

BEDSIDE-TO-BENCH REPORT



Identification of a new cryptic PML-RAR α fusion gene without t(15;17) and biallelic CEBPA mutation in a case of acute promyelocytic leukemia: a case detected only by RT-PCR but not cytogenetics and FISH

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ABSTRACT

Acute promyelocytic leukemia (APL) is characterized by the presence of promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) fusion gene, which is formed following the specific chromosomal translocation t(15;17)(q22;q21). However, cases with PML-RAR α generated by occult t(15;17) which are negative by both cytogenetics and fluorescence in situ hybridization (FISH), are difficult to diagnose, leading to impaired treatment effectiveness. In the present study, we reported a case of a 66-year-old male patient, and bone marrow morphology, flow cytometry and cytogenetics did not support the diagnosis of APL. Molecular techniques, such as reverse-transcription polymerase chain reaction (RT-PCR), showed the existence of a cryptic PML-RAR α fusion gene, and sequence analysis revealed a new variable isoform. Hotspot gene mutation analysis showed a biallelic CEBPA mutation. He received IA chemotherapy and all-trans retinoic acid (ATRA) treatment, and finally achieved complete remission. This case report provided valuable insights into the relevance of the correct identification of atypical PML-RAR α fusion gene and biallelic CEBPA mutation. Moreover, combination of IA chemotherapy and ATRA treatment suggested a good clinical effect in this atypical PML-RAR α .

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Introduction

The promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) fusion gene, occurring in about 95% patients with acute promyelocytic leukemia (APL) by the chromosomal translocation t(15;17)(q22;q21), is considered to be responsible for the arrest of granulopoiesis by directly inhibiting the transcription of retinoic acid target genes.^{1,2} All-trans retinoid acid (ATRA), the first drug found to cause disease regression specifically in APL patients, interacts with the ligand-binding domain present on the RAR α moiety of the chimeric oncoprotein and causes both its transcriptional activation and proteolytic degradation, leading to granulocyte differentiation of APL cells.^{3,4} Therefore, it is very important to detect the PML-RAR α fusion gene in patients with acute myeloid leukemia (AML). In clinical diagnosis of APL, PML-RAR α fusion gene can be detected by several molecular methods based on conventional karyotyping, fluorescence in situ hybridization (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR).^{5,6} However, rare cases with PML-RAR α rearrangements, generated by occult t(15;17) which are negative by both cytogenetics and FISH, are difficult to diagnose, significantly impairing the treatment effectiveness.^{7–11}

In the present study, we reported an AML patient with biallelic CEBPA mutation which was diagnosed as FAB M5 type by morphological analysis. In addition, the diagnosis of APL was supported by a cryptic PML-RAR α translocation detected only by nested RT-PCR but not cytogenetics, FISH and real-time RT-PCR.

The patient achieved complete remission (CR) after IA chemotherapy and ATRA-induced differentiation therapy.

Materials and methods

Patient and description

A 66-year-old Chinese male patient presented to our center due to petechiae or bruises on the lower limbs that lasted for half a month with intermittent fever and coughing. The computed tomography (CT) scan examination revealed slight high-density pulmonary parenchyma plaques in left lower lobe. The B type ultrasonography showed renal cyst (left) and benign prostate hyperplasia. He was diagnosed with pulmonary tuberculosis (TB) and given anti-TB drugs. Physical examination showed that he had mild anemia and multiple petechiae or bruises on the lower limbs. [Table 1](#) summarizes the peripheral blood examinations of the patient, including complete blood count, blood coagulation profile and blood chemistry analysis.

Bone marrow cell morphology

The bone marrow sample was spread on slides and stained by Wright-Giemsa stain (Baso Diagnostic Inc., China). A total of 200 bone marrow cells were analyzed by light microscopy (Olympus BX51, Olympus Corporation Tokyo, Japan). Cytochemical examinations, including peroxidase (POX) reaction, periodic acid Schiff

Table 1. Clinicopathologic features of the patient.

	Patient	Normal range
Complete Blood Count		
WBC $\times 10^9/L$	2.95	4–10
Blasts in peripheral blood (%)	68	<0.01
Hb (g/L)	78	120–150
Platelets $\times 10^9/L$	7	100–300
Blood Coagulation Profile		
PT (s)	14.1	9.8–12.1
APTT (s)	27.7	22.7–31.8
TT (s)	14.1	14.0–21.0
Fbg (g/L)	3.8	1.8–3.5
D-dimer (mg/L)	0.56	0.01–0.55
Blood Chemistry Analysis		
TBil ($\mu\text{mol/L}$)	31.6	3.42–20.5
DBil ($\mu\text{mol/L}$)	10.6	0–6.84
LDH (U/L)	353	120–250

(PAS) reaction and nonspecific esterase (NSE) reaction, were carried out according to manufacturer's instructions (Baso Diagnostic Inc., China)

Flow cytometry

The immunophenotyping of bone marrow cells was conducted by a five-color flow cytometry (CYTOMICS FC500, Beckman Coulter, Inc., Brea, CA, USA). Each cluster of differentiation (CD) on gated cells was examined with monoclonal antibodies (Beckman Coulter, Inc., Brea, CA, USA), and it was defined as positive when over 20% of the gated cells showed fluorescence above the staining background of controls by a clinical laboratory physician.

Fish

DCDF PML-RAR α (Vysis Inc., Downers Grove, IL, USA) FISH was performed on bone marrow cells prepared as routine FISH methods. The result was determined by a clinical laboratory physician using an Olympus BX51 fluorescence microscope (Olympus Corporation Tokyo, Japan). At least 200 cells with well-delineated signals were evaluated. The cutoff level was set as 1% according to mean \pm standard deviation in normal controls.

Molecular analysis

Acute non-lymphoblastic leukemia fusion genes, such as MLL/AF, MLL/AF9, MLL/AF10, MLL/AF17, MLL/ELL, AML/ETO, DEK/CAN, dupMLL, TLS/ERG, EVII, PML/RARA, PLZF/RARA, NPM/RARA, NPM/MLF1, BCR/ABL1(p190), BCR/ABL1(p210), CBF β /MYH11 and HOX11, were detected using multiplex nested RT-PCR technique by Kindstar Global Co., Ltd., China. Hotspot mutation regions of genes, such as FLT3-ITD, NPM1 (exon 11), c-KIT-D816, DNMT3A-R882, CEBPA, RUNX1 (exon 2–6) and TP53 (exon 5–8), were detected by Sanger sequencing using an AB 3500XL sequencer by Kindstar Global Co., Ltd., China.

Results

Bone marrow cell morphology, flow cytometry and FISH do not support the diagnosis of APL

Bone marrow cell morphology analysis showed marked hyperplasia in nuclear cells. It contained 51.5% abnormal blast cells, including 6% monoblasts and 45.5% promonocytes.

These cells were of uneven sizes and shapes, exhibiting irregular nuclear shapes and misty nucleoli (Figure 1a). Moreover, the blasts were positive for POX (data not shown). Chromosome analysis using GTG-banding indicated a karyotype of 46, XY (Figure 1b). FISH signals from the PML-RARA dual-color dual-fusion probe showed that normal cell patterns consisted of two red and two green signals (Figure 1c). Flow cytometry revealed that the AML cells positively expressed CD34, CD38, CD7, CD33, CD13, MPO, CD117 and HLA-DR, but negatively expressed CD19, CD10, CD3, CD4 and CD8 (Figure 1d).

Atypical PML-RAR α and biallelic CEBPA mutation

Although cytogenetic and FISH analyses demonstrated the absence of t(15;17)(q24;q21) and PML-RAR α in the patient, nested RT-PCR with PML-RAR α specific primer and electrophoresis showed a specific band, which was slightly smaller than L-type PML-RAR α (Figure 2a). We speculated that it was a new variant of PML-RAR α . To validate such a hypothesis, the sequence of PCR amplicon showed PML breakpoint within exon 6 and RAR α breakpoint within intron 2 (Figure 2b). Compared with the L-type PML-RAR α , the variant detected in our current patient lacked a 139-bp fragment in exon 6 of PML, and there was a 19-bp increase in length in intron 2 of RAR α (Figure 2c). Myeloid leukemia-related hotspot gene mutation analysis showed that the patient had a biallelic mutation of CEBPA gene in the transactivation domains (TADs) at its N-terminus as well as a DNA-binding and dimerization bZIP structure at its C-terminus (c.92_93 insT in TAD region and c.930_931 insACG in bZIP region) (Figure 3).

Therapy and clinical outcome

Figure 4 shows the detailed therapeutic regimen. This patient was first treated with IA chemotherapy (idarubicin, 8 mg/m²/day for 3 days and cytarabine, 100 mg/m²/day for 7 days). After treated with IA for 4 days, 40 mg/day ATRA was added to regimen when the atypical PML-RAR α was detected. When the first IA regimen was completed, the patient continued to receive ATRA treatment for 30 days. His hemogram of peripheral blood returned to normal levels (Hb 10⁸ g/L, WBC 7.28 $\times 10^9/L$, PLT 153 $\times 10^9/L$). The analysis of bone marrow cell morphology showed CR, and minimal residual disease (MRD) monitoring using flow cytometry was 0.00%. He remained CR until the last follow-up on December 25, 2018.

Discussion

To the best of our knowledge, the present case was the first case describing an AML containing a new cryptic atypical bcr2 transcript of PML-RAR α and a biallelic CEBPA mutation. However, this case did not show typical morphological, immunological and cytogenetic features of APL, and the therapy of ATRA plus cytotoxic chemotherapy was effective. In recent years, some patients with atypical PML-RAR α transcripts have been reported.^{12–16} In the reported cases, the cell morphological and immunological characteristics are

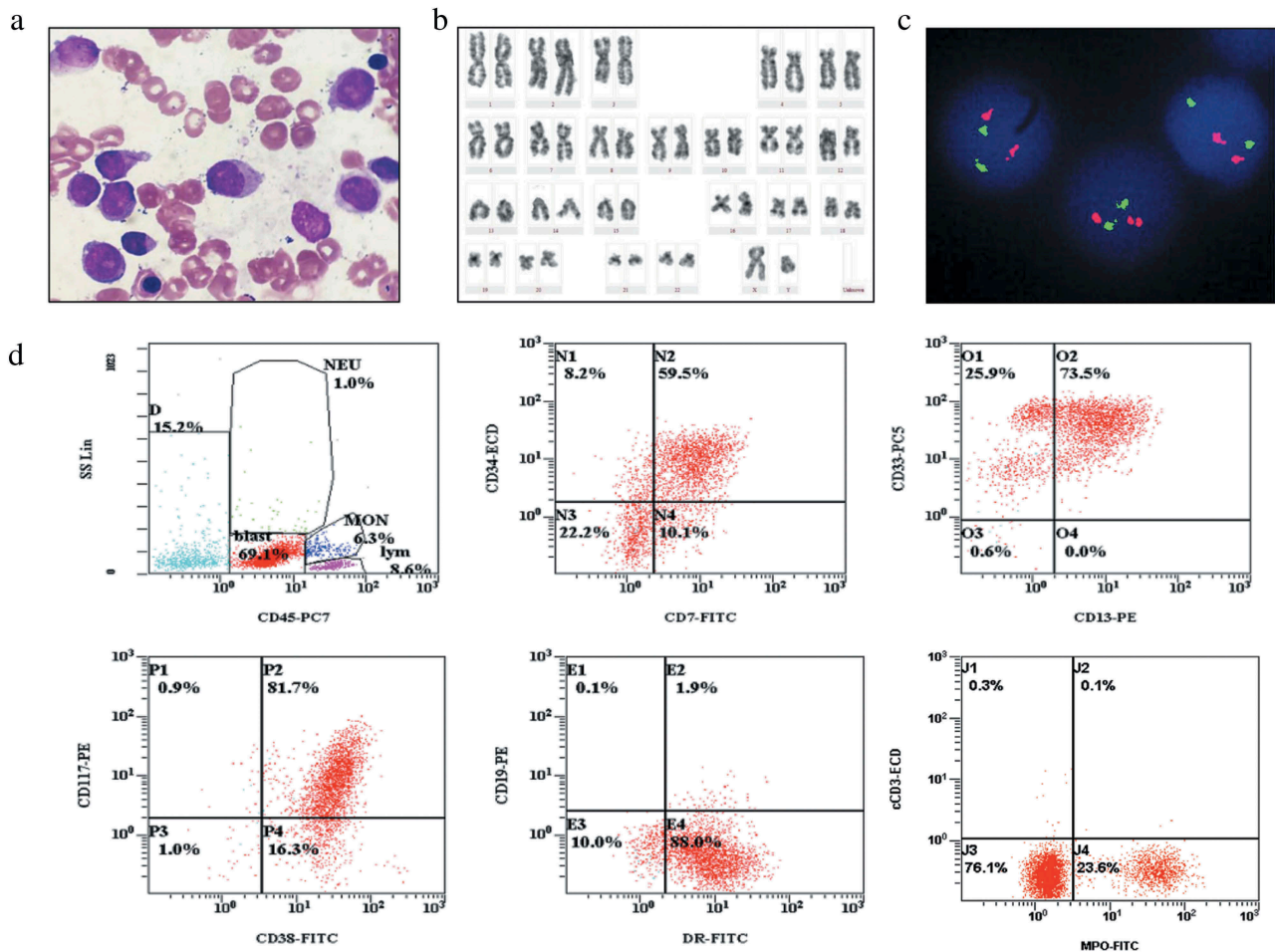


Figure 1. The results of cell morphology, cytogenetics, FISH and flow cytometry of the bone marrow cells. (A) Bone marrow smear exhibiting abnormal, uneven cell sizes and shapes with irregular nuclear shapes and misty nucleoli blast cells. (B) G-banding karyotype of a bone marrow cell exhibiting 46, XY[20]. (C) Dual-color FISH analysis with PML/RAR α -specific probes 15q22 (red) and 17q21 (green) exhibiting two red and two green signals as a normal signal type. (D) Flow cytometry indicated that the leukemia cells positively expressed CD34, CD38, CD13, CD33, CD117, CD7, HLA-DR and MPO.

consistent with typical APL. However, in our case, the bone marrow cell morphology of the patient showed FAB M5 type with irregular nuclear shapes and misty nucleoli, and weakly positive POX cytochemical staining. Flow cytometry analysis showed that the leukemia cells expressed CD34, CD7, CD13, CD33, CD117, CD38, HLA-DR and MPO, which was not inconsistent with the immune-phenotype of typical APL. Therefore, we speculated that these features might be related with CEBPA double mutation in the patient.

CEBPA mutations are found in approximately 10% of AML cases.^{17,18} Most CEBPA-mutated AML cases exhibit two mutations, which frequently include TADs at its N-terminus as well as a DNA-binding and dimerization bZIP structure at its C-terminus.^{18–20} Recently, Mannelli F et al. have found that CEBPA-double-mutated AML displays a unique phenotypic profile.¹⁹ They have analyzed 259 AML cases and found 16 patients with biallelic mutation. Moreover, the blast cells of these patients always express CD34, CD7, CD117 and HLA-DR. Furthermore, some other studies have also found that the expression of CD7 in AML is usually associated with loss of wild-type CEBPA due to mutations or silencing by epigenetic mechanisms.^{21–24} It suggested

that the immune phenotype of our patient was related to the CEBPA mutation, even if no reports about the CEBPA mutation have been found in other APL patients.

Since no case reports of PML-RAR α and CEBPA mutations have been described, no currently available treatment regimen can be used as reference. When an APL is suspected, the clinicians always give ATRA treatment to patients even before a definitive diagnosis is made. Once genetic diagnosis is confirmed, full induction treatment should be started. For the non-high risk patients (WBC count $<10 \times 10^9/L$), the ATRA+ATO combination is the first choice for induction therapy. When the patient's WBC is more than $10 \times 10^9/L$, induction therapy is always combined with anthracycline-based chemotherapy according to therapy-induced differentiation.^{25,26} In 2016, World Health Organization (WHO) included AML with double-mutated CEBPA as a provisional prognostic category.^{27,28} Since our patient had no typical APL specificity in cell morphology, immune-phenotype and cytogenetics, we treated the patient with anthracycline-based chemotherapy at the initial stage of diagnosis although the patient's WBC was below $10 \times 10^9/L$. We added ATRA to the traditional chemotherapy to induce

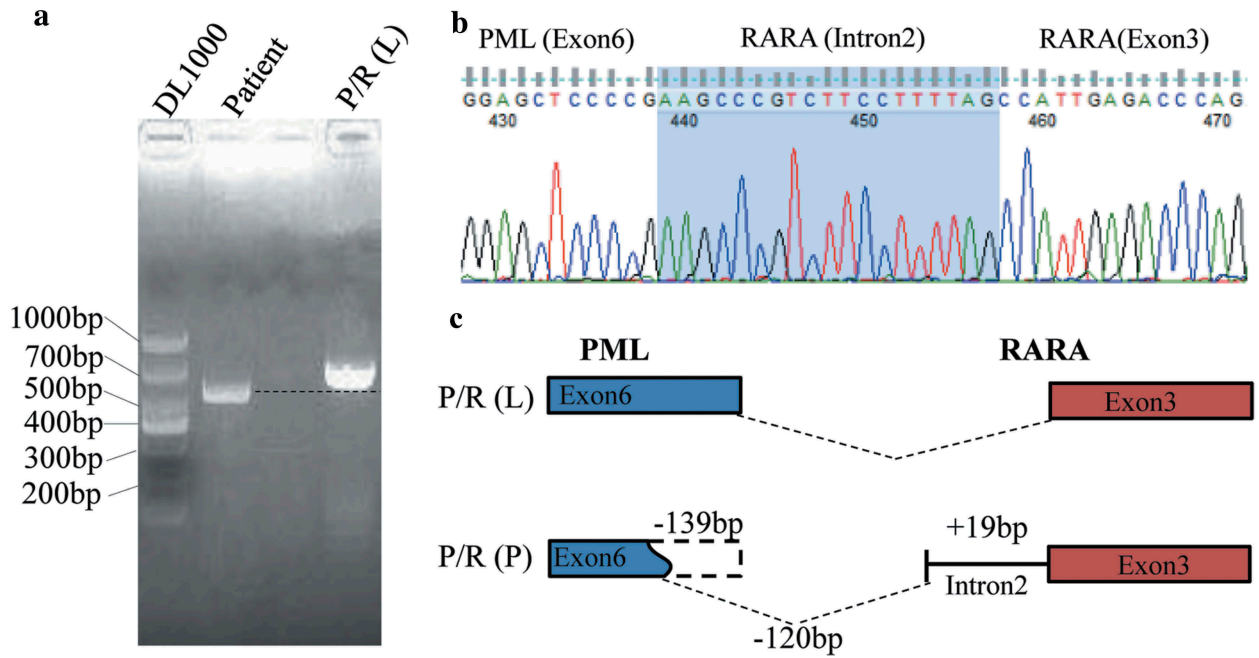


Figure 2. Detection of the PML-RAR α using the semi-quantitative PCR and DNA sequencing method. (A) Agarose gel electrophoresis showed a specific slightly smaller band in the patient compared with L-type PML-RAR α . (B) DNA sequencing analysis of PCR product. (C) The schema chart of the new PML-RAR α transcript.

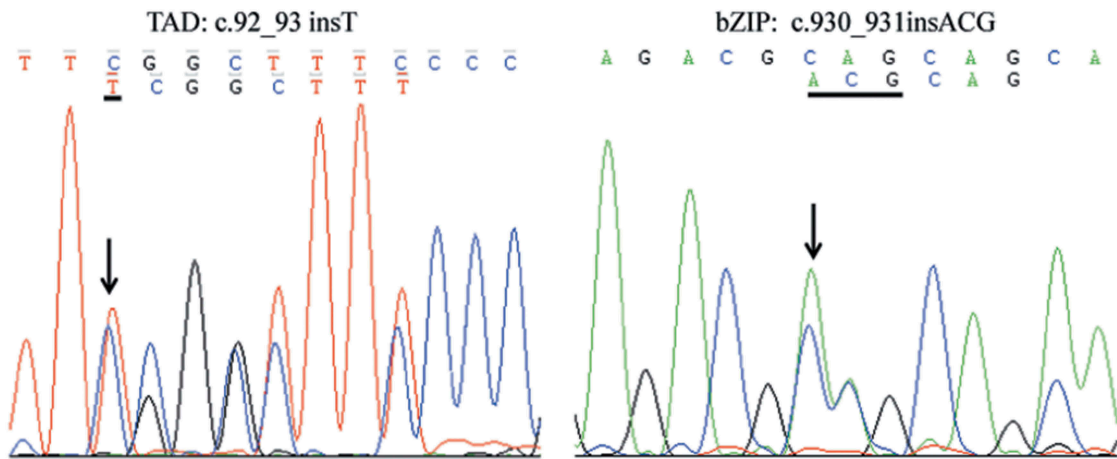


Figure 3. Gene mutation of CEBPA in TAD and bZIP domain detected by Sanger sequencing showed a c.92_93 insT and c.930_931 insACG mutation.

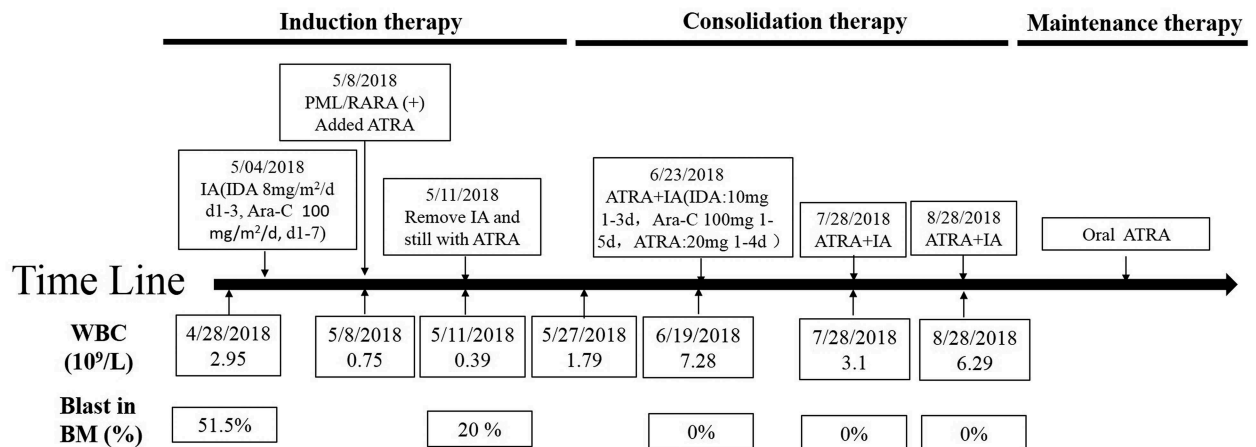


Figure 4. Timeline of the patient's therapy.

differentiation when the molecular diagnosis confirmed that the patient had PML-RAR α fusion gene and CEBPA double mutations. After treated for 7 days, the patient continued to receive ATRA treatment until he achieved CR. Fortunately, the patient did not have DIC during the course of treatment, which provided a guarantee for our later use of ATRA treatment.

Recently, there were some other similar cases like the current report in the literature. The karyotype is normal, dual-fusion PML-RAR α FISH is initially classified as normal, the presence of sis identified by one of the molecular techniques, and the ATRA treatment is effective in these patients. The new case in our present report did not show typical morphological, immunological and cytogenetic features of APL, allowing us to recognize the importance of morphology, immunophenotyping, cytogenetics, molecular cell biology (MICM) in leukemia diagnosis. Collectively, relying on a single diagnostic method often leads to misdiagnosis or even wrong diagnosis.

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Authors' contributions

Zhang Zhanglin and Li Fei conceived and designed the study. Zhang Zhanglin Xu Yawen and Jiang Mei performed the experiments. Zhang zhanglin and Li Fei wrote the paper. Zhang Zhanglin, Xu Yawen, Jiang Mei, Kong Fancong, Chen Zhiwei and Liu Shuyuan interpreted the data and provided final approval of the version to be published. All authors read and approved the manuscript.

Ethics approval and consent to participate

The patient provided written informed consent for the publication of this study and the study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China).

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