Atypical acute promyelocytic leukemia with tripartite fusion gene *PML::RARG::LINE-L2a* is resistant to ATRA but sensitive to arsenic-based therapy

RARG-related atypical acute promyelocytic leukemia (*RARG*-aAPL) exhibits unique clinical features, morphology, immunotyping, cytogenetics, and genomic and transcriptomic landscape.[1] Unlike the canonical *PML::RARA*-positive APL (*PML::RARA*-APL), *RARG*-aAPL is resistant to all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO), and it is associated with a generally poor clinical outcome.[1] Recently, Zhou *et al*. reported that all cases of *RARG*-aAPL exhibit a novel form of previously unexpected tripartite *RARG* fusions, resulting in truncation of the helices 11_12 of its ligand binding domain (LBD-H11_12), which renders ATRA unresponsiveness.[2] However, Zhou's study included all seven *RARG* 5′ fusion partner genes but did not include *PML*, the canonical fusion partner that forms the bipartite *PML::RARA* fusion gene in APL.

PML-related *RARG*-aAPL is much rarer, with only one case reported by Ha *et al*., which demonstrated resistance to ATRA.[3] However, their study identified only *PML::RARG* splicing and did not explore whether *RARG* also underwent 3′ fusion in that case. While ATO monotherapy cures over 70% of *PML::RARA*-APL cases through targeting the PML portion of the chimeric protein,[4,5] there are no reports on the treatment efficacy of ATO in *PML*-related *RARG*-aAPL. Here, we present a case of *PML*-related *RARG*-aAPL with a confirmed tripartite fusion, demonstrating resistance to ATRA but sensitivity to arsenic-based therapy. This study was approved by the institutional review board of the Zhongnan Hospital, Wuhan University (2024019K).

A 27-year-old pregnant woman presented on October 23, 2023 with severe anemia, skin bruising, gum bleeding, darkened stool, and dizziness persisting for several weeks. Laboratory tests revealed hemoglobin level of 83 g/L, white blood cell count of 1.40×10^9/L, and platelet count of 8×10^9/L. Coagulation investigation showed fibrinogen levels of 165 mg/dL (reference range, 238-498 mg/dL) and D-dimer levels of 3,181 ng/mL (reference range, 0-500 ng/mL). After delivering a healthy baby girl via emergency cesarean section, the bone marrow (BM) aspiration revealed hyperproliferative state with 81.5% blast cells, predominantly abnormal hypergranular promyelocytes with occasional Auer rods (Figure 1A). The blasts, including the aberrant promyelocytes, strongly expressed myeloperoxidase (Figure 1B). Immunophenotyping showed positivity for CD13, CD33, CD117, and cytoplasmic myeloperoxidase, while being negative for HLA-DR, CD34, CD38, CD15, CD14, and lymphoid markers (Figure 1C).

Dual-color dual-fusion fluorescence *in situ* hybridization (FISH) with *PML* and *RARA* probes failed to detect *PML::RARA* fusion in BM samples, while discrepant fluorescence signal sizes for *PML* were observed in most interphase nuclei (Figure 1D). Reverse transcription ploymerase chain reaction (PCR) confirmed the absence of common leukemia fusion genes, including *PML*::*RARA*, *PLZF*::*RARA*, and *NUMA1*::*RARA*. Karyotyping identified a clonal t(12;15)(q13;q22) translocation, affecting regions containing the *PML* and *RARG* genes (Figure 1E). However, attempts to detect *PML*::*RARG* transcripts using previously reported primers were unsuccessful.[3]

To identify the potential fusion gene, we performed whole transcriptome sequencing on BM samples and discovered a fusion between *PML* exon 6 and *RARG* exon 4, as identified through Arriba software analysis (*Online Supplementary Figure S1A*). Notably, the expression of RARG exon 10 was significantly lower compared to other exons (*Online Supplementary Figure S1B*). Manual inspection with Integrative Genomics Viewer revealed a fusion between *RARG* exon 9 and the transposable element *LINE-L2a* (*Online Supplementary Figure S1C*), suggesting the presence of a tripartite *PML*::*RARG*::*LINE-L2a* fusion. PCR amplification yielded a predicted 1,100-bp fusion fragment in the patient's BM sample but not in a *PML*::*RARA*-APL control cases (Figure 1F), and Sanger sequencing further confirmed the in-*cis* tripartite *PML*::*RARG*::*LINE-L2a* fusion (Figure 1G).

The patient was initially treated with ATRA (20 mg twice daily) and ATO (10 mg per day) based on a presumptive diagnosis of APL. Daunorubicin (60 mg per day, days 9-11) was administered to mitigate granulocyte release. Following the confirmation of *RARG* fusion, venetoclax (200 mg per day) was introduced on day 22. Morphological and immunological assessments on day 20 showed differentiation of leukemia cells, prompting the continuation of the ATRA and ATO regimen alongside venetoclax. After 36 days of induction therapy, the patient was discharged and prescribed oral ATRA. A follow-up BM examination confirmed sustained remission. The patient subsequently underwent six cycles of moderate-dose cytarabine consolidation therapy and remained in complete remission at the last follow-up (Figure 2A).

To date, eight *RARG* 5′ fusion partners (*NUP98, CPSF6, NPM1, PRPF19, HNRNPC, HNRNPM, SART3*, and *PML*) have been identified in *RARG-αAPL*.[2,3,6-11] Zhou *et al*. recently reported an unexpected finding of *RARG* 3′ splicing in all 21 cases studied, involving all seven known *RARG* 5′ fusion partners, except for *PML*, and elucidated the pivotal role of *RARG* 3′

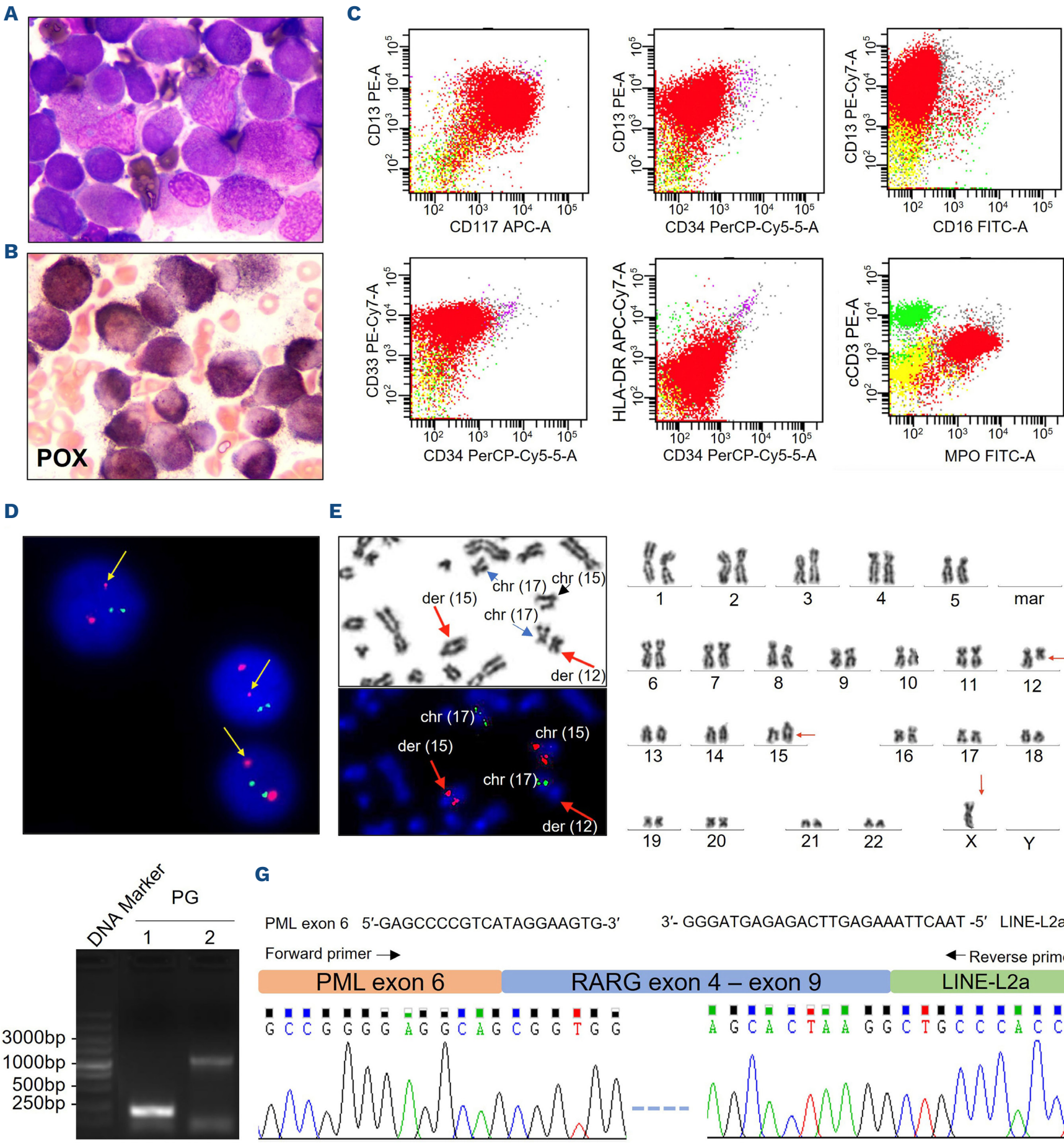
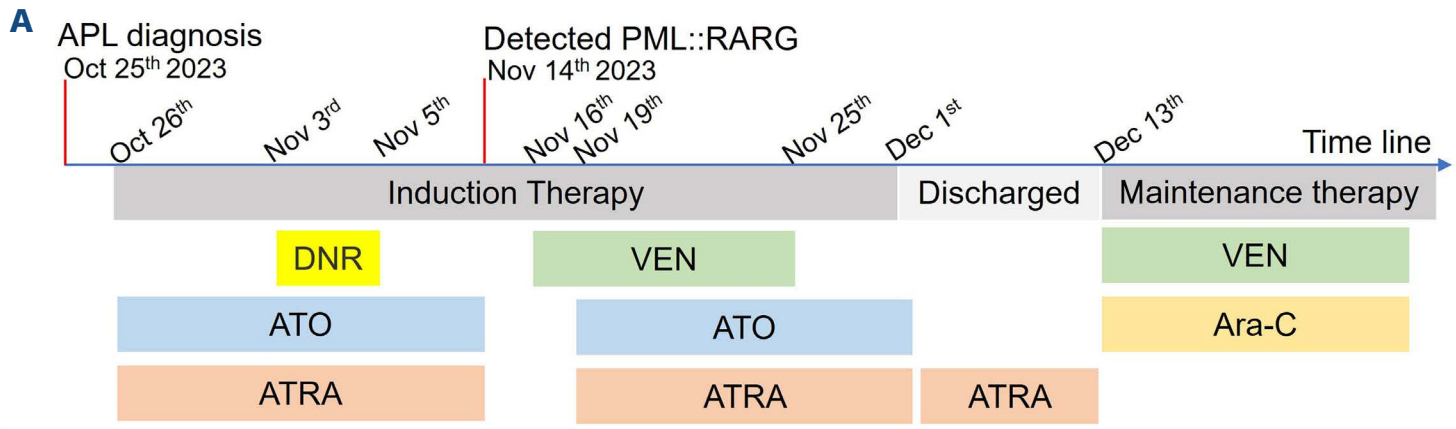
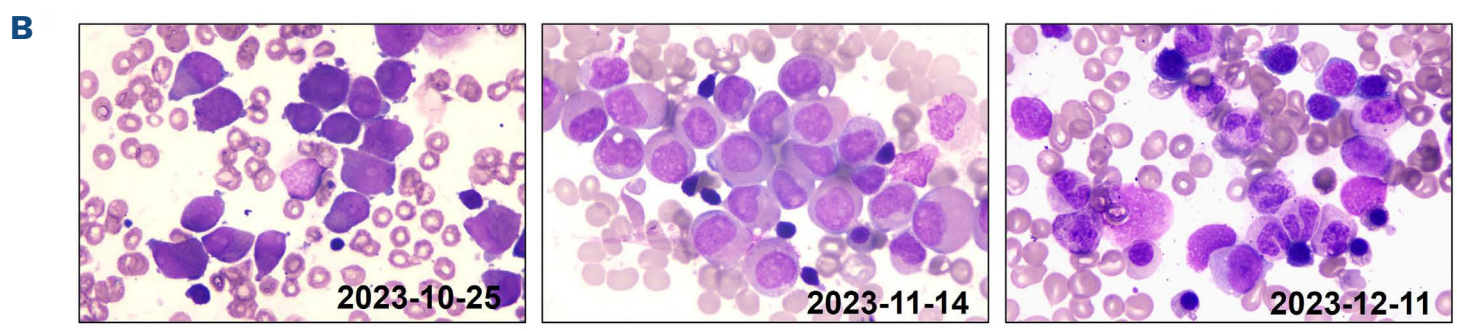
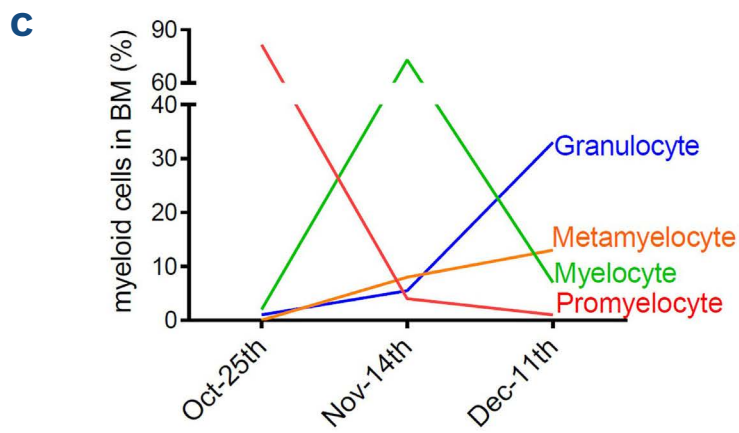
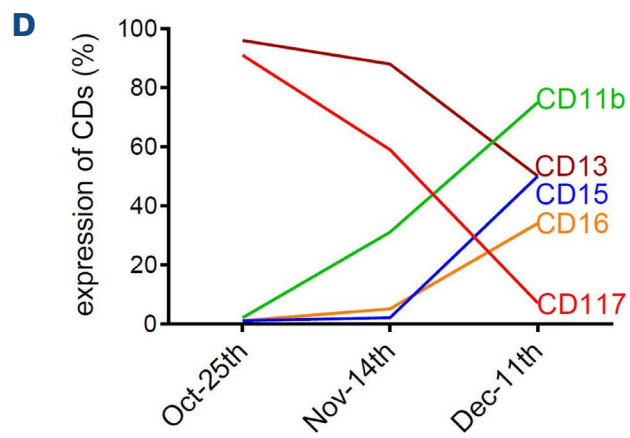


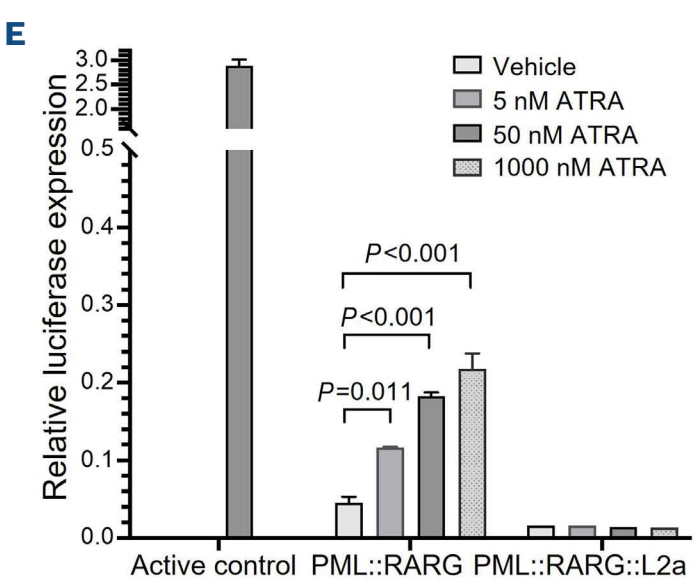
Figure 1. Morphological, cytochemical, immunophenotypic, cytogenetic and molecular biology testing assessment of the patient. (A) Bone marrow (BM) smear showed abnormal promyelocytes with hypergranulated cytoplasm of coarse purple red granules, irregular nuclei, loose chromatin, and visible nucleoli (Wright-Giemsa stain, ×1,000). (B) Cytochemical staining shows strong positivity of myeloperoxidase in leukemia cells of BM samples (×1,000). (C) Flow cytometric analysis showed that the leukemia cells expressed CD33, CD13, CD117 and MPO, and did not express CD34, CD7, CD38, HLA-DR, or other myeloid and lymphoid markers. (D) Dual-color dual-fusion fluorescence *in situ* hybridization (FISH) with *PML* and *RARA* probes failed to detect the fusion signal in the BM sample, but significant reduction of one *PML* signal was observed in majority of interphase nuclei (the red signal pointed by the arrow). (E) Cytogenetic analysis shows that the patient had chromosomal translocation t(12;15)(q13.1;q24.1). Metaphase FISH revealed abnormal *PML* signals present on the derived chromosome 15. (F) Electrophoresis of reverse transcription polymerase chain reaction (RT-PCR) products showed the presence of approximately 210 bp and 1,100 bp fragments in the patient with *PML* forward primer and *RARG* reverse primer or *LINE-L2a* reverse primer, but specific fragments were not detected in *PML::RARA*-positive patient. Lane 1 was amplified with *PML* and *RARG* primers, and lane 2 with *PML* and *LINE-L2a* primers. PG represented for *PML:: RARG* patient. (G) Sanger sequencing analysis of the 1,100 bp RT-PCR product verified the presence of PML-RARG-LINE-L2a tripartite fusion transcript.

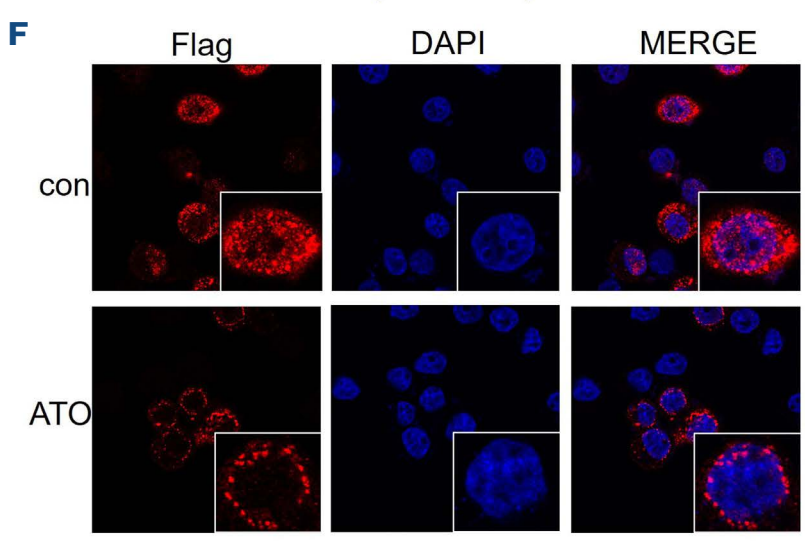


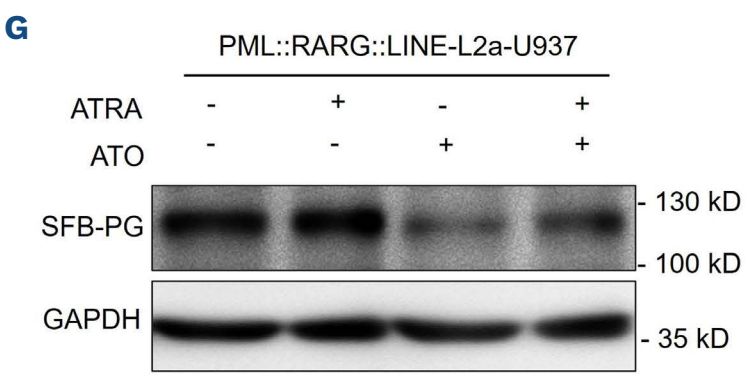












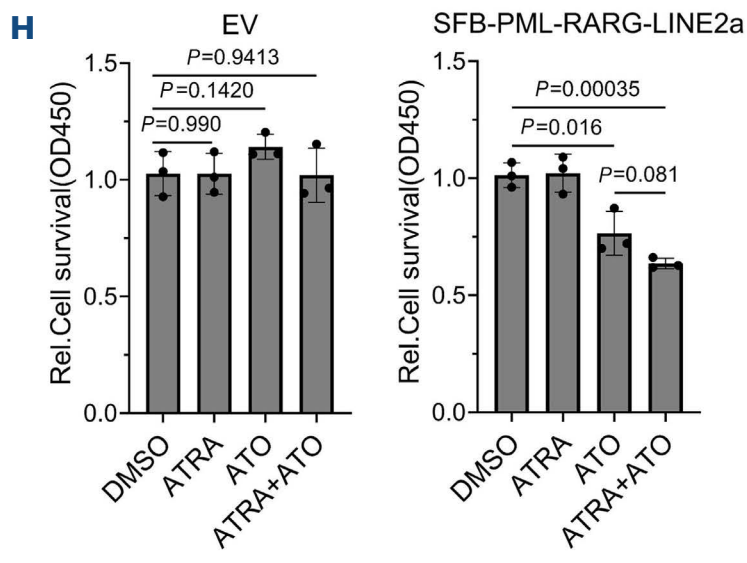


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Figure 2. Clinical patient course and therapeutic effect analysis. (A) Timeline of the patient's therapy. (B) Morphological changes in bone marrow (BM) during treatment. Majority of abnormal promyelocytes in the patient's BM at the initial diagnosis (2023-10-25), leukemia cells differentiated into myelocytes after using arsenic trioxide (ATO) + all-*trans* retinoic acid (ATRA) for 20 days (2023-11-14), and the BM cells became normal after 1 induction therapy (2023-12-13). (C) The number of myeloid cells in different stages during of the clinical course of the patient. (D) Quantitative analysis of myeloid differentiation marker during the clinical course of the patient. (E) The response of bipartite and tripartite fusion proteins to ATRA. Bipartite PML::RARG and tripartite PML::RARG::LINE-L2a fusion proteins simulated with different concentrations of ATRA and the RARE response element reactivity was analyzed by luciferase reporter gene assay. (F) The response of PML::RARG::LINE-L2a fusion protein for ATO with immunofluorescence analysis. The fusion protein was present in the nucleus and cytoplasm, mainly dispersed around the nucleus, fusion protein aggregated into small bodies around the nucleus after ATO treating 12 hours. (G) The effects of ATRA and ATO on the expression of PML::RARG::LINE-L2a fusion protein in U937 cells with western blot analysis. The fusion protein decreased significantly after ATO treating 72 hours, ATRA had no effect on the expression of fusion protein. (H) Proliferation of U937 cells with PML::RARG::LINE-L2a under ATRA and ATO treatment. ATO significantly inhibited proliferation of U937 cells with PML:: RARG::LINE-L2a but not vector control, ATRA had no effect on either. DNR: daunorubicin; VEN: venetoclax; Ara-C: cytarabine.

splicing in APL leukemogenesis and ATRA resistance.[2] Only one case of *PML*-related *RARG*-aAPL has been reported, with splicing sites located at PML exon 3 and *RARG* exon 1 or exon 2.[3] We identified a novel *RARG* splicing involving its exon 4, underscoring the variability of its 5′ splicing sites, which is consistent with Zhou's findings. Additionally, the involvement of the transposon *LINE-L2a* as the 3′ fusion partner in our case mirrors Zhou's observation of its frequent occurrence in *RARG* fusions.

ATRA and ATO are well-established differentiation agents for APL treatment.[12] Our patient underwent a 20-day induction regimen with ATRA and ATO before the *PML*::*RARG*::*LINE-L2a* fusion was identified. Morphological examination confirmed the differentiation of abnormal promyelocytes into myelocytes (Figure 2B, C), and immunological testing demonstrated a significant decrease of CD117 and increase in myeloid differentiation marker CD11B, CD15 and CD16 (Figure 2D). These findings indicated that the patient responded to ATRA and ATO combination therapy, supporting the continuation and completion of induction chemotherapy with this regimen. Ha *et al*. previously reported that their patient exhibited no early response after 18 days of ATRA treatment.[3] Zhou *et al*. have explained that truncation of the RARG LBD-H11_12, resulting from tripartite fusion, leads to ATRA unresponsiveness.[2] Therefore, we hypothesize that ATO played a predominant role in inducing differentiation in our patient.

We cloned the fusion gene from leukemia cells of our patient and determined that the *PML*::*RARG*::*LINE-L2a* fusion did not respond to ATRA, as demonstrated by luciferase activity experiment (Figure 2E). ATO-driven remission in *PML*::*RARA*-APL relies on the degradation of chimeric protein and subsequent reformation of PML nuclear bodies.[13,14] To further investigate, we transfected the *PML*::*RARG*::*LINE-L2a* fusion into Hela cells to analyze the localization of the chimeric protein and its responsiveness to ATO. Confocal microscopy revealed that the chimeric protein localized to both the nucleus and cytoplasm, with a primary distribution around the nucleus. After 12 hours of ATO treatment, the chimeric protein was reduced in the cytoplasm and aggregated into small nuclear bodies surrounding the nucleus (Figure 2F). In addition, we overexpressed PML::RARG::LINE-L2a in U937 cells and found that the chimeric protein promoted the proliferation of U937 cells. ATRA had no effect on the expression of chimeric protein and cell proliferation in both vector and fusion gene expressing cells, while ATO resulted in a decrease of the chimeric protein (Figure 2G) and growth inhibition (Figure 2H) of the fusion gene expressing U937 cells but not the vector control. These experimental results confirm that ATO, but not ATRA, is effective in aAPL patients with tripartite *PML*::*RARG*::*LINE-L2a* fusion. Our *in vitro* models, while replicating the drug sensitivity patterns seen clinically, do not fully delineate the molecular mechanisms underlying this response. Future studies will be essential to dissect the causal relationships between the tripartite fusion structure and therapeutic susceptibility.

While the primary focus of this study was to evaluate the efficacy of ATRA and ATO in this specific genetic context, we acknowledge the possibility that anthracycline and venetoclax may have contributed to the observed clinical response. Recently, Feng Wang *et al*. found that RARG-aAPL may be sensitive to venetoclax because BCL2 is crucial for the maintenance of RARG-aAPL,[15] which also supports the important role of venetoclax in the treatment of our patient. Future studies with controlled combination regimens will help further delineate the roles of individual agents.

In conclusion, we confirmed that the *RARG* fusion exists in the tripartite form in cases where *PML* serves as the 5′ fusion partner. The tripartite fusion *PML*::*RARG*::*LINE-L2a* leads to truncation of the RARG LBD-H11_12, rendering ATRA unresponsiveness. However, these patients can still benefit from arsenic-based therapy due to the involvement of *PML* as the 5′ fusion partner.

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Disclosures

No conflicts of interest to disclose.

Contributions

ZZ and HL wrote the manuscript. ZZ conceptualized and designed the study. FZ provided valuable discussion. XLin, XZ and HL were responsible for laboratory experiments and bioinformatic analysis. SW, YY, YZ, PL, HS, JH, LL, XLiu, DZ, YL and HX provided study materials or recruited patient.

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Data-sharing statement

Data are available on request from the corresponding author ZZ. For RNA-sequencing original data of the patient contact the corresponding author HL.

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