A novel *SART3::RARG* fusion gene in acute myeloid leukemia with acute promyelocytic leukemia phenotype and differentiation escape to retinoic acid

Acute promyelocytic leukemia (APL) is a unique subtype of acute myeloid leukemia (AML) that features promyelocytic leukemia cells, prominent coagulopathy, and the promyelocytic leukemia *(PML)::*retinoic acid receptor α (*RARA*) fusion gene derived from translocation t(15;17).[1] The introduction of all-trans retinoic acid (ATRA) and later arsenic trioxide (ATO) has revolutionized the treatment of this disease, which is now highly curable.[1] In approximately 5% of the cases presenting APL phenotype, *PML::RARA* was negative. By far, translocations involving *RARA* and 16 other partner genes have been reported with different sensitivity to ATRA.[2] Rearrangement involving the other two members of the retinoic acid receptor family, retinoic acid receptor β (*RARB*) or retinoic acid receptor γ (*RARG*), has been reported including *TBLR1::RARB*, *NUP98::RARG*, *CPSF6::RARG*, *PML::RARG*, *NPM1::RARG::NPM1*, *HNRNPC::RARG* and *RARG::HNRNPM*.[2-5] All of these patients demonstrated ATRA resistance. Here, we report a novel *SART3::RARG* fusion gene in a case of APL-like leukemia (APLL) showing differentiation escape to ATRA. A comprehensive analysis of the patient and preliminary function assays for *SART3::RARG* were performed. Our work not only enriches our knowledge of APL-like leukemia (APLL), but also lends support to the proposal that *RARG* rearrangement represent a distinct subtype of AML that needs early recognition and proper management.

A 46-year-old previously healthy man was admitted because of gum bleeding and spontaneous ecchymosis. His initial complete blood count showed a white blood cell (WBC) count of 2.0×10^9/L, hemoglobin level of 99 g/L, and platelet count of 80×10^9/L. Prominent coagulopathy was noted with prothrombin time of 20.1 seconds (s) (reference 10-14 s), activated partial thromboplastin time of 60.6 s (reference 28-45 s), fibrinogen level of 0.33 g/l (reference 2-4 g/L), fibrinogen/fibrin degradation product levels 27.67 mg/L (reference, 0-5 mg/L), and D-dimer levels 4.37 mg/L (reference, 0-0.5mg/L). Bone marrow (BM) smear showed hypercellularity with 81.7% abnormal promyelocytes (Figure 1A). The blasts featured abundant cytoplasmic coarse granules, lobulated or kidney-shaped nuclear, and occasional nucleoli, reminiscent of APL. Auer rods were absent. Myeloperoxidase (MPO) staining was negative somehow (Figure 1B). These cells were positive for CD13, CD33, and CD117, partially positive for MPO, CD64, and CD15, but negative for CD34, HLA-DR, CD11b, CD14, and other lymphoid-related markers by flow cytometry (Figure 1E). Fluorescence *in situ* hybridization (FISH) using a *PML::RARA* dual-color dual-fusion probe showed no fusion signals (Figure 1C). Multiplex reverse transcription polymerase chain reaction (RT-PCR) detecting 43 leukemia-related fusion genes including *PML::RARA* were negative. G-banding karyotype analysis of BM cells revealed a clonal -Y in 16 of 20 metaphase analyzed (Figure 1C). ATRA (20 mg, twice daily) was administered on suspicion of APL for a week without improvement of coagulopathy. He was transferred to our hospital on the 8th day. We continued ATRA treatment and reevaluated his BM morphology and immunophenotype, where there were no signs of differentiation. Meanwhile, his WBC count increased to 6.4×10^9/L, and he complained of bone pain. Despite strong transfusion supportive care, he died of diffuse alveolar hemorrhage on the 11th day.

Given the absence of *PML::RARA*, RNA sequencing, single-nucleotide polymorphism (SNP) array, and whole exome sequencing (WES) were performed using the patient's BM sample to characterize underlying molecular aberrations. The study was approved by the Second Xiangya Hospital Institutional Review Board, Central South University. Informed consent or assent was obtained in accordance with the Declaration of Helsinki. RNA sequencing revealed that *SART3* exon 18 was fused to *RARG* exon 3 (Figures 2A). In order to validate this novel fusion, RT-PCR was performed with the forward primer 5'-TCAAAGTGGCAATCAGCAACC-3' (at *SART3* exon 18), and reverse primer 5'-AGCCTGGGAGGCTCCGTA-3' (at *RARG* exon 3). An expected 160 bp band was visualized on agarose gel electrophoresis (Figure 2B) (GenBank accession number ON681589). Using the same primers, a 1,156 bp transcript was amplified from the genomic DNA of BM sample, which localized the genomic breakpoint at *SART3* intron 18 (Chr12: 108917694) and *RARG* intron 2 (Chr12: 53622069) (Figure 2C) (GenBank accession number ON681590). The reciprocal *RARG::SART3* fusion was not detected. The fusion was in-frame and encode a 1,406-amino acid chimera (Figure 2E). Immunoblotting of BM mononuclear cell lysate using an anti-RARG antibody (Cell Signaling Technology) confirmed the presence of SART3::RARG as a band (~170 kDa) was visualized in addition to the wild-type RARG band (~55 kDa) (Figure 2D).

Furthermore, SNP array revealed a mosaic loss of chromosome Y in accordance with karyotype (*Online Supplementary Figure S1A*). Besides, a 2.26 Mb microdeletion in

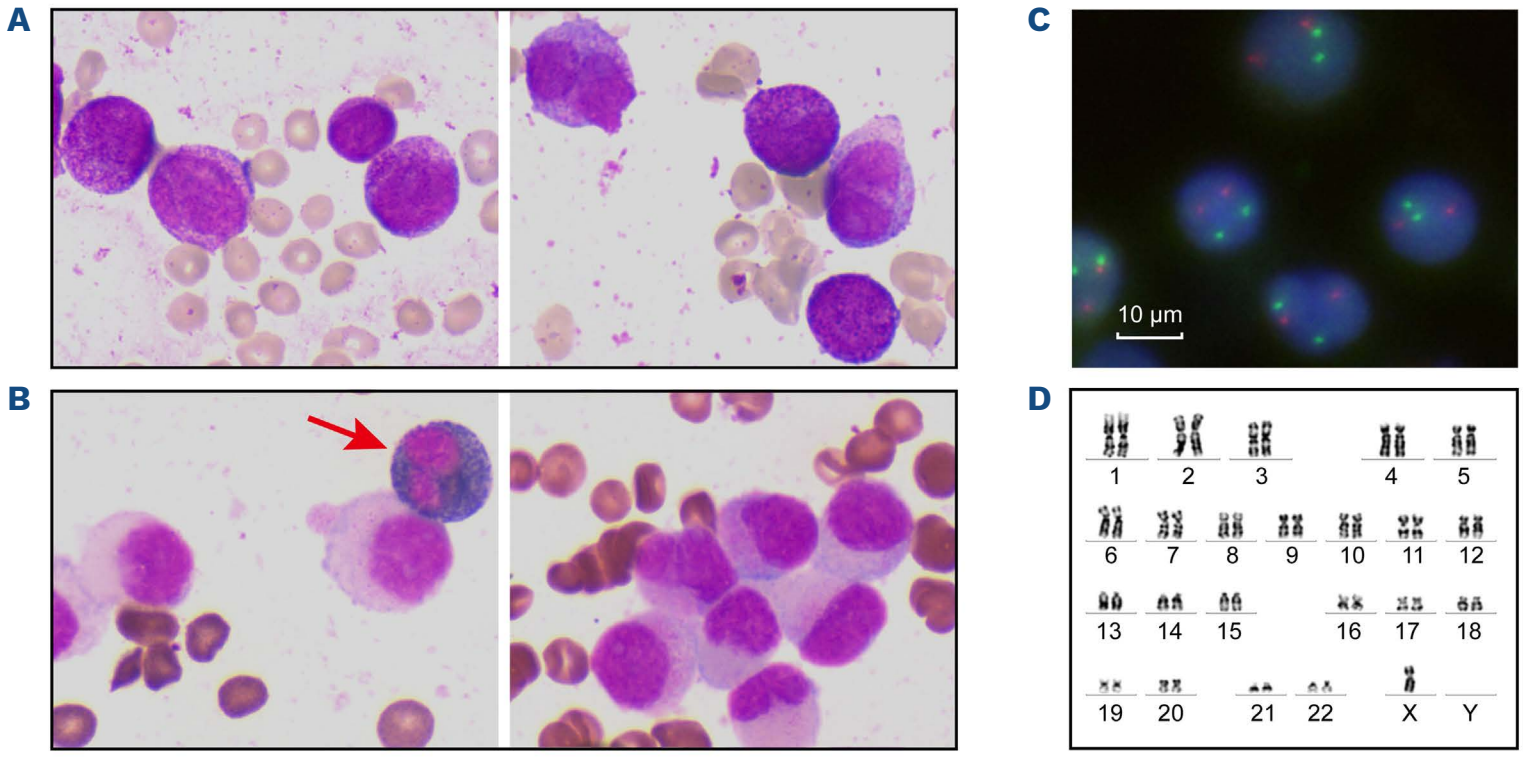


Figure 1. Morphologic, immunophenotypic, and cytogenetic features of the *SART3::RARG*-positive blasts. (A) Wright-Giemsa staining of bone marrow smear showing hypergranular promyelocytes without Auer rods. Original magnification X1,000. (B) Cytochemical evidence for myeloperoxidase negativity of blast cells (as compared with neutrophils; arrows). Original magnification X1,000. (C) Interphase fluorescence *in situ* hybridization using the *PML::RARA* dual-color, dual-fusion translocation probe revealed the absence of *PML::RARA*. (D) Karyotypic analysis of the bone marrow revealed a karyotype of 45,X,-Y[16]/46,XY[4]. (E) Immunophenotype of the patient with *SART3::RARG*-positive AML. Dot plots of flow cytometry data of bone marrow aspiration sample. Red cluster shows the blast population.

12q21.2, a 1.99 Mb microdeletion in 12q23.3q24.11 encompassing *SART3* (*Online Supplementary Figure S1B*), and a uniparental disomy of a 5.42 Mb segment at 17q21.2q21.32 (*Online Supplementary Figure S1C*) were detected. Since *SART3* and *RARG* located at 12q23.3 and 12q13.13, respectively, the *SART3::RARG* fusion gene may result from the microdeletions or cryptic translocations in 12q. In addition, WES identified two somatic mutations, *WT1* c.1114-2A>G (variant allele frequency [VAF] 27%) (*Online Supplementary Figure S2A*) and *KDM6A* p.Pro1107AlafsTer46 (VAF 90.5%) (*Online Supplementary Figure S2B*), which were verified by RT-PCR and Sanger sequencing. Moreover, three *MPO* mutations (*Online Supplementary Table S1*) were detected by WES, which might have impaired the enzymatic and immunological activity of MPO in a way of compound heterozygous mutations, leading to negative MPO staining and reduced MPO immunophenotype in this case.[6]

Retinoic acid receptors (RAR) are members of nuclear hormone receptors, and regulate cell growth, differentiation,

and cell death in response to retinoic acid/ligand binding. Their modular structure features two main functional domains: DNA-binding domain (DBD) and ligand-binding domain (LBD). The three RAR family members, RARA, RARB, and RARG, share high sequence homology but show distinct transcriptional properties.[7] During hematopoiesis, RARG is a potent ligand-dependent transactivator that governs hemopoietic stem/progenitor cells self-renewal, whereas RARA exerts ATRA-reversible basal repressive functions favoring myeloid differentiation.[7] SART3 (spliceosome-associated factor 3, U4/U6 recycling protein), is a nuclear RNA-binding protein regulates pre-mRNA splicing, translesion DNA synthesis, and histone chaperoning.[8,9] It plays important roles in viral and host gene transcription, embryonic development, and hematopoiesis.[8-10] Structurally, SART3 consist of multiple half-a-

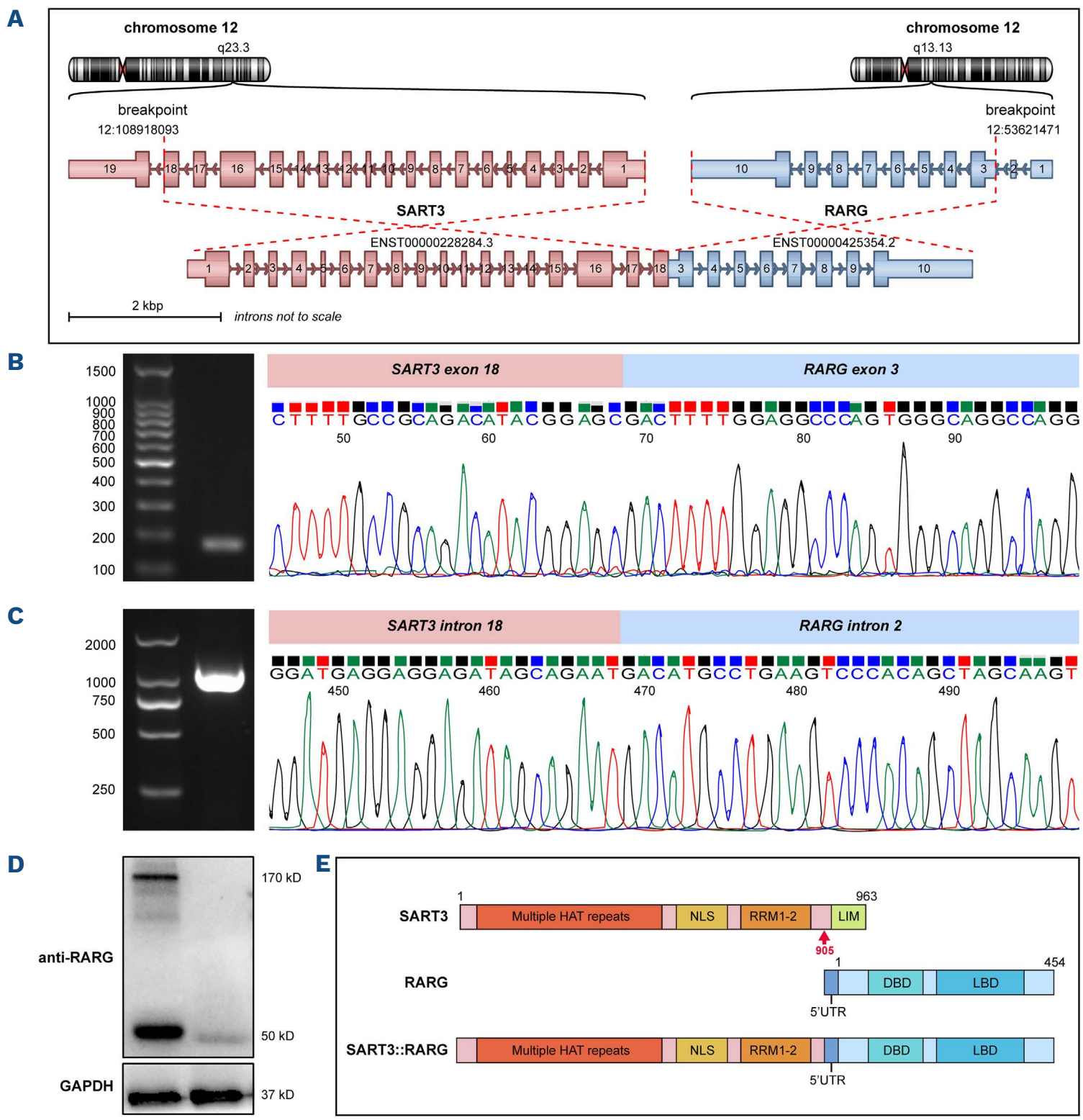
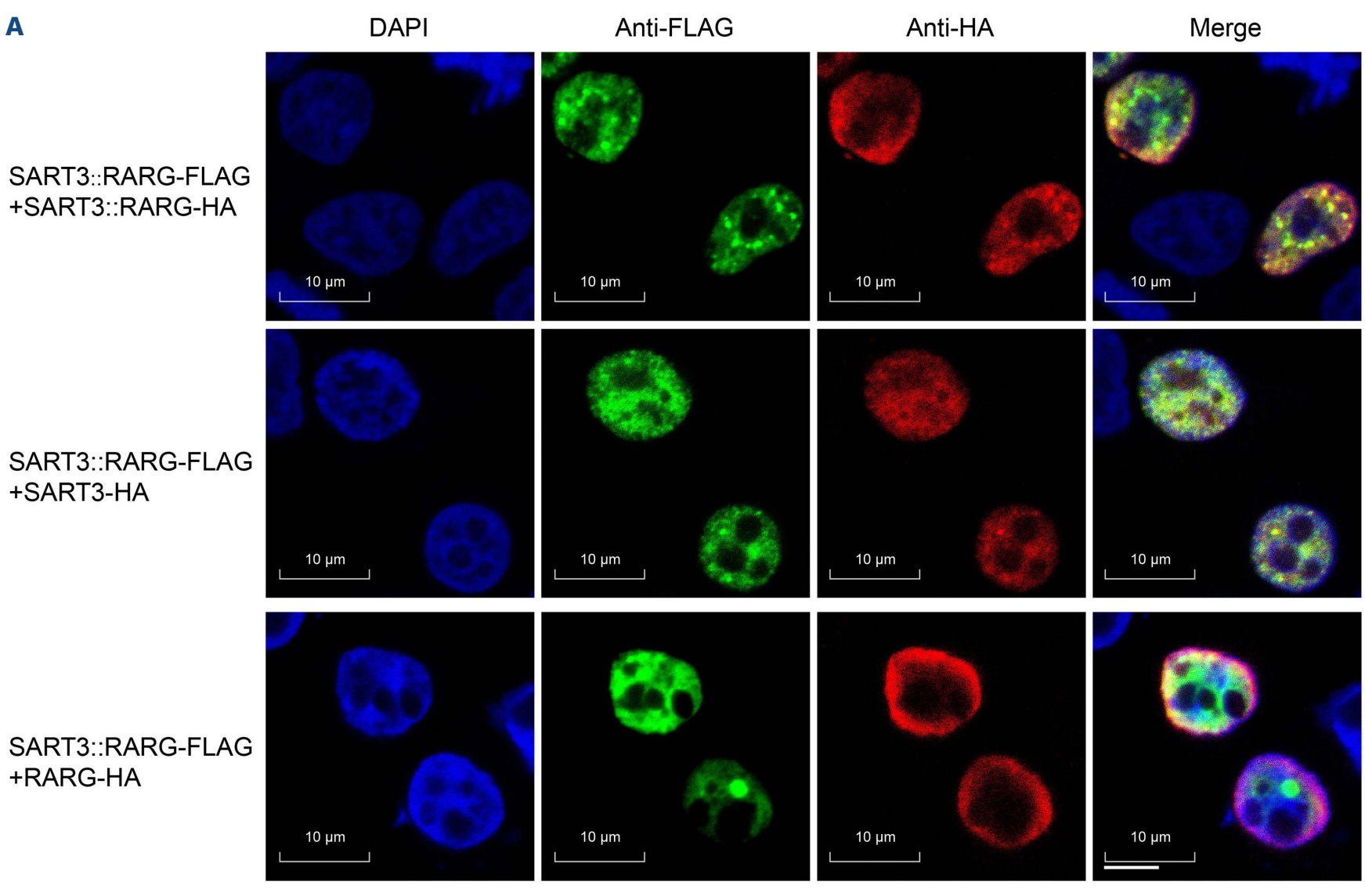


Figure 2. Molecular characterization of the *SART3::RARG* fusion. (A) RNA sequencing analysis revealed the fusion between exon 18 of *SART3* and exon 3 of *RARG*. (B) Reverse transcription polymerase chain reaction (RT-PCR) using bone marrow (BM) cDNA confirmed the *SART3::RARG* fusion transcript when a 160 bp band was visualized. Partial nucleotide sequences surrounding the junction was shown. (C) RT-PCR for *SART3::RARG* using BM genomic DNA amplified a band of 1,156 bp. Partial nucleotide sequences surrounding the genomic breakpoint was shown. (D) Western blotting of BM mononuclear cell lysate using anti-RARG antibody showed two bands corresponding to the wild-type RARG (~55 kDa) and the SART3::RARG chimera (~170 kDa) in the patient. 1: the patient with *SART3::RARG*; 2: a classic APL patient with *PML::RARA*. (E) Schematic diagram of SART3, RARG, and the SART3::RARG fusion protein. The fusion breakpoint was highlighted with a red arrow. DBD: DNA binding domain; HAT: half-a-tetratricopeptide repeats; LBD: ligand binding domain; LIM: LSm-interacting motif; NLS: nuclear localization sequence; RRM1-2: RNA recognition motifs 1-2; 5' UTR: 5' untranslated region.

tetratricopeptide (HAT) repeats, a nuclear localization signal (NLS) sequence, two RNA recognition motifs (RRM), and a LSm-interaction motif (LIM) at the C-terminus (Figure 2E). *SART3::PDGFRB* was reported in a patient with eosinophilia-associated myeloproliferative neoplasm.[11] The SART3::RARG chimera retained the HAT repeats, NLS, and RRMs of SART3, an intact RARG, and additional 46 amino acids due to inclusion of partial RARG 5' untranslated region (UTR) (Figure 2E). The loss of 58 amino acids in the carboxyl terminal of SART3, including the LIM domain, may impair SART3's function as an mRNA splicing regulator.

As shown by immunofluorescence experiments, the subcellular localization of SART3::RARG was primarily intranuclear, diffusely distributed and specifically aggregated in large bright dots (Figure 3A, the second column) similar to that of SART3[12] but distinct from RARG. Homo-dimerization is a common feature of RARA fusion chimeras as well as NUP98::RARG to gain oncogenic potential.[13] As shown in Figure 3A, FLAG-tagged SART3::RARG co-localized with either HA-tagged SART3::RARG or SART3, but not RARG. Co-immunoprecipitation results confirmed the self-association of SART3::RARG and its heterodimerization with SART3, but not with RARG (Figure 3B), suggest that homodimerization of SART3::RARG is mediated by the SART3 portion, likely the HAT repeats.[9] Furthermore, dual luciferase reporter assays revealed that SART3::RARG acts as a dominant-negative RARG mutant (Figure 3C) similar to NUP98::RARG[13]. In response to ATRA, the transcriptional activity of RARA, PML::RARA, and RARG increased dramatically whereas SART3::RARG showed a blunt ATRA response comparable to ATRA-insensitive ZBTB16::RARA and GTF2I::RARA[14] (Figure 3D).

Including *SART3::RARG*, seven different *RARG* fusion genes have been reported.[2-5] Although the breakpoint in *RARG* varied, occurring at exon 1, 2, 3, or 4 for *X::RARG* and at intron 9 for *RARG::X*, all these RARG fusion chimeras retain the DBD and LBD of RARG. From cases reported in the literature, these RARG-rearranged AML were predominantly East Asians, and exhibited APL morphology and immunophenotype, bleeding diathesis, but were insensitive to ATRA. Response to anthracycline plus cytarabine "7+3" induction regimen varied, while incorporating homoharringtonine into reinduction chemotherapy succeed in achieving complete remission. Due to its rarity, the incidence and prognosis of RARG-rearranged AML remain to be determined. The early death of our patient makes it hard to conclude clinical ATRA resistance. However, the lack of an early response suggests ATRA insensitivity. It is reported that CD11b positivity and correction of coagulation disorders are early signs of ATRA's efficacy.[1,15] In our



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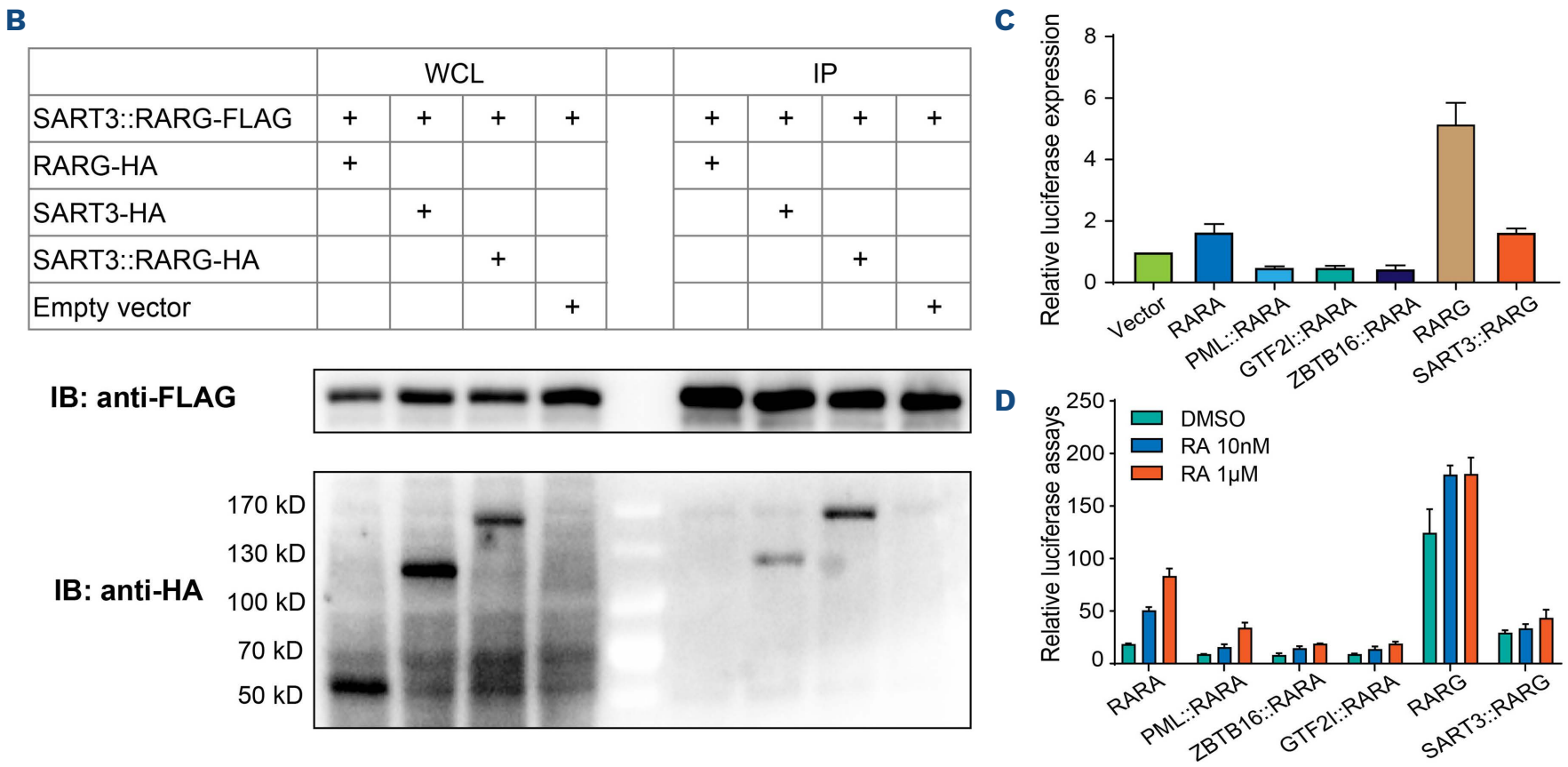

Figure 3. Functional assays for the *SART3::RARG* fusion chimera. (A) Subcellular localization/colocalization of SART3::RARG, SART3, and RARG. 293T cells were co-transfected with indicated pcDNA3.1 expression plasmids. Immunofluorescence was performed with anti-FLAG and anti-HA antibodies. The nuclei were visualized with 4', 6-diamidino-2-phenylindole (DAPI: blue, the first column), FLAG-tagged SART3::RARG was stained with Alexa Fluor 488 (green, the second column), and HA-tagged proteins were stained with Alexa Fluor 594 (red, the third column). The 4th column shows composite images: yellow fluorescence indicates co-localization. Original magnification X630. (B) SART3::RARG interacts with itself or SART3, but not RARG. PcDNA3.1 expression plasmids of SART3::RARG-FLAG was co-transfected into 293T cells with either empty vector or HA-tagged expression vector, as shown in the table. Immunoblotting of whole cell lysate (WCL) confirmed successful transfection and expression (left). Cell lysate were immunoprecipitated (IP) using anti-FLAG antibodies, and analyzed by immunoblotting (right). SART3::RARG-HA and SART3-HA, but not RARG-HA, could be detected after immunoprecipitation with anti-FLAG antibodies. (C) SART3::RARG act as a negative mutant of RARG. 293T cells were transfected with 4×RARE pGL3 reporter vector, pRL-TK, and together with a vector containing one of the following genes: *RARA*, *PML::RARA*, *GTF2I::RARA*, *ZBTB16::RARA*, *RARG*, or *SART3::RARG*. Relative firefly luciferase expression of cell lysate was normalized to Renilla luciferase. The expression of the vector control was set to 1. The error bars represent the mean of an experiment performed in triplicate. (D) SART3::RARG responds poorly to ATRA. 293T cells transfected with the indicated constructs were treated with dimethyl sulfoxide (DMSO), 10 nm ATRA, or 1 μM ATRA for 48 hours. Relative firefly luciferase expression of cell lysate was normalized to Renilla luciferase. N=3 independent experiments. All data are presented as mean ± standard deviation.

patient, 7 days' of ATRA therapy failed to upregulate CD11b expression, which were seen as a sign of granulocytic differentiation,[15] suggested differentiation escape to ATRA. A total of 10 days' ATRA treatment together with strong transfusion-supported care, did not bring improvement of coagulopathy, suggested ATRA ineffectiveness. Given the observation that other *RARG*-rearranged patients were insensitive to ATRA, we propose ATRA withdraw and switching to AML-like approaches in *RARG*-rearranged APLL. Early identification and strong transfusion supportive care are crucial to avoid early death, whereas optimal treatment requires further investigation.

In summary, we have identified a novel *SART3::RARG* fusion gene in a patient with APL phenotype. Our finding enriched the understanding of APLL. Further studies are needed to elucidate the critical role of *SART3::RARG* and *RARG* dysregulation in leukemogenesis and for guiding treatment approaches.

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Disclosures

No conflicts of interest to disclose.

Contributions

JL and GZ designed the study, performed the study, and wrote the manuscript. YZ, JL and YX collected data, and interpreted the data.

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Data-sharing statement

Questions regarding data sharing should be addressed to the corresponding author. For original data, please contact zgsllzy@163.com, or lijiji26@163.com.

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