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Acute promyelocytic leukemia with *TTMV::RARA* fusion potentially responds to all-trans retinoic acid/arsenic trioxide treatment

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Running heads: APL with *TTMV::RARA* responds to ATRA+ATO

Author's contributions: QX collected the clinical information of the patient and wrote the main text; YP and SS analyzed bioinformatics; YZ, TS and XK provided some of the patient's clinical information; YW gave suggestions for revisions; HHZ was responsible for initiated the whole work.

Data-sharing statement: The raw RNA-seq and whole-genome sequencing (WGS) data that support the findings of this study has been uploaded to the GSA-Human database of the CNCB (https://www.cncb.ac.cn/) with the accession identifier HRA007666 (https://www.cncb.ac.cn/search/specific?dbId=hra&q=HRA007666). The data are not publicly available due to privacy or ethical restrictions. Researchers can request the raw data according to the GSA-Human protocol. The complete mRNA of *TTMV::RARA* fusion has been deposited in the GenBank database (the accession

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Word count: 1500

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Over 95% of patients diagnosed with acute promyelocytic leukemia (APL) exhibit the fusion of the promyelocytic leukemia (*PML*) gene with the retinoic acid receptor alpha (*RARA*) gene, and significantly respond to all-trans retinoic acid (ATRA) combined with arsenic trioxide (ATO).[1,2] Conversely, less than 5% of morphologically classified APL cases harbor alternative partner genes besides *PML*, which impacts the efficacy of ATRA+ATO, thereby defining variant APL.[3]

Torque teno mini virus (TTMV) is prevalent and symbiotically exists with human. Recent research indicates that TTMV could integrate with *RARA*, potentially resulting in the development of a distinct type of variant APL.[4] To date, there have been eleven cases reported, including nine children and two adults.[4-10] However, the pathogenetic mechanisms of *TTMV::RARA* in acute myeloid leukemia (AML) remain uncertain, as does its response to ATRA+ATO. We firstly showed an APL case with *TTMV::RARA* achieving a long-term complete remission (CR) induced by ATRA+ATO, offering an insight into managing this disease and distinguishing it from other variant APL.

The patient, a 15-year-old boy, initially presented to a local hospital with a persistent headache and weakness. Laboratory findings revealed leukocytosis (white blood cell count: 40.85×10^9/L), anemia (hemoglobin: 109 g/L), and thrombocytopenia (platelet count: 47×10^9/L). Coagulation tests demonstrated prolonged prothrombin time (19.6 seconds) and activated partial thromboplastin time (32.6 seconds), as well as elevated D-dimer (38.44 mg/L) and hypofibrinogenemia (0.52 g/L). The bone marrow (BM) morphology showed a predominant population consisting of classical hyper-granular promyelocytes (81%) and exhibiting intense reactivity to myeloperoxidase staining (+++) (Figure 1A). According to the AI-PAL model utilizing age and blood parameters (https://alcazerv.shinyapps.io/AIPAL/), our case was predicted to be APL with high probability (93.7%, Figure 1B). Flow cytometry identified 93% of granulocytes with abnormal immunophenotype (CD13+, CD33+, CD123+, CD34-, HLA-DR low/-; Figure 1C). Karyotype was 46,XY[20] (Figure 1D). Real-time PCR showed negative expression of *PML::RARA*, while the targeted next-generation sequencing (including 72 myeloid-associated genes) revealed mutations in *KRAS* (p.G12D, variant allele frequency, VAF 1.06%), *NRAS* (p.G12D, VAF 37%), *BRAF* (p.G469A, VAF 1.15%) and *ARID1B* (p.E2051K, VAF 47%) genes. The Principal Component Analysis (PCA) of gene expression confirmed that our case was more

similar to APL than to non-M3 AML (Figure 1E).

Given the clinical suspicion of hyper-granular (or typical) APL, the initial leukoreduction treatment consisted of hydroxyurea and low-dose cytotoxicity drug, followed by four courses of ATRA (20mg, twice daily, days 1-14) + ATO (10mg, daily, days 1-28). The patient achieved the first CR (CR1) after the first cycle and maintained CR status for six months. Subsequently, the patient presented with fever and hip pain, along with leukocytosis (14.73×10^9/L) and central nervous system (CNS) involvement. Flow cytometry revealed 78.7% abnormal promyelocytes in BM, showing a changed karyotype (46,XY,t(3;20)(q26.2;q11)[5]/46,idem,del(9)(p21)[13]/46,XY[2]). Due to relapse, the patient underwent the salvage induction therapy consisting of ATRA (20mg, twice daily, days 1-14), idarubicin (10mg, daily, days 1, 3, 7), and cytarabine (200mg, daily, days 1-7), resulting in secondary CR. Following two additional cycles of venetoclax, homoharringtonine, cytarabine, and granulocyte-colony stimulating factor, the disease relapsed once more and the patient succumbed to progressive disease within two months before undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT).

After the relapse following four courses of ATRA+ATO, the whole transcript sequencing (WTS) of BM was carried out, revealing no *PML::RARA*. Samples were collected with informed consent in accordance with the Helsinki Declaration. The study was approved by the Ethics Committee of Beijing Chao-Yang Hospital (2024-KE-515). Based on the recent literature reporting *TTMV::RARA* [4,5,7,11], it was postulated that *TTMV* genetic material had integrated at this locus. A total of 33 reads from RNA sequencing successfully aligned to *RARA* exon 3 and *TTMV* genome, exhibiting a mapping length ≥ 50 base pairs (bp) and an identity ≥ 90%, indicating the presence of *TTMV* genetic material within the genome of this case. To further investigate this phenomenon, transcripts in close proximity to *RARA* gene were assembled.

An assembled transcript sequence was found to partially align with *RARA* exon 3-9 (NC_000017.11:40,348,316-40,356,914) (Figure 2A). Additionally, a 568 bp sequence located at the 5' end, which could not be mapped to the human hg38 reference

genome, was determined as part of the *TTMV* genome (Figure 2B). Substantial alignment of the integrated *TTMV* sequence was noted with the complete genome of *TTMV* isolate SAfiA-453-9 (ACCESSION: MN770239).[12] Two mapping blocks demonstrated 82% and 67% identity across the entire 568 bp sequence (Figure 2B). The insertion of the *TTMV* sequence was located at chr17:40,347,785 (hg38), with the integrated sequence spanning 568 nucleotides. Notably, the final 226 nucleotides correspond to the coding region that aligns in-frame with *RARA* exon 3 (Figure 2C). The complete *TTMV::RARA* transcript spans 2354 bp, with an estimated 1437 bp coding sequence. The coding sequence and the *TTMV* insertion breakpoint were further confirmed by PCR, sanger sequencing, WTS, and the whole-genome sequencing (WGS) (Figure 3). While MN770239 lacks detailed annotation of *TTMV* open reading frame (ORF) 2, the overlap with ORF1 is consistent with earlier findings.[13] The conserved TTMV ORF2 5′ untranslated region (UTR) sequence "CGAATGGCTGAGTTT" was identified at nucleotides 267-281 in our assembled *TTMV* transcript sequence, followed by a 226 nucleotides segment initiating with the start codon (ATG). Additionally, the conserved motif "$WX_7HX_3CXCX_5H$" of TTMV ORF2 was also found in the *TTMV::RARA* transcript (Figure 2C). According to recent reports, the *TTMV* sequence consistently appears in ORF2.

This study contributes to the existing literature by presenting additional one documented APL case with *TTMV::RARA*. Most reported cases were characterized by hyper-granular promyelocytes, displaying morphological and immunophenotypic similarities to typical APL. CNS involvement, which is uncommon in APL with *PML::RARA*, was present in four of six cases with *TTMV::RARA*. Specifically, three cases demonstrated CNS involvement at diagnosis [9,10], while our case displayed it following relapse. Among the four cases undergoing ATRA + chemotherapy (idarubicin or "7+3" regimen), two demonstrated sustained CR durations lasting 8-15 months.[4,6,10] Particularly, our case demonstrated a six-month CR duration during the four courses of ATRA+ATO. *TTMV::RARA* was not initially considered in our case, yet classic APL characteristics were observed. An empirical ATRA+ATO treatment was thereby implemented, achieving unexpected CR1, possibly due to the anti-RARA properties of ATRA, with ATO synergistically enhancing its anti-leukemic effects. ATO exhibits anti-proliferative and pro-apoptotic effects in leukemia via cell cycle arrest, apoptosis and autophagy induction, as well as reactive oxygen species generation

and caspase activation.[14] ATRA+ATO also inhibits the Nrf2 antioxidant pathway, thereby increasing cytotoxicity in AML.[15] Ongoing trials involving this combination are currently underway in non-M3 AML (NCT03031249, NCT05297123). Given the anti-leukemia effects of ATO, further research on ATRA+ATO in APL with *TTMV::RARA* is warranted.

The case from Wang[9], with high-risk factors like complex karyotype and extramedullary involvement, failed to achieve remission with ATRA+ATO. In contrast, our case lacked such factors before treatment, suggesting that ATRA+ATO could be considered as a first-line option for treating APL with *TTMV::RARA* without high-risk features, rather than as a salvage therapy.

The efficacy of allo-HSCT for APL with *TTMV::RARA* is noteworthy. Of eight patients receiving allo-HSCT, four underwent allo-HSCT in CR1, with three maintaining CR for 0.63 to 8 years[10] and one recurring after 13 months[9]. The other four underwent allo-HSCT post-relapse, with two surviving for 4 years and 20 months[7,10], respectively, but two relapsing after 7 months and 50 days[4,6]. Allo-HSCT in CR1 showed low relapse and prolonged remission. While post-relapse patients had lower survival, some still achieved CR. Further research with larger cases and longer follow-up is still needed.

Prior studies showed that the retained lengths of *RARA* intron 2 in fusion events range from 13 to 38 nucleotides. However, *RARA* intron 2 was absent in the fusion transcript of our case (Figure 2C). It indicates a higher level of complexity in the *TTMV::RARA* fusion event and suggests that the *RARA* intron 2 may not be an essential causative factor. Additionally, each case exhibits distinct partner genes integrated with *RARA*, potentially resulting from differential splicing patterns, necessitating further investigation.

Due to the widespread presence of TTMV, there is a potential for additional cases with *TTMV::RARA* to remain undetected. The development of a specialized bioinformatics approach designed for the diverse *TTMV* genome sequence could facilitate the identification of *TTMV::RARA* through the WTS analysis. Additionally, the establishment of a reliable PCR protocol would be essential for detecting

TTMV::RARA.

Furthermore, additional research is necessary to elucidate the oncogenic mechanisms associated with *TTMV::RARA* fusion protein and identify other instances of viral genome integration with human genes that contribute to hematological malignancies. Given the high prevalence of TTMV in population, it is imperative to ascertain whether the fusion event between *TTMV* and *RARA* occurs randomly or is influenced by genetic susceptibility factors. A comprehensive understanding of these potential susceptibility factors is essential for preventing virus-induced malignant transformations. It is noteworthy that, unlike previous APL subtypes which were solely of human origin, APL with *TTMV::RARA* is characterized by a viral component. This warrants a deeper investigation into the mechanisms by which TTMV integrates into human genome.

Conclusively, APL with *TTMV::RARA* represents a unique variant APL with a novel oncogenic mechanism and distinct clinical and molecular features, offering new insights into APL.

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FIGURE LEGEND

FIGURE 1. Morphologic features, immunophenotypes, karyotype and expression profile of the variant acute promyelocytic leukemia (APL) case with *TTMV::RARA*.

(A) Giemsa staining of the bone marrow (BM) aspiration specimen at first diagnosis. The promyelocytes had a round or irregular shape with off-center ovoid nuclei, sometimes binucleated. Their chromatin was loosely dispersed, with inconspicuous nucleoli. The cytoplasm, variable in amount and bluish-gray, contained coarse, dense granules, prominent both inside and outside the cytoplasm.

(B) The AI-PAL model included large training (n=477), internal testing (n=202) and external validation (n=731) sets, with 10 clinical parameters. According to AI-PAL model, our case was predicted to be APL with high probability (93.7%). ALL, acute lymphocytic leukemia.

(C) Immunophenotype profiling of BM was characterized by CD13+, CD33+, CD123+, CD34- and HLA-DRlow/-.

(D) Karyotype showing a normal chromosomal constitution.

(E) The gene expression data from 12 APL cases and 45 cases with non-M3 acute myeloid leukemia (AML) in the Beat AML1.0 dataset (No. NCT03013998) were randomly selected and integrated with our case with *TTMV::RARA* for the Principal Component Analysis (PCA). The resulting plots of the first two principal components (PC1 and PC2) revealed that our case was positioned closer to the APL cluster along the PC1 axis, with the high variance explained by PC1 (66.4%) suggesting a similar gene expression profile between our case and APL, rather than non-M3 AML (PC2).

FIGURE 2. Sequence features and integration schematic of *TTMV::RARA* fusion transcript.

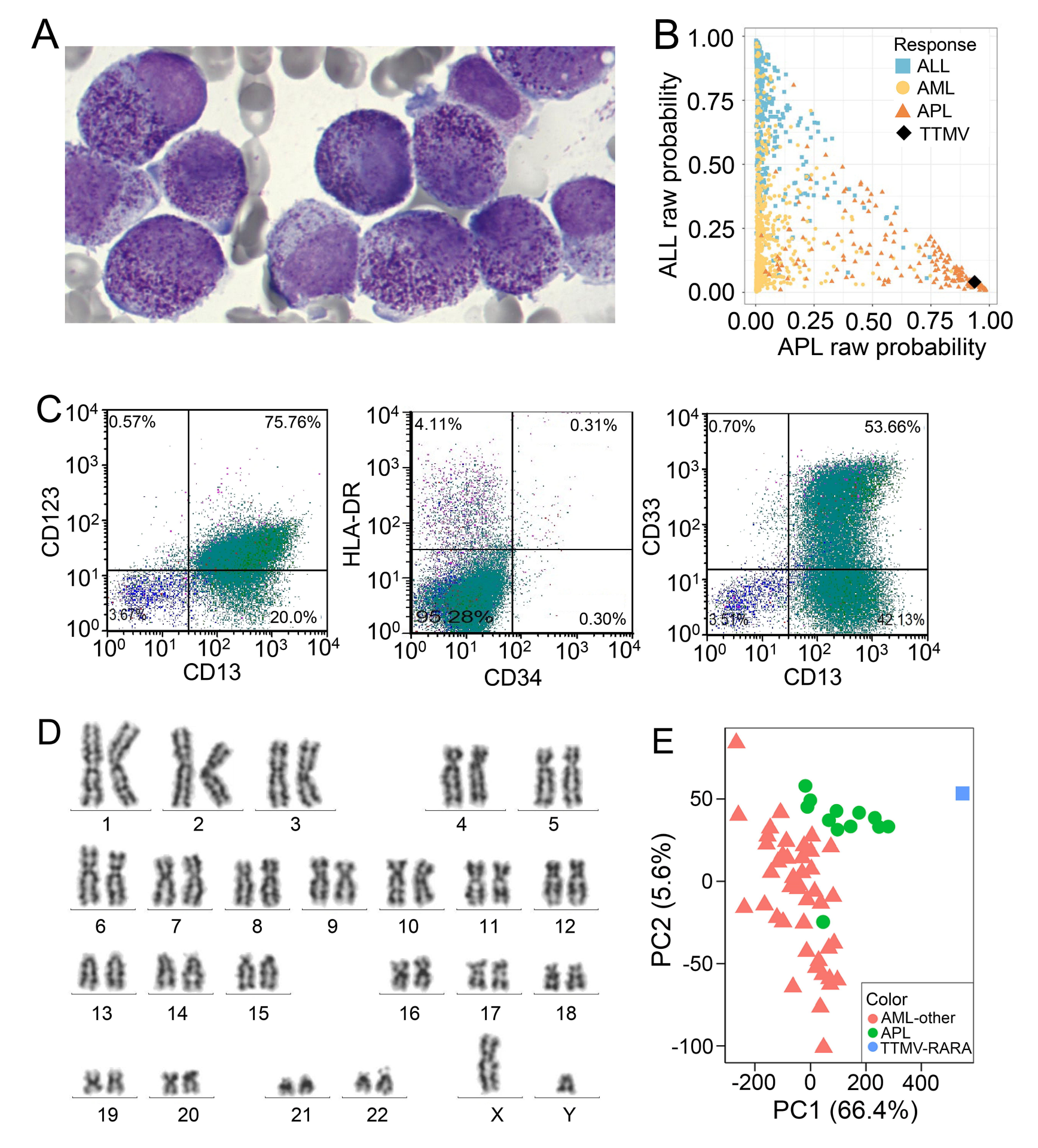
(A) The coverage details of the assembled transcript over the *RARA* gene on the hg38 genome reference.

(B) Coverage of *TTMV* sequence in this case over the *TTMV* isolate SAfiA-453-9 complete genome was assessed.

(C) Schematic Representation of Integration. The integration schematic depicts *TTMV* open reading frame 2 (ORF2) and an upstream untranslated region (UTR) integrated into *RARA*. The chimeric transcript includes a *TTMV* sequence joined to *RARA* exon

3-9. The conserved UTR sequence “CGAATGGCTGAGTTT” was observed at the end of UTR sequence. A start codon (ATG) at the 5’ end of *TTMV* ORF2 may predominate the initial translation phase.

FIGURE 3. Confirmation of *TTMV::RARA* fusion by PCR, sanger sequencing, the whole transcript sequencing, and the whole-genome sequencing data.
(A) Detection of the *TTMV::RARA* chimeric fusion in cDNA.
(B) Sanger sequencing electropherograms of the downstream and upstream breakpoints of *TTMV* integration on the cDNA.
(C) Detection of the *TTMV* integration site at the RNA level. The Integrative Genomics Viewer (IGV) screenshot of *TTMV::RARA* reads indicated that a total of 33 reads from RNA sequencing successfully aligned to *RARA* exon 3 and *TTMV* genome, exhibiting a mapping length ≥ 50 bp and an identity ≥ 90% at the RNA level, after the removal of PCR duplicates. The starting base of *RARA* exon 3 is marked by a dashed black line.
(D) Detection of the *TTMV* integration site at the DNA level was performed using IGV. The integration site at chr17:40,347,785 was marked by a dashed black line. The read coverage on either side of this breakpoint (chr17:40,347,785) showed a notable shift, indicating the occurrence of a chimeric event. bp, base pairs.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APL raw probability
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75.76%
3.67%
20.0%
HLA-DR
CD34
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95.28%
0.30%
CD33
0.70%
53.66%
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PC1 (66.4%)
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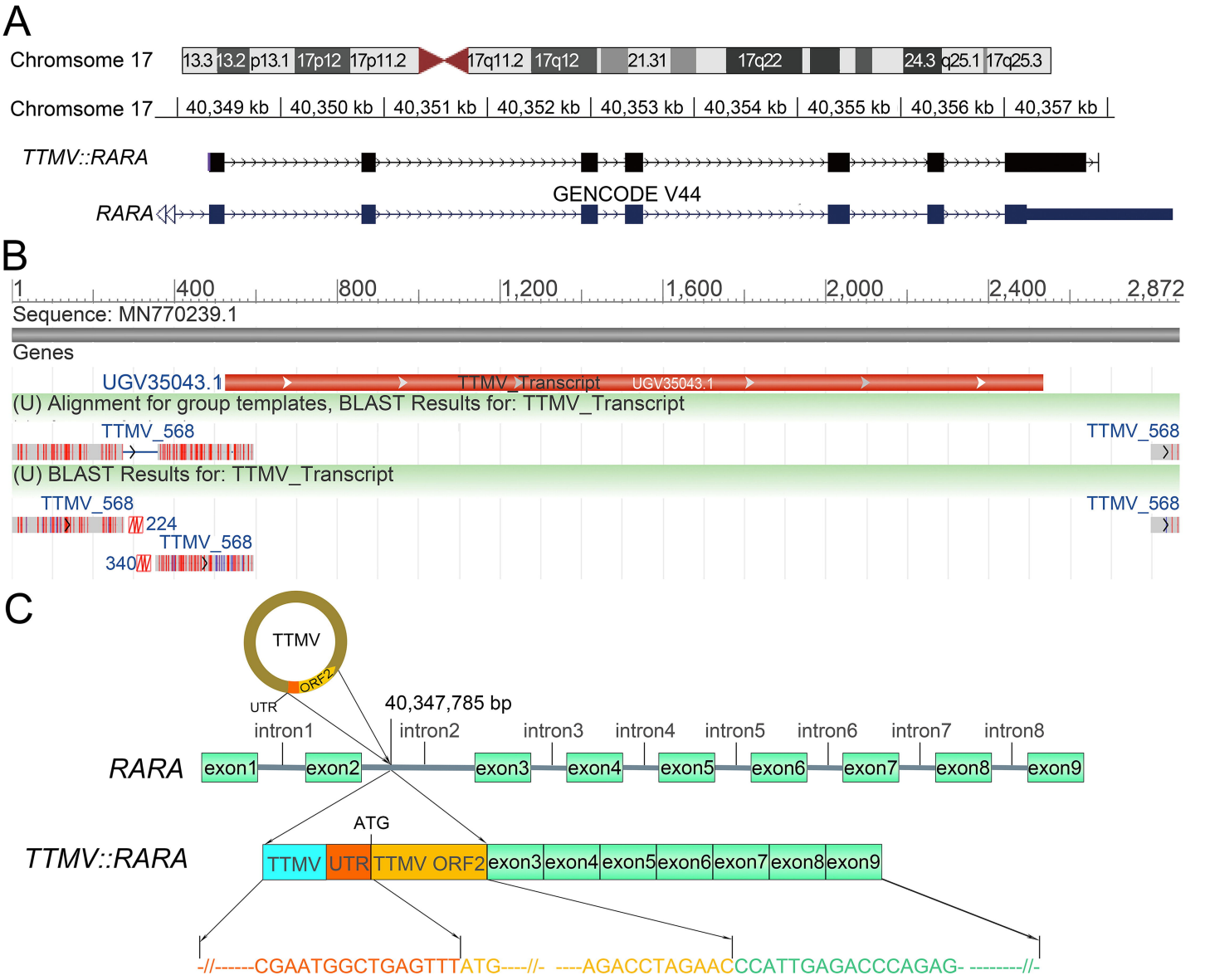
AML-other
APL
TTMV-RARA


A
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Chromsome 17
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GENCODE V44
RARA
B
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TTMV_Transcript UGV35043.1
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TTMV_568
TTMV_568
(U) BLAST Results for: TTMV_Transcript
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RARA
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ATG
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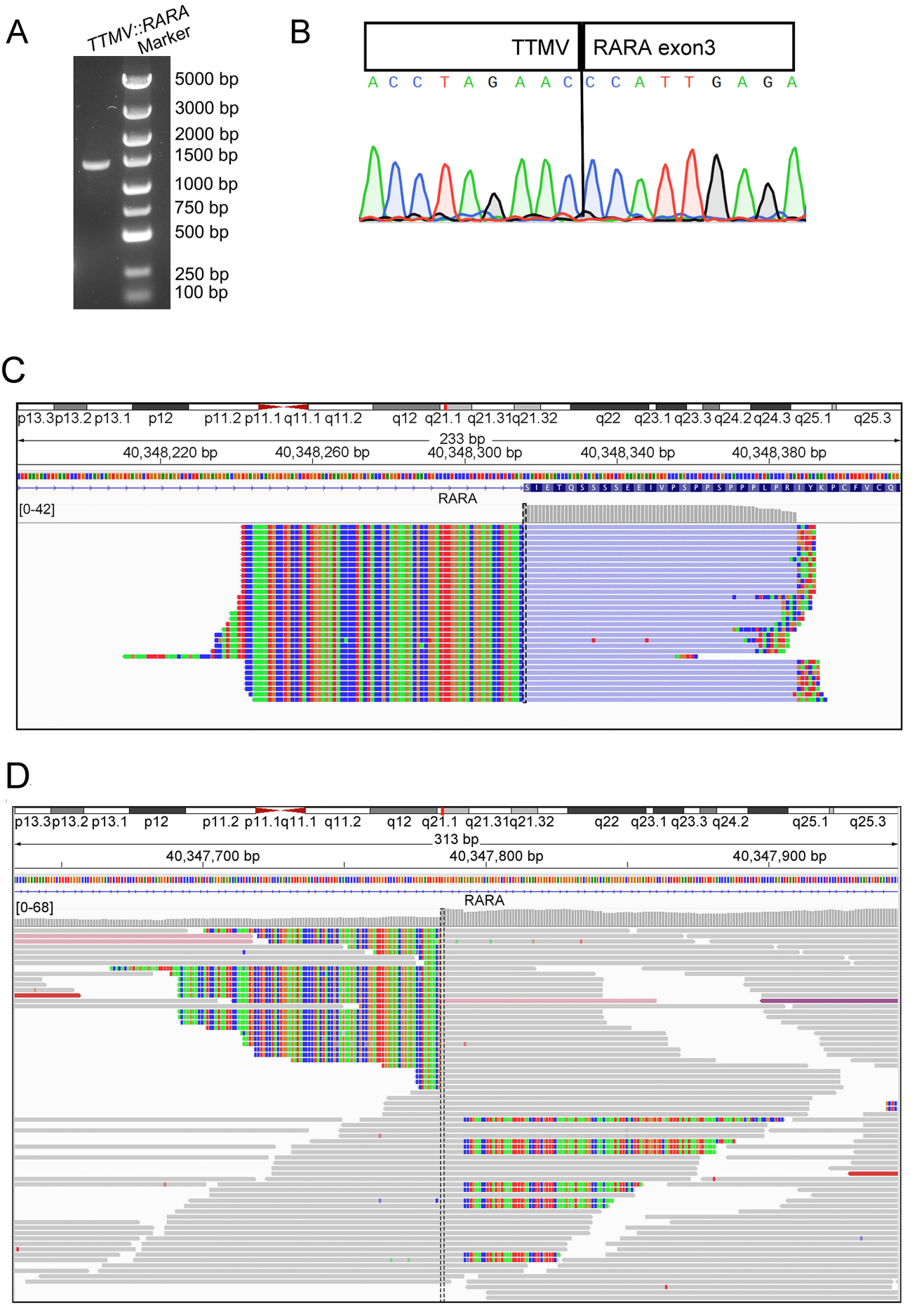
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