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Original Article

Molecular Heterogeneity in Acute Promyelocytic Leukemia - a Single Center Experience from India

Nikhil Rabade[1#], Goutham Raval[1#], Shruti Chaudhary[1#], PG Subramanian[1], Rohan Kodgule[1], Swapnali Joshi[1], Prashant Tembhare[1], Syed K. Hasan[1], Hasmukh Jain[2], Manju Sengar[2], Gaurav Narula[2], Shripad Banavali[2], Pratibha Amare Kadam[3], Dhanalaxmi Shetty[3], Sumeet Gujral[1] and Nikhil Patkar[1].

[1] Hematopathology laboratory, Department of Pathology, Tata Memorial Centre, Mumbai.
[2] Department of Medical Oncology, Tata Memorial Centre, Mumbai.
[3] Department of Cancer Cytogenetics, Tata Memorial Centre, Mumbai.

[#] These authors contributed equally to this work.

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Abstract. Atypical breakpoints and variant APL cases involving alternative chromosomal aberrations are seen in a small subset of acute promyelocytic leukemia (APL) patients. Over seven different partner genes for RARA have been described. Although rare, these variants prove to be a diagnostic challenge and require a combination of advanced cytogenetic and molecular techniques for accurate characterization. Heterogeneity occurs not only at the molecular level but also at clinico-pathological level influencing treatment response and outcome. In this case series, we describe the molecular heterogeneity of APL with a focus on seven variant APL cases from a single tertiary cancer center in India over a period of two and a half years.

We discuss five cases with *ZBTB16-RARA* fusion and two novel *PML-RARA* variants, including a Bcr3 variant involving fusion of *PML* exon4 and *RARA* exon3 with an additional 40 nucleotides originating from *RARA* intron2, another involving exon 6 of *PML* and exon 3 of *RARA* with addition of 126 nucleotides, which mapped to the central portion of *RARA* intron 2. To the best of our knowledge, this is the first case series of this kind from India.

Keywords: Acute promyelocytic leukemia, Molecular subtypes, APL variants, ZBT16-RARA fusion, APL rare translocations.

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Correspondence to: Dr. Nikhil Patkar. Clinician Scientist and Assistant Professor. Hematopathology lab, ground floor, CCE building, Tata Memorial center, ACTREC, Kharghar, Navi Mumbai. E-mail: nvpatkar@gmail.com

Introduction. APL results from balanced reciprocal translocation, t(15;17)(q22;q12) which leads to fusion of promyelocytic leukemia (*PML*) and retinoic acid receptor alpha (*RARA*) genes.[1,2] The fusion is commonly caused by breakpoints in intron 2 of *RARA* and any one of the three breakpoint cluster regions in *PML*, intron 6 (Bcr1), exon 6 (Bcr2) or intron 3 (Bcr3) leading to long, variable and short isoforms respectively.[3] The *PML-RARA* fusion protein not only leads to block in differentiation and leukemic transformation in APL but also mediates the response to all-trans retinoic acid (ATRA) therapy.[1,2]

Approximately 8% of APL cases lack the characteristic t(15;17)(q22;q12) translocation on cytogenetic testing.[3] These include cases with

cryptic translocations or those with variant *RARA* translocations. Variant translocations involve fusion of *RARA* with one of eleven partner genes which include *PLZF or ZBTB16* (chromosome 11q23), *NPM* (5q35), *NuMa (*11q13), *STAT5b* (17q21.1-21.2), *PRKAR1A* (17q24), *FIP1L1* (4q12) and *BCOR* (X).[4] Recently *OBFC2A, NABP1* and *IRF2BP2* have also been reported to fuse with *RARA*. *ZBTB16-RARA* fusion is a rare entity seen in a minority of patients with APL. Unlike classical APL, variants involving *ZBTB16* lack the differentiation response to ATRA therapy and carry an unfavorable prognosis.[5]

Identification of the above mention fusion genes requires a combination of FISH, conventional karyotyping and molecular techniques, which are not readily available in most diagnostic laboratories in India. The scarcity of diagnostic centers possessing these advanced techniques, coupled with the rarity of this disease has resulted in lack of data describing such cases from India. Thus, we illustrate the molecular heterogeneity of APL from a tertiary cancer center in India over a period of two and a half years. We describe the clinicopathological features of variant APL cases along with two novel *PML-RARA* variants.

Materials and Methods. Cellular morphology was assessed by Wright-stained peripheral blood/bone marrow aspirate smears. A ten-color antibody panel was used for RQ-PCR flow cytometric immunophenotyping (FCI). Bone marrow was processed using bulk lyse-stain-wash method and analyzed on Beckman Coulter Navios flow cytometer (Beckman Coulter, USA) using the Kaluza analysis software. FISH was performed on interphase cells using a dual fusion probe for detecting *PML-RARA* fusion (Abbott Molecular LSI), and a *RARA* break apart rearrangement probe (Abbott Molecular LSI) for *RARA* variant detection.

RNA extraction and cDNA synthesis. RNA was extracted from bone marrow samples after red blood cell (RBC) lysis using RNA blood mini kit (Qiagen, Germany). cDNA was obtained from RNA using the 'High capacity' reverse transcriptase cDNA synthesis kit (Applied Biosystems, CA, USA)

Fusion transcript detection and Real-time quantitative PCR (RT-qPCR). ZBTB16-RARA fusion transcript was identified using a previously described real-time quantitative PCR(RT-qPCR) assay on a Roche Light Cycler 96.[6] The delta-delta Ct method was applied to determine the post-induction *ZBTB16-RARA* fusion transcript copy number.[7] Each sample was run in triplicate along with *ABL1* as the control gene.

The *PML-RARA* fusion transcript was identified by using the reverse transcriptase PCR (RT-PCR) BIOMED-1 protocol as described by van Dongen et al..[8] Plasmid standards for the novel *PML-RARA* variant, ranging from 10^6 to 10^1 were prepared in-house by cloning RT-PCR amplicons (see below). We followed Europe against cancer (EAC) guidelines for primer, probe design and RT-qPCR assay for quantifying *PML-RARA* fusion transcripts.[9]

Sequencing and cloning. The *PML-RARA* variant RT-PCR products were purified with a SapExo solution (Life Technologies, CA, USA) and incubated at 37 degrees C for 15 min followed by 80C for 15 mins. Purified products were cloned into a pJET 1.2 vector by using the Clone JET PCR Cloning kit (Thermo Scientific, USA) to give a final volume of 20 ul of cloned product. TOP HAT DH5alpha competent cells (Invitrogen, CA, USA) were transformed with 10ul of cloned product and selected for recombinants by antibiotic selection. DNA from the resultant clones was extracted by using a QIAprep Spin Miniprep Kit (Qiagen, Germany) and quantified by NanoDrop 2000 spectrophotometer. The PCR product and the cloned product were sequenced by Sanger sequencing. The following sequences from Ensemble were used as reference: PML-001 (ENST00000268058.7) and RARA-007 (ENST00000425707.7).

Long-range genomic DNA PCR. Long-range PCR for the *PML-RARA* variant was performed by using TaKaRa LA Taq kit according to the manufacturer's protocol. Briefly, 2 ul of genomic DNA (gDNA) was used as a template and subjected to PCR at 94C for 1 min, 98C for 10 sec & 68C for 15 mins (30 cycles) and 72C for 10 mins. One forward primer spanning PML exon 3 and eight reverse primers for RARA intron 2 were used for PCR.[10] The PCR products were run on a 1% agarose gel and DNA from the required band

was extracted by using QIAquick Gel Extraction kit (Qiagen, Germany). The extracted DNA was subjected to Sanger sequencing using the same primers.

Results. A total of 180 new cases of APL were diagnosed at our institute between January 2015 and June 2017. These included 34 pediatric and 146 adult cases with a median age of 10 years (range 2 – 16 years) and 34 years (range 17 – 71 years) respectively. There were 20 males and 14 females (male: female ratio – 1.4:1) in the pediatric age group, 78 males and 68 females (male: female ratio – 1.1:1) in the adult age group. Distribution of cases as per the transcript type is discussed in **Figure 1**. The purview of this case series is in five cases with *ZBTB16-RARA* fusion and two *PML-RARA* variants along with their clinical and morphological details.

Table 1. Clinico-pathological features of APL cases.

		Case 1 ZBTB16-RARA	Case 2 ZBTB16-RARA	Case 3 ZBTB16-RARA	Case 4 ZBTB16-RARA	Case 5 ZBTB16-RARA	Case 6 Novel Bcr3 variant	Case 7 Bcr2 variant
Age (years) & Sex		15, Male	38, Male	45, Male	36, Male	22, Male	35, Male	27, Female
Hemoglobin gm/dl		9.9	NA	NA	11.8	4.3	8.2	10.3
Total leukocyte count X10^9/L		64.94			4.86	76.99	20.00	19.88
Platelets X10^9/L		63			109	124	150	29
PT(seconds)		18.8 (control 13.4)			16.1 (control 14)	16.4 (control 12)	22.2 (control 13.4)	16.8 (control 13.4)
INR (normal range 0.8-1.2)		1.48			1.17	1.2	1.8	1.3
APTT (seconds)		29.9 (control 29.5)			26.1 (control 28.5)	26.1 (control 28.5)	31.6 (control 29.5)	23.5 (control 29.5)
Fibrinogen mg/dl (normal range 180-320)		386.5			NA	316.3	259.6	187.6
Morphology		Abnormal promyelocytes with a regular round to oval nuclei, without the classical bilobed appearance, dense cytoplasmic granularity and absence of Auer rods. Few Pelger-like neutrophils were also observed along with maturing myeloid cells.	Abnormal promyelocytes with regular nuclei, scanty cytoplasmic granularity, presence of Auer rods and Pelger-like neutrophils.	Similar to case 1	Similar to case 1	Similar to case 1. Additionally, hypogranular myeloid cells are present.	Abnormal promyelocytes with characteristic bilobed nuclei, presence of abundant cytoplasmic granules and strong cytochemical MPO positivity with lack of Auer rods and presence of differentiating myeloid series cells	Similar to case 6 Auer rods present but no differentiating myeloid cells seen
Flow Markers	CD34	Negative			Negative	Negative	Negative	Negative
	CD117	Negative			Intermediate	Negative	Subset	Intermediate
	HLADr	Negative			Negative	Negative	Negative	Negative
	CD13	Intermediate			Intermediate	Bright	Heterogeneous	Heterogeneous
	CD33	Bright			Bright	Bright	Bright	Bright
	CD56	Negative			Negative	Dim	Negative	Negative
	CD15	Dim			Dim	Dim	Heterogeneous	Dim
	CD64	Dim			Dim	Dim	Intermediate	Intermediate
Baseline Ct values		21.42	23.65	21.64	21.39	23.64	24.7	-
Post induction Ct values		26.78	25.02	--	--	29.21 (delta delta Ct value 0.01)	37.2 (<10 copies)	No identifiable PML-RARA copies

Post induction FISH	Variant RARA positive in 52% cells				Variant RARA positive in 5% cells	Negative	Negative
Therapy regimens							
Induction	ATO 0.15mg/kg x 45 days + Daunorubicin 60mg/m^2 x 3 days	ATO 0.15mg/kg x 45 days	ATO 0.15mg/kg x 45 days	Daunorubicin 60mg/m^2 x 3 days + Cytarabine 100mg/m^2 x 7 days	ATO 0.15mg/kg x 11 days followed by 3+7 Induction Daunorubicin 60mg/m^2 x 3 days + Cytarabine 100mg/m^2 x 7 days	ATO 0.15mg/kg x 45 days.	Same as case 6 But stopped at day 38
Consolidation	High does cytarabine 12gm/m^2 (3 cycles)	NA	NA	High does cytarabine 12gm/m^2(3 cycles)	High does cytarabine 12gm/m^2 (3 cycles)	Daunorubicin 60mg/m^2 x 3 days (3 cycles) + ATRA 45mg/m^2 in divided doses x60 days	Same as case 6
Maintenance	ATRA 45mg/m^2 in divided doses x 15 days once every 3 months (6 cycles)	NA	NA	NA	NA	ATRA 45mg/m^2 in divided doses x 15 days once every 3 months (6 cycles) 6-MP 50mg once daily Methotrexate 2.5mg 6 tablets a week	Same as case 6
Follow up	On maintenance therapy. In complete remission (variant RARA negative; no detectable ZBTB16 RARA transcripts)	Expired post induction	Lost to follow up	On palliative therapy	On consolidation therapy	On maintenance therapy	On maintenance therapy

*NA – Not applicable

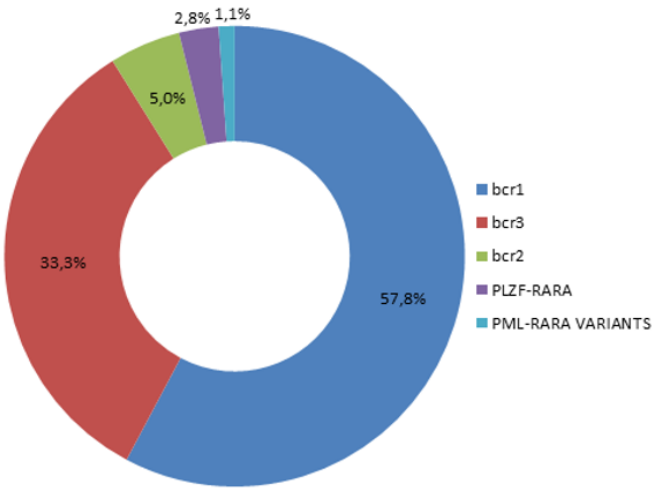

Figure 1. Distribution of APL cases as per the fusion transcript type.

Case 1. A 15-year-old boy complained of abdominal pain, weakness and intermittent fever. Laboratory investigation values are depicted in **Table 1**. Bone marrow examination revealed abnormal promyelocytes with regular, round to oval nuclei, (lacking the classical bilobed appearance) dense cytoplasmic granularity, the absence of Auer rods but strong cytochemical myeloperoxidase (MPO) positivity (**Figure 2**, **A & B**). Few Pelger-like neutrophils were also observed along with maturing myeloid cells. The patient did not develop any bleeding manifestations. FCI revealed cells having high side scatter and dim CD45 expression, heterogeneous CD13, and homogeneous bright CD33, along with lack of CD34, HLA-DR, and CD56 expression. FISH was negative for t(15;17). The RARA break-apart probe detected a variant *RARA* translocation. PCR for *PML-RARA* fusion transcripts was negative as well. *ZBTB16* as the partner gene for variant *RARA* was confirmed with PCR. The patient received induction with Idarubicin and arsenic trioxide (ATO). Forty-five days post induction remission was not achieved.

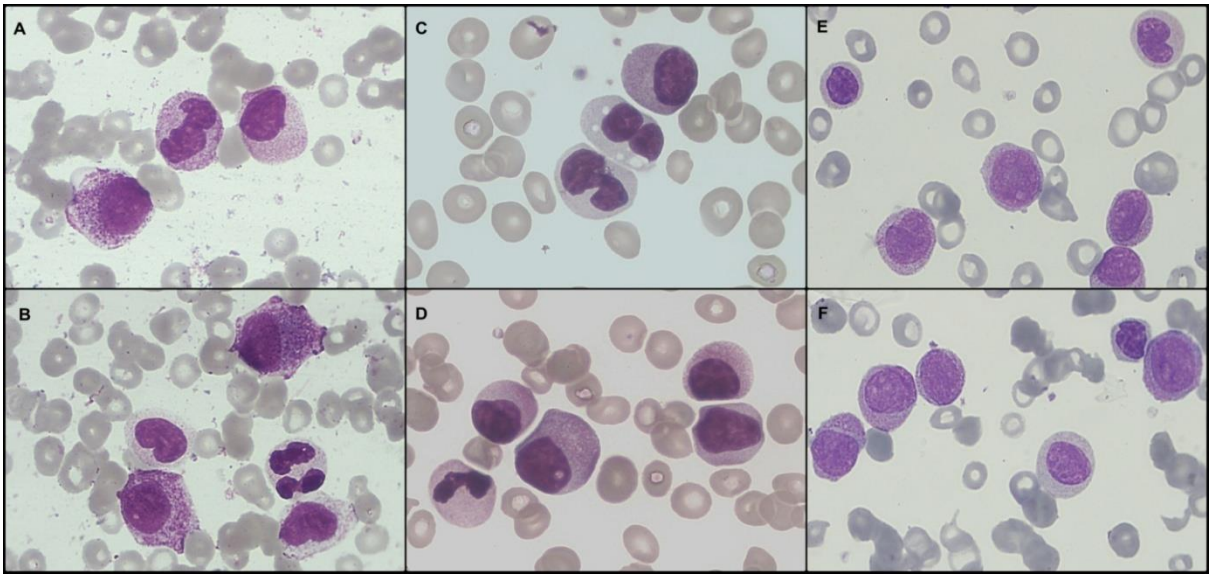

Figure 2. A&B – Case 1. Abnormal promyelocytes with rounded nuclei and dense cytoplasmic granularity and hypolobated neutrophils. **C&D** – Case 2. Relatively scanty cytoplasmic granularity of the promyelocytes. **E&F** – Case 5. Hypolobulated and hypogranular myeloid cells along with abnormal promyelocytes.

FISH showed variant *RARA* positivity in 52% cells. RT-qPCR showed mean threshold cycle (Ct) value of 26.78 against a baseline Ct value of 21.42 for *ZBTB16-RARA* and 21.03 for *ABL1*. The patient further received three cycles of high dose cytarabine (HiDAC) following which complete remission was achieved with no detectable *ZBTB16-RARA* fusion transcripts.

Case 2. A 38-year-old man with complaints of easy fatigability, dyspnea, and intermittent fever was referred with a suspicion of APL for molecular testing. FISH detected a variant *RARA* translocation which was confirmed to be *ZBTB16-RARA* by PCR. However, the morphological features differed from the previous case. The abnormal promyelocytes with regular nuclei showed the presence of Auer rods but had abundant cytoplasm with scanty granularity along with the presence of Pelger-like neutrophils (**Figure 2, C & D**).

The patient received ATO based induction therapy but responded poorly and failed to achieve remission. The post induction Ct value (25.02) by RT-qPCR was similar to the baseline one (23.65). However, the patient died shortly after completing two months of therapy.

Case 3. A 45-year-old man was referred for molecular testing to confirm the morphological suspicion of APL. Fever and easy fatigability were the only presenting symptoms. Morphological features were similar to case 1, as highlighted by the presence of abnormal promyelocytes containing round nuclei with regular nuclear contours, abundant granulated cytoplasm and a lack of Auer rods. In contrast to classical APL cases maturing myeloid cells were also present along with a few hypolobated or Pelger-like neutrophils. Dual fusion FISH for *PML-RARA* was negative, but the break-apart probe revealed *RARA* variant translocation. *ZBTB16-RARA* fusion was confirmed by PCR. RT-qPCR showed a baseline mean Ct value of 21.64. Although the patient was started on induction with ATO, he was lost to follow up subsequently.

Case 4. The patient is a 36-year-old gentleman with relapsed APL referred to our institute for further management. A detailed history revealed that the patient was first presented with fever and rash in February 2016 and morphological features on peripheral smear raised the suspicion of APL. However, *PML-RARA* was not detected by molecular testing (details of *PML-RARA* or variant *RARA* testing not available). The patient was started on induction therapy with daunorubicin and cytarabine followed by three cycles of HiDAC. The patient achieved remission after the 1st cycle of HiDAC. He also received two cycles of decitabine and arsenic trioxide before being referred to us. The patient relapsed in January 2017 with confirmation of t(11;17) by FISH. On presentation, the patient was asymptomatic and clinical examination did not show any abnormality. CBC and FCI findings are detailed in

Table 1. Bone marrow smears showed 22% abnormal promyelocytes and 33% differentiating myeloid cells. Morphological features of the abnormal promyelocytes were similar to case 1. Immunophenotype was similar to case 1 except the CD117 expression in the present case. PCR was used to confirm the presence of *ZBTB16-RARA* fusion. Baseline Ct value by RT-qPCR was 21.39. The patient was counseled for bone marrow transplantation but was put on palliative care, given non-affordability for transplantation.

Case 5. The patient was a 22-year-old student with a history of fever and body ache for the past 2-3 months. Pallor and tenderness over the rib cage were the only positive findings on clinical examination. Laboratory findings are detailed in **Table 1**. Peripheral smear examination showed 90% abnormal promyelocytes with strong cytochemical myeloperoxidase positivity. Promyelocyte morphology resembled case 1. In addition, maturing myeloid cells, hypogranular and hypolobated neutrophils were also present (**Figure 2, E & F**). The patient was immediately started on ATO based on the morphological suspicion of APL. The patient did not develop any bleeding manifestations. The immunophenotype was similar to case 1 and 4, however, unlike those cases, CD56 was dim (**Table 1**). FISH did not show t(15;17), but the break-apart probe revealed *RARA* variant translocation. After 11 days of ATO and following confirmation of *ZBTB16-RARA* fusion by PCR, the patient was shifted to daunorubicin and cytarabine-based induction therapy. Remission was not achieved post induction (**Table 1**), and the patient was started on consolidation therapy with HiDAC.

Case 6. A 35-year-old male was referred to our Institute with complaints of high grade, intermittent fever and back pain for one month. Laboratory features are detailed in **Table 1**. Peripheral smear revealed 29% abnormal promyelocytes with characteristic morphology and presence of differentiating myeloid series cells (**Figure 3**). The patient did not develop any bleeding manifestations, in spite of elevated PT and APTT values. Fluorescence in situ hybridization using a dual fusion probe confirmed the presence of *PML-RARA* fusion in 93% of cells. The patient was classified as high risk as per the

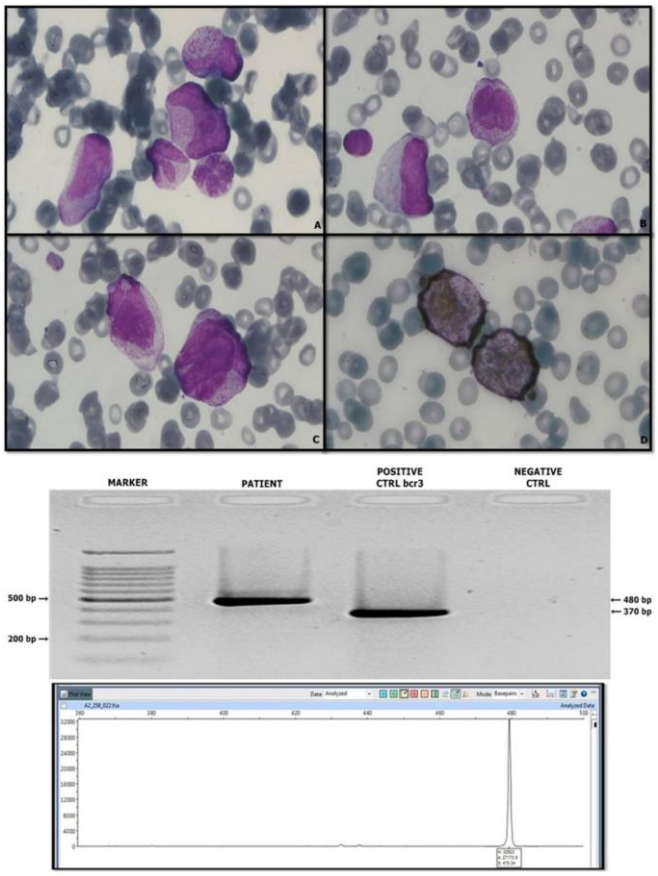

Figure 3. Cellular Morphology and PML-RARA fusion transcript detection. Abnormal promyelocytes with characteristic bilobed nuclei (B) and presence of abundant cytoplasmic granules which showed strong cytochemical myeloperoxidase positivity (D) is seen in the uppermost panel. Some unusual myeloid differentiation is also seen (A & C). PCR amplicon size (480 bp) of novel Bcr3 PML-RARA transcript on agarose gel (middle panel) and capillary (lower panel) electrophoresis.

Sanz criteria[11] and started on induction therapy with ATO. Post-induction bone marrow examination for response assessment revealed that the patient had achieved morphological and cytogenetic remission. Consolidation therapy included three cycles of daunorubicin and ATRA. The patient is currently doing well and is in maintenance therapy.

Agarose gel electrophoresis for *PML-RARA* transcript identification revealed a PCR amplicon size of approximately 480bp, which was larger than the expected Bcr3 fusion transcript size (~370bp) (**Figure 4**). The PCR amplicons were further subjected to Sanger sequencing and revealed an atypical, novel fusion pattern between exon4 of *PML* and exon 3 of *RARA* genes (**Figure 4**). Interestingly, an additional 40 nucleotides, mapped to intron 2 of *RARA*, were also coded as part of the mRNA fusion transcript (**Figure 4**). The addition of exon 4 and 40 nucleotides from intron 2 explains the increase in the size of the Bcr

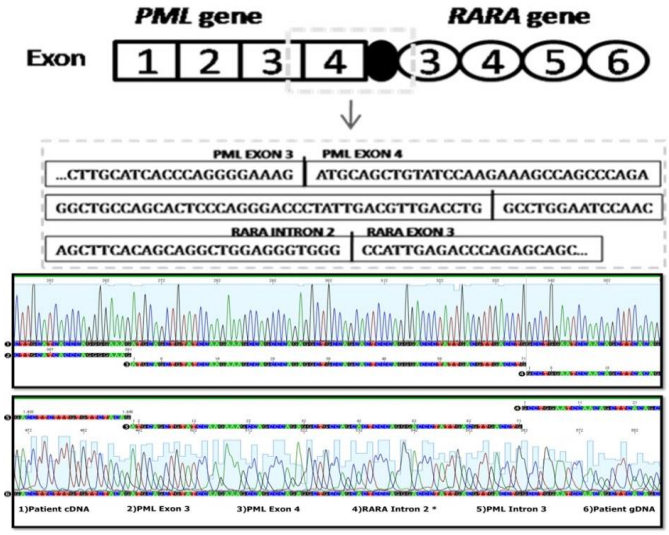

Figure 4. Characterization of novel Bcr3 PML-RARA variant by Sanger sequencing (Case 6). Schematic representation of novel PML-RARA variant transcript is shown in the upper panel. PML gene exons are shown the squares and RARA gene exons are shown in the circles. Insertion of 40 nucleotides from RARA intron2 is shown as a shaded black circle. Exact patient sequences are shown in dashed box below. The cDNA and gDNA (lower panels) from the patient were sequenced by Sanger sequencing and aligned to the exonic and/or intronic sequences of PML and RARA genes.

3 transcript type by 111 base pairs (corresponding to the addition of 37 amino acids in the protein).

Thus, this novel Bcr3 variant transcript is in-frame and codes for a fusion protein which is longer than expected *PML-RARA* fusion transcript product. Cloning of the PCR product into a vector and Sanger sequencing confirmed the above sequencing results (**Figure 3**). The same plasmid was serially diluted from 10^6 to 10^1 copies to be used as RT-qPCR standards. Baseline RT-qPCR revealed high copies (13070) with amplification occurring at a Ct value of 24.7. Post-induction bone marrow evaluation, after 48 days of ATO therapy, revealed fewer than ten copies corresponding to a Ct value of 37.2. Long-range genomic DNA PCR followed by Sanger sequencing identified a breakpoint immediately downstream to exon 4 of *PML* gene (*PML* nucleotide no: 74024927) and distal to the 11,789th base (*RARA* nucleotide no: 40343186) within intron 2 of *RARA* gene (**Figure 4**). The first 40 nucleotides of RARA intron 2 (starting from 11,790th base) alone were coded in the protein and were part of this novel fusion transcript.

Case 7. A 26-year-old female was referred with a history of headache and right iliac fossa pain for 2 months. Clinical examination revealed an ill-defined, palpable mass in the right iliac fossa. There was no history or evidence of any bleeding tendency on clinical examination. Abdominal ultrasonography revealed the presence of a 3.3x2.2 cm hemorrhagic cyst within the right ovary. Peripheral smear examination showed 92% abnormal promyelocytes with classical morphology, strong MPO positivity and the presence of Auer rods. The patient was classified as high risk as per Sanz criteria[11] and started on ATO based on the morphological suspicion of APL. FCI revealed typical features of APL; with high side scatter, lack of CD34 and HLA-DR expression, homogeneous bright CD33 and heterogeneous CD13 expression (detailed in **table 1**). Dual fusion, dual color FISH showed evidence of *PML-RARA* fusion. RT-PCR for transcript identification yielded an amplicon of ~ 100 bases, higher than that of the expected Bcr1 transcript (390 bases). Sanger sequencing revealed breakpoints within exon 6 of *PML* and exon 3 of *RARA* (*RARA* nucleotide no: 40351910) with the addition of 126 nucleotides, which mapped to the central portion of *RARA* intron 2 (**Figure 5**). Therefore the patient was classified as a Bcr 2 variant. *PML* exon 6 was truncated at nucleotide 248, 11 base pairs (bp) upstream of the normal exon 6/intron 6 boundary (*PML* nucleotide no: 74033403). The insertion of 126 nucleotides from intron 2 can explain the increase in size of the transcript type on electrophoresis. This novel Bcr 2 variant transcript is in-frame and codes for a longer *PML-RARA* fusion transcript product.

Induction therapy was withheld after 38 days due to suspicion of ATO induced hepatotoxicity. However, investigations revealed acute viral

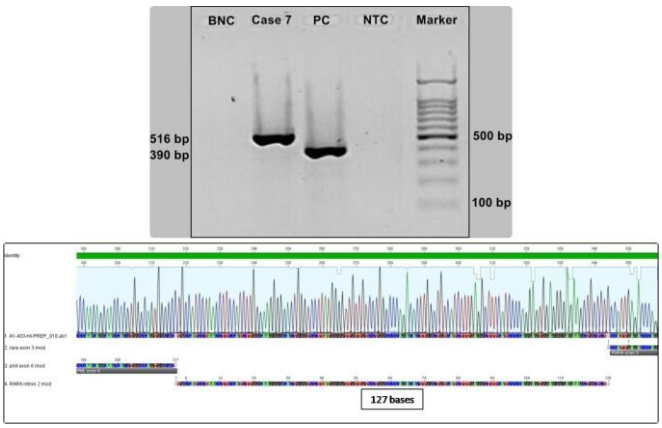

Figure 5. Characterization of Bcr 2 PML-RARA variant (Case 7). Agarose gel electrophoresis (upper panel) of our case (Case 7) along with known positive control (PC), biologically negative control (BNC) and a non-template control (NTC). Sanger sequencing (lower panel) of the same case shows fusion of PML exon 6 with RARA exon 3 and addition of 126 nucleotides from RARA intron 2.

hepatitis due to hepatitis E virus (IgM HEV positive). Bone marrow examination for response evaluation revealed complete morphological and cytogenetic remission. RT-qPCR for *PML-RARA* was also negative. The patient further received consolidation with ATRA, daunorubicin and was started on maintenance therapy. RT-qPCR post consolidation did not detect any *PML-RARA* copies.

Discussion. This case series highlights the heterogeneity in molecular characteristics of APL cases at a tertiary cancer center in India. APL's variant involving *ZBTB16* is rare with a frequency of approximately 0.8% described in world literature.[4] However, the frequency of occurrence of *ZBTB16-RARA* in our study was much higher than previously reported (2.8%). Distinct morphological and immunophenotypic features have been described in association with *ZBTB16-RARA*,[12] which include round to oval nuclei with a regular nuclear membrane, hypogranular cytoplasm, lack of Auer rods or Faggot cells and increased number of Pelger-like neutrophils. Given these findings, Sainty et al. have proposed a new morphological variant of APL, i.e. 'M3r' with a threshold of more than 30% cells with above-described morphology as a defining feature. Dense cytoplasmic granularity was seen in all but one of our cases. Pelger-like neutrophils were consistent in three of the five cases and the fifth case harbored hypogranular, almost dyspoietic looking myeloid cells. Interestingly, these morphological features are a combination of classical APL and *ZBTB16-RARA* APL findings. Even within this rare subset of APL cases, we observed a wide variation in the morphological features. In addition, lack of CD56 expression (except case 5) was also the only exception to the immunophenotype associated with *ZBTB16-RARA*. Although Sainty et al. have reported strong CD56 expression in four and weak expression in one out of a total 6 cases with *ZBTB16-RARA* fusion, we found weak CD56 expression in just one of our cases.[12] The morphology and immunophenotypic features of *NPM1* mutated AML; such as low or absent expression of immaturity associated antigens such as CD34 and HLA-DR, can also be associated with APL, especially with *ZBTB16-RARA* fusion.[13,14] None of our cases expressed CD34 or HLA-DR. Hence, relying on morphology and immunophenotype alone, differentiation between the two entities can be difficult and requires molecular diagnostic techniques for confirmation. CD117 was not consistently expressed in our cases as well, with only one of three *ZBTB16-RARA* cases being positive. The novel *PML-RARA* Bcr3 variant described here showed classical APL morphology but with the presence of differentiating myeloid cells, a feature which is unusual in classic APL cases.

Bcr 1 and Bcr 3 breakpoints are seen in 55% and 40% of APL cases respectively. Two Indian studies have reported contrasting findings with bcr1 and bcr3 frequencies. Whereas Chatterjee et al.[15] have reported lower bcr1(42.7%) but higher bcr2(14.8%) frequencies; Sazwal et al. (64%)[16] and Dutta et al. (72.7%)[17] have reported over-representation of the bcr3 subtype in Indian patients. The frequency of bcr1 (58%), bcr3 (33%) and bcr2 (5%) subtypes detected at our center was similar to previously published western literature.[3] Breakpoints in Bcr2 leading to the variable (V) isoform are detected in up to 5% patients and have been reported to be more common in the pediatric age group. The Bcr2 variant results from a breakpoint in exon 6 of *PML* (rarely exon 5) and may show insertion of genomic DNA from *RARA* intron 2. The size of the inserted *RARA* intron 2 segments may vary between 3 to 127 nucleotides.[18,19] The loss of distal part of *PML* exon 6 has been reported to negatively influence the response to treatment.[18,19] We report an adult, Bcr 2 variant case showing the addition of 126 nucleotides between *PML* exon 6 and *RARA* exon 3 (**Figure 5**). Our patient responded well to ATO based induction and in spite of complications showed a good response and remains in complete remission.

We also report a unique Bcr3 variant with breakpoint at the junction of exon 4/intron 4 and intron 2 of *RARA* along with the addition of 40 nucleotides originating from a central portion of *RARA* intron 2 (**Figure 4**). To the best of our knowledge, such a case has not been reported previously. Jeziskova et al. also report a unique case with similar breakpoint but with a smaller intronic insertion (9 nucleotides).[20] Our patient showed a slightly delayed response to ATO based therapy, achieving complete molecular remission post consolidation as compared to the patient described by Jeziskova et al., who was in complete

molecular remission post-ATRA based induction therapy.

Classical and cytogenetic variant APL's have distinct natural histories. APL with *PML-RARA* fusion has a favorable outcome when treated with ATRA or arsenic trioxide. Pharmacological doses of retinoic acid (in the form of ATRA) unbind the N-Cor corepressor from RAR-RXR (*RARA*-retinoic X receptor) complex and allow transcription of target genes, ultimately leading to myeloid differentiation. The inability of ATRA to dissociate *ZBTB16* from the corepressor complex and epigenetic factors such as Polycomb group complexes contribute to the resistance of *ZBTB16-RARA* APL to conventional therapy.[21] The reduction in *ZBTB16-RARA* copy number in our cases was determined using relative quantification based RT-qPCR or the delta-delta Ct method. There was no significant change in our treated patients between the baseline and post-induction Ct-values, signifying resistance to ATRA/ATO based therapy. The efficacy of combination therapy with anthracyclines and cytarabine along with ATRA in inducing remission has been previously reported.[22] Rohr et al. describe two cases in which the patients did not achieve remission following induction with ATRA and anthracycline combination but achieved partial remission after reinduction in one of the cases.[23] Even though our case (case 1) was not in remission post induction, no *ZBTB16-RARA* transcripts were detected by RT-qPCR post consolidation with high dose cytarabine.

Although the clinical features and risk of developing life-threatening disseminated intravascular coagulation is similar to classical APL, *ZBTB16-RARA*, however, shows distinct morphological features and a lack of response to ATRA/ATO.[5,12] *ZBTB16-RARA* diagnosis offers a challenge that can be met with a combination of morphological and molecular testing. We report a series of five cases of *ZBTB16-RARA* associated APL from India with unusual morphological and immunophenotypic features. Cases with atypical breakpoints in *PML-RARA* represent sporadic events. We also report a unique Bcr3 *PML-RARA* variant hitherto unreported in the literature.

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