Transcriptional features of acute leukemia with promyelocytic differentiation lacking retinoic acid receptor rearrangements

Acute promyelocytic leukemia (APL) is typically characterized by the rearrangement of *RARA*, the most common of which is the *PML-RARA* fusion gene. The emergence of all-*trans* retinoic acid and arsenic trioxide has led to a reversal of disease prognosis. However, there are a few cases of acute myeloid leukemia in which the cell morphology, immunology, and even clinical manifestations are similar to classical APL; however, no *RARA* fusion gene has been detected. This type of leukemia is also known as acute promyelocytic-like leukemia (APLL). Retinoic acid receptors (RAR) include three members: RARA, RARB, and RARG. They are evolutionarily highly conserved, and their sequences and functions are remarkably similar. Moreover, retinoic acid X receptors (RXR) are closely related to RAR and usually form heterodimers to perform functions together. The first fusion gene harboring *RARG* (*NUP98-RARG*) in APLL was discovered in 2011.[1] Subsequently, *PML-RARG*, *CPSF6-RARG*, *HNRNPC-RARG*, and *NPM1-RARG-NPM1* have been identified.[2–7] Osumi *et al.* reported on five Japanese patients and identified the *RARB*-involved fusion gene.[8] Nevertheless, there are other patients with promyelocytic differentiation unrelated to RAR rearrangements, suggesting the complexity of APLL genome abnormalities.[9,10] Here, we performed transcriptome sequencing (RNA-seq) in four such patients, analyzed the characteristics of their expression profiles, identified novel fusion genes other than RAR, and focused on splicing alterations of RAR and RXR. Of the four patients (*Online Supplementary Table S1*), two were men and two were women. Their ages ranged from 8 to 71 years old. All patients had more than two blood morphology experts for the diagnosis of the cell morphology (*Online Supplementary Figure S1*). Karyotype analysis and reverse transcription polymerase chain reaction (RT-PCR) of the fusion gene harboring *RARA* were performed, and there was no evidence of *RARA* rearrangement. Total RNA was extracted from the bone marrow or peripheral blood mononuclear cells using TRIzol and stored at -80℃. Illumina HiSeq 3000 and BGISEQ-500 sequencers were used for RNA-seq in paired-end mode. The HISAT software was employed to compare clean reads to the reference human genome hg19/GRCh37, with an average comparison rate of 90.64% for each sample. StringTie software was used to reconstruct the transcript of each sample, followed by Cuffcompare to compare the reconstructed transcript with reference annotation information to obtain new transcripts. Chimeric transcripts from each sample were extracted using SOAPfuse. The RSEM package was used to calculate the expression levels of the transcripts. RT-PCR and Sanger sequencing were performed to verify the results. Furthermore, the Cancer Genome Atlas-Acute Myeloid Leukemia (TCGA-LAML) cohort was downloaded as a control group, whose expression profile was compared with that of three cases (with new transcripts of RAR or RXR).

A total of six gene fusion events were detected by RNA-seq in case 1, among which *KSR1-LGALS9* and *GPBP1L1-CCDC17* were verified by RT-PCR and Sanger sequencing. The pattern diagram and Sanger sequencing of the *KSR1-LGALS9* fusion are shown in Figure 1A and the *Online Supplementary Figure S2A*, respectively. The break point was between exon 29 of *KSR1* and exon 2 of *LGALS9*, and the reading frame was not shifted. The break point of *GPBP1L1-CCDC17* was flanked by exon 15 of *GPBP1L1* and exon 1 of *CCDC17*. The break point of *GPBP1L1* was within the stop codon TAG, that is, the deletion of the sequence following the AG bases. The break point of *CCDC17* is located in the 5'-UTR (untranslated region) of exon 1 (Figure 1B; *Online Supplementary Figure S2B, F*). Moreover, a large number of novel transcripts of numerous genes were identified, in which we detected a new transcript of the *RXRA* gene with 8.72 fragments per kilobase per million (FPKM). The variant consisted of 11 exons, and exon 1 was located in the intron region of the *RXRA* gene sequence released by NCBI and Ensembl genome databases, with a total of 63 bp. Its whole predicted sequence is shown in the *Online Supplementary Document A*. Figure 2A shows the Sanger sequencing at the junction of exons 1 and 2. In addition, we identified three new transcripts of the *RARB* gene (*Online Supplementary Document A*). The PCR verification was not performed since the FPKM value could not be measured. The three new *RARB* transcripts are composed of known exons.

A total of 37 gene fusion events were detected in case 2. A novel fusion gene, *GLYCTK-DNAH1*, was validated. Exon 3 of *GLYCTK* was fused to exon 5 of *DNAH1* in-frame (Figure 1C; *Online Supplementary Figure S2C*). Interestingly, *GPBP1L1-CCDC17* was also found in case 2. No novel splicing variant was discovered in the RAR or RXR.

In case 3, 16 gene fusion events were detected. Three novel *NUP98-HOXD8* variants were identified, one of which was confirmed (Figure 1D). The break point was flanked

by exon 11 of *NUP98* and exon 2 of *HOXD8* in-frame (*Online Supplementary Figure S2D*). In addition, RNA-seq results showed that the exon 10 sequence of *NUP98* was not consistent with the known sequences, and it was similar to the exon 10 sequence of the ENST00000397013.2 transcript, with two more bases (GT) than the latter at the 3' end, which can be considered as an alternative 3' end. A novel *RARB* transcript was identified (0.63 FPKM). There were six exons in the variant, of which exon 6 was located in the intron region of the released *RARB* gene sequence, with a total of 412 bp (Figure 2B; *Online Supplementary Document A*).

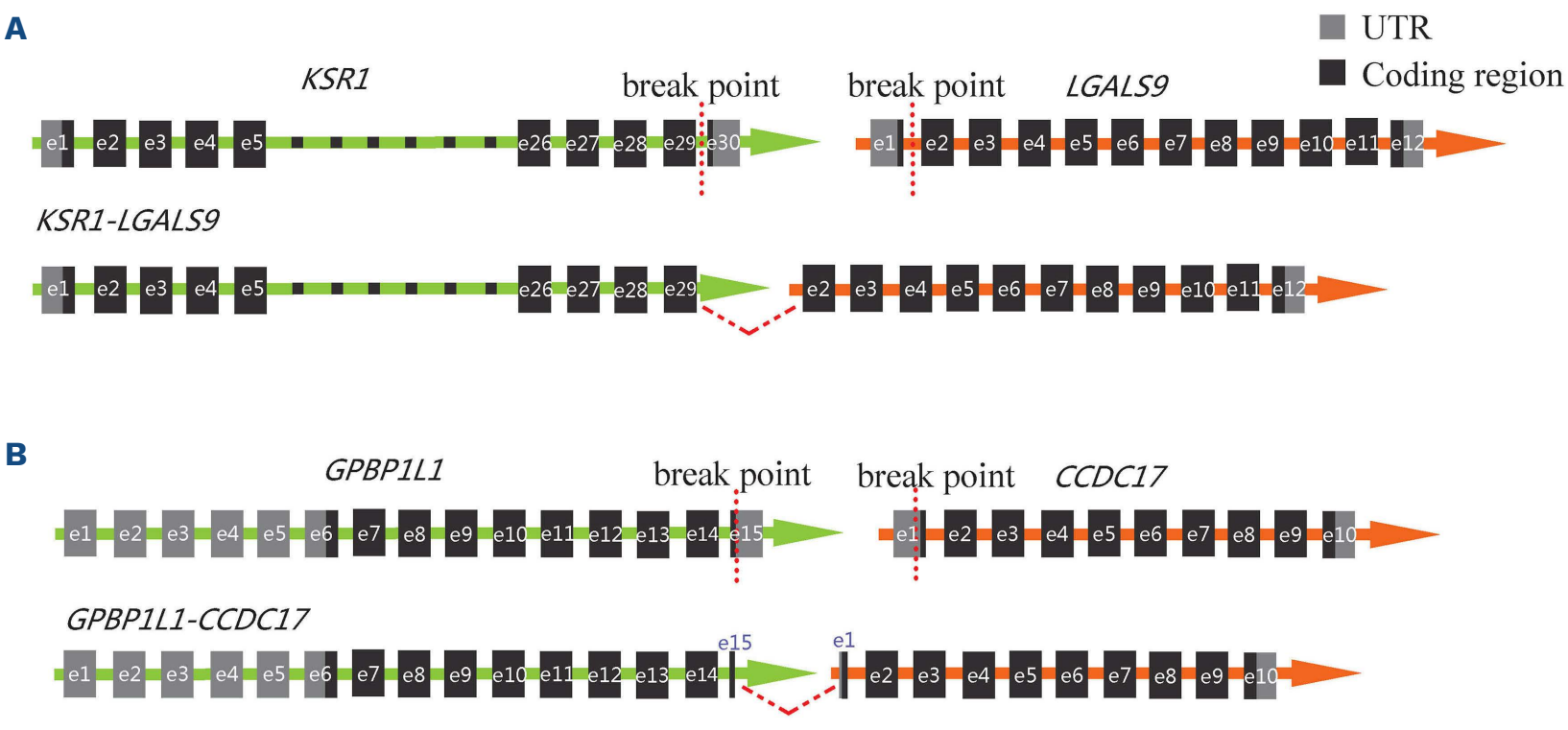

Figure 1. Schematic of novel fusion genes, splicing variants and expression profile. (A-E) List of fusion genes. The small black square indicates that the display of some exons is omitted.

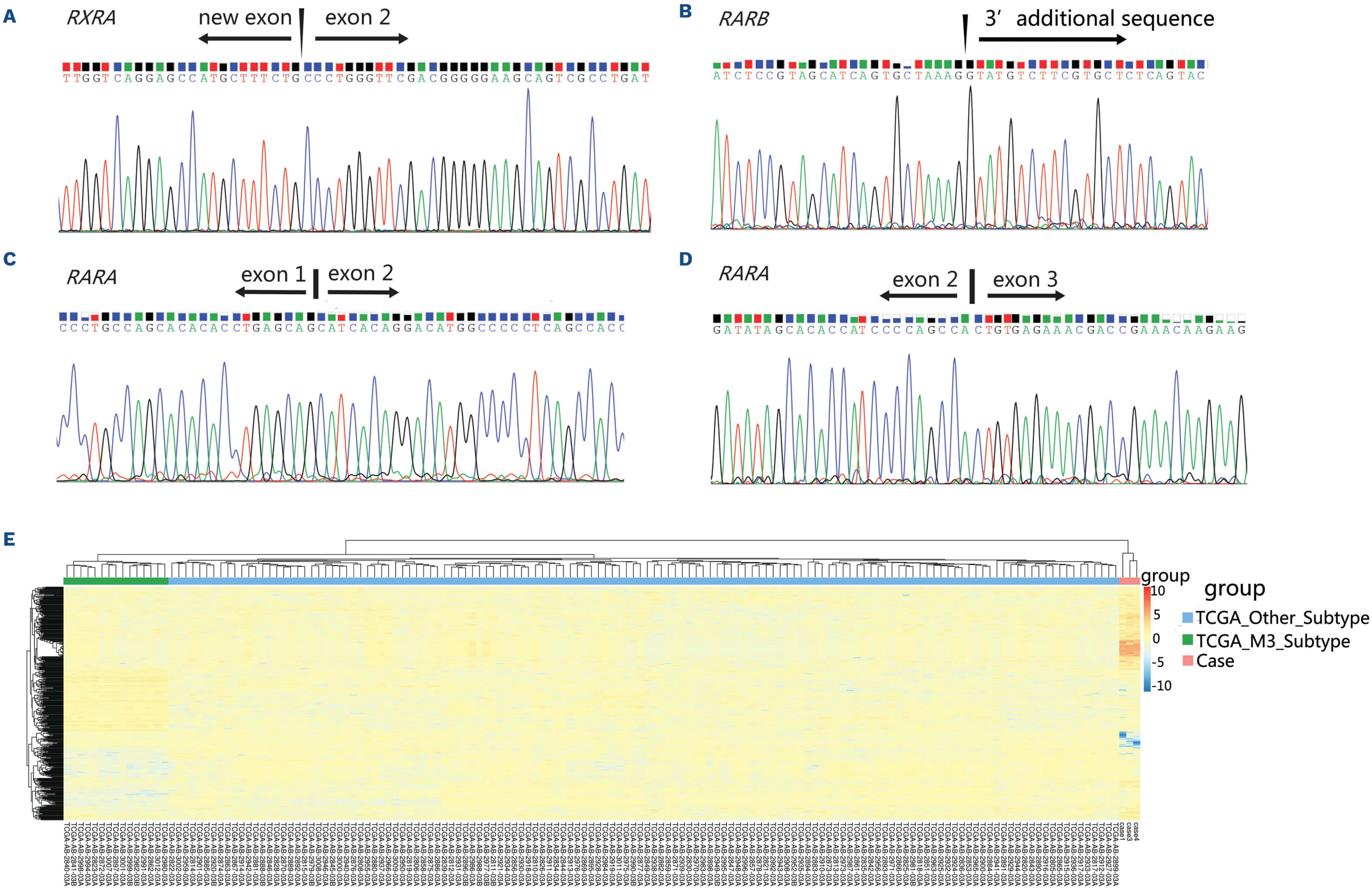

Figure 2. Sequences of retinoic acid receptor/retinoic acid X receptor novel transcripts and heat map of expression profiles. (A-D) Sanger sequencing of novel transcripts of *RXRA*, *RARB*, and *RARA*. In novel transcript of *RARA*, the sequence from exon 1 to exon 3 was used as a template for the polymerase chain reaction (PCR). Panel (C) is the junction sequence of exon 1 and exon 2, and panel (D) is that of exon 2 and exon 3. (E) Expression profile comparison. We downloaded TCGA-LAML cohort as a control group, which was compared to the expression profile of 3 cases in the experimental group (with new transcripts of the retinoic acid receptors [RAR] and retinoic acid X receptor [RXR]). The TCGA-LAML RPKM (reads per kilobase per million mapped reads) expression data was combined with the data of 3 cases, then they were clustered into 3 groups, namely experimental group (case), TCGA library classic promyelocytic leukemia group (TCGA_M3_Subtype), and TCGA library non-promyelocytic leukemia group (TCGA_Other_Subtypes). Limma R package was employed to calculate the differential gene expression, and top 420 up- and down-regulated genes were merged to define signatures.

Two *CFD-GNA15* variants were found in case 4, one of which was verified. Exon 4 of *CFD* was spliced with exon 7 of *GNA15*, which caused a reading frame shift in *GNA15* (Figure 1E; *Online Supplementary Figure S2E*). Moreover, we detected a new transcript of *RARA* gene (0.49 FPKM). There were seven exons in the variant, all of which were known (Figure 2C, D; *Online Supplementary Document A*). Intriguingly, a new transcript of *RARB* (0.09 FPKM), identical to case 3, was also identified in case 4.

As mentioned above, fusion genes involving RAR or RXR were absent in some APLL, whereas new fusions formed by other genes were found.[9-11] Similarly, the results detected should fall into this category. Some of these chimeric transcripts are fused by adjacent genes located on the same chromosome, which can be observed in the cis-splicing of adjacent genes. Whether these novel fusion genes directly or indirectly affect retinoic acid-related transcriptional regulation or block leukemic cell differentiation to the promyelocytic stage by other pathways needs to be further explored.

We also detected new splicing variants of RXR and RAR members in three cases, including *RXRA*, *RARA*, and *RARB*. RXR can form homodimers or heterodimers with RAR, which are important transcriptional regulators. Activated by ligands (all-*trans*- or 9-*cis*-retinoic acid), they bind to target response elements to regulate gene expression in various biological processes. *RXRA* is the most abundant subtype of RXR in bone marrow cells and its expression varies according to the differentiation stage of the hematopoietic process. *RARA* is found in normal myeloid cells; however, *RARB* is rarely expressed in the bone marrow. Alternative splicing of mRNA is a common cellular process that leads to proteomic complexity in advanced eukaryotes and regulates gene expression patterns that dominate cell fate. Alternative splicing can occur in the UTR or coding region, resulting in corresponding functional alterations. In recent years, abnormal alternative splicing has been observed in various types of tumors. Abnormal splicing may be caused by gene mutations or epigenetic or spliceosome changes, and participates in the pathogenesis of multiple human diseases.

Through comparison with the TCGA-LAML database, we described the expression profiles of three cases (cases 1, 3, and 4). Figure 2E shows a heat map of the expression profile. In addition, *Online Supplementary Figure S2G, H* shows gene ontology (GO) and KEGG enrichment analyses. The expression profiles of classic APL (M3) cases in TCGA cohort were consistent and significantly different from those of other AML cases (non-M3), which is likely due to its unique fusion gene. Additionally, the gene expression profiles of other RAR-rearranged APLL might be similar to those of classic APL, which has been confirmed by the discovery of the *RARG-CPSF6* fusion gene.[5] Non-M3 cases bear their characteristics, which can be attributed to the diversity of types. Since the clinical data in the TCGA database were not detailed, we did not perform further groupings. Inconsistent with the *RARG-CPSF6*-positive APLL, the expression profiles of the experimental group were far from those of the M3 group. Moreover, the expression profiles of the experimental group were different from those of the non-M3 group and were unique. This may be explained by the absence of RAR-involved fusion genes in the experimental group. Hence, it can be deduced that the differentiation mechanism of APLL lacking RAR rearrangements is different from that of RAR-rearranged APL, which may be more complicated and involve distinctive biological functions and pathways.

In summary, we described the transcriptome features of APLL cases lacking RAR rearrangements. In these cases, fusion genes other than RAR, as well as distinct variants of the RAR and RXR members, exist. Profiling suggests a complex molecular mechanism of the disease, which deserves further investigation.

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https://doi.org/10.3324/haematol.2022.282426

Received: December 6, 2022.
Accepted: May 8, 2023.
Early view: May 18, 2023.

Disclosures

No conflicts of interest to disclose.

Contributions

JP, ZS and XL designed the studies and wrote the paper. YZ, WW and XL were involved in the management of the patients and providing clinical data. ZS, JY and XW performed the molecular studies. All authors read and approved the manuscript.

Funding

This work was supported by the Fundamental Research Funds for the Central Universities, 2022JC025.

Data-sharing statement

All data included in this study are available upon request by contacting the corresponding author.

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