Europe (Lehmann et al., 2011; Jacomo et al., 2007). Age ($\geq$55) was one factor contributing to early death (De Botton et al., 1998). Three of our high-risk bcr3 patients who died were young (<50) and the one with an age of 71 years was intermediate risk but with pathogenic mutations in *FLT3* and *DNMT3A* genes, that could have contributed in part to ATRA resistance, due to disruption of nuclear bodies and preventing PML-RARα degradation (Esnault et al., 2019). It is interesting to note that *FLT3* mutations are frequently identified in patients with newly diagnosed APL, are associated with higher initial WBC and short PML-RARα isoform bcr3 (Picharski et al., 2019; Xue et al., 2008; Jimenez et al., 2020). ATRA syndrome is another most common and potentially life-threatening complication in APL treatment (Jimenez et al., 2020). Both bcr3 and bcr1 patients developed ATRA syndrome with clinical symptoms of pulmonary infiltrates, embolism, long QT interval, vasculitis, persistent fever and urinary tract infections. However, bcr3 patients with early death within less than a month of diagnosis did not even complete or receive the treatment and others who developed ATRA syndrome are still in event free survival suggesting that bcr3 isoform expression at diagnosis may have a direct role in the patient outcome. It is possible that the mechanism in which bcr3 isoform is generated such as from a derivative chromosome 17 due to submicroscopic insertion of the *PML* gene into *RARα* locus [ins (Tallman et al., 2002)(17;15)] as reported in one case (Wang et al., 2009). In a transgenic mouse model, bcr3 isoform significantly increased the penetrance of APL in mice expressing *Pml-Rarα* suggesting its role in leukemogenesis (Pollock et al., 1999). Early death due to CNS hemorrhage, pulmonary bleeding, sepsis and multiorgan failure have been reported for APL (Lehmann et al., 2011). However, CNS involvement in APL patients with respect to the *PML-RARα* isoform in question has not been reported. We here show for the first time, evidence of abnormal imaging findings in bcr3 patients with CNS involvement in the form of intracranial hemorrhage and ischemic micro-angiopathy in addition to hepatomegaly, bilateral perihilar opacities, atelectasis, pleural effusions and pulmonary embolism. Radiological assessment through CT-brain to rule-out intracranial haemorrhage was clinically indicated in bcr3 patients who presented with severe headache, abnormal promyelocytes in the PB and bleeding complications. Clinical presentation in bcr1 patients did not warrant radiological assessment, and whenever indicated and performed in patients with associated co-morbidities, was unremarkable. This observation is quite significant since 4 in 8 bcr3 and none in 19 bcr1 patients presented with haemorrhage. Alternatively, these data indicate that bcr3 isoform of PML-RARα may predict CNS involvement and warrant immediate radiological assessment to prevent complications in these patients. Furthermore, CNS involvement in bcr3 patients may be an important consideration for the use of CNS prophylaxis in these APL patients. According to the recent recommendation from an expert panel of European LeukemiaNet, CNS prophylaxis should be restricted to patients with WBC counts > 10 $\times$ 10 e9/L, or to for those who have CNS hemorrhage (Gameiro et al., 2001). CNS involvement in bcr3 patients with high WBC counts would make them ideal candidates for CNS prophylaxis.

In conclusion, *PML-RARα* isoform expression at diagnosis in selective patients has an impact on CNS involvement, prolonged qPCR positivity, molecular relapse and disease course over time, and may even lead to early mortality due to haemorrhage. Thus, timely reporting of the specific PML-RARα isoform by clinical laboratories and CNS assessment by radiology can prevent complications leading to death in some APL patients. Whether *PML-RARα* isoform expression at diagnosis should be incorporated into APL risk-stratification system and help devise personalized treatment regimens, use of CNS prophylaxis to prevent early thrombo-hemorrhagic death remains an open question for future studies. If this hypothesis holds true, then clinical laboratories need to devise standardized reporting methodologies for *PML-RARα* isoform expression at diagnosis and follow-up, and not just report 'Detected/Not detected' as is the current practice. In Saudi Arabia, we have started this practice at King Fahad Medical City and we encourage other laboratories performing such testing to unify the reporting system.

Authorship List

PZ AA (KFMC) and MA (KSU) are the Principal Investigators of the project from respective institutions, performed data analyses, involved in conceptual design, writing and editing of the manuscript.

HO, is a Master of Science student involved in project.

MS is a co-supervisor and co-PI of the Masters student involved in editing the manuscript.

SB is a Biostatistician at KFMC who performed statistical analysis.

AAS is a Consultant Hematopathologist (MD physician) who analysed and interpreted morphology.

SBH is an assistant professor of Clinical Haematology/Cancer and Genomic Sciences at KSU, who was involved in editing the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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