Novel reciprocal fusion genes involving *HNRNPC* and *RARG* in acute promyelocytic leukemia lacking *RARA* rearrangement

Acute promyelocytic leukemia (APL) is a unique subtype of acute leukemia characterized by retinoic acid receptor alpha gene (*RARA*) rearrangements (*RARA*-rearranged APL), of which *PML-RARa* is the typical one. As an altered retinoic acid receptor, PML-RARA owns the ability to transmit oncogenic signals within the cell. PML-RARA competitively controls the DNA sequences of retinoic acid-responsive element (RARE) sites and has a negative effect on transcription, inhibits the activation of physiological ligands, and leads to the arrest of promyelocytic differentiation.[1] However, there are some rare patients lacking *RARA* rearrangement although they share the same morphology and immunocytochemistry features with *RARA* -rearranged APL. The classification of such leukemias remains in chaos and they have been also termed as acute promyelocytic-like leukemias (APLL). For years the underlying molecular aberrations of APLL remained unrevealed, but in recent years, *RARG* and *RARB* rearrangenments have been found in APLL.[2-6] *RARA*, *RARB* and *RARG* belong to the nuclear receptor superfamily, and their sequences and functions are highly similar. In the present study we identified a couple of novel reciprocal fusion genes involving the heterogeneous nuclear ribonucleoprotein C (HNRNPC) gene and the retinoic acid receptor gamma (*RARG*) gene in APLL. To our knowledges, the reciprocal fusions were identified in human disease for the first time.

A 43-year-old man was referred to our hospital with fever and a sore throat. Laboratory investigations revealed the following patient characteristics: (1) hemoglobin level, 125 g/L; (2) platelet count, 57×10^9/L; and (3) white blood cell count 12×10^9L (1% blasts and 86% abnormal promyelocytes). No signs of coagulopathy were evident. Morphologic analysis of the bone marrow aspirate showed 86.5% microgranular atypical promyelocytes (Figure 1A). Analysis from flow cytometry showed that the blasts were positive for CD33, CD13, CD45, and cMPO and negative for CD14, CD34, CD16, CD56, HLA-DR, B- or T-cell markers. Thus, the patient started all-trans retinoic acid (ATRA, 60 mg/day) and arsenious acid (10 mg/day) treatment immediately.

Afterwards, chromosomal analysis revealed the majority of metaphases were der(14)ins(14;?) (q24;?)t(14;17?)(q24;q11.2?),der17t(14;17?)(q24;q11.2?). Fluorescence *in situ* hybridization did not identify the *PML/RARA* rearrangement. A series of reverse transcription PCR (RT-PCR) assays amplified the *HOX11* gene and alternative transcripts of 31 fusion genes, including *PML-RARA, NPM-RARA, PLZF-RARA*, and *BCR-ABL*, which were all negative. Sanger sequencing detected no *FLT3* mutation.

A combined chemotherapy (daunorubicin plus cytarabine) was administrated immediately following ATRA, then, because *RARA* rearrangment was lacking, arsenious acid (days 1-7) was suspended. After three weeks of ATRA therapy no response was observed in blood cell counts, and no evidence suggested differentiation syndrome. A second bone marrow biopsy (Figure 1B) was subsequently peformed and the blasts were almost unchanged (87.5%) which resulted in the withdrawal of ATRA. Next, the patient received a new course of induction chemotherapy alone (homoharringtonine, daunorubicin and cytarabine, HDA) and he achieved a complete response (CR) this time. Afterwards, he received five cycles of chemotherapy, of which three were consolidated therapies (HAD, HA, and mitoxantrone plus cytarabine) and two were cytarabine intensive treatments. Unfortunately, the leukemia relapsed 1 year later, and two cycles of re-induction chemotherapy, MAC (mitoxantrone, cytarabine and cyclophosphamide) followed by FLAG regiment, were sequentially administrated. Yet the blast counts in the bone marrow remained almost at the same level as at the disease onset (87.2%). Subsequently, ATRA in combination with arsenious acid therapy of sufficient dose and duration was resumed and continued for four weeks. However, all treatments failed to produce any effects. The patient eventually died from sepsis.

To identify molecular alterations, transcriptome sequencing (RNA-seq) analysis was performed using peripheral blood mononuclear cells which were obtained in the patient' relapse phase. Total RNA (4.6 μg) was used to synthesize cDNA libraries, and rRNA were removed

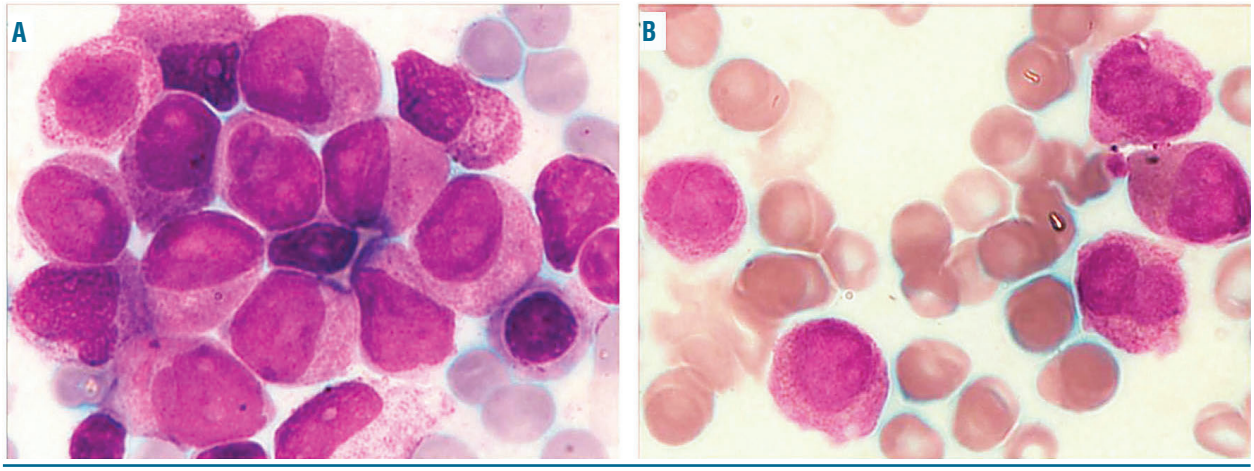

Figure 1. Morphology analysis of bone marrow smear. (A) Bone marrow aspirate at the first consultation. The abnormal promyelocytes are shown, featured with abundant cytoplasm, azurophilic microgranules, and concaved or irregular nucleinuclei. (B) Bone marrow aspirate evaluation of the initial treatment after three weeks. Response was not observed in the cell morphology. (Wright-Giemsa staining, 1,000x magnification).

with the Ribo-Zero™ rRNA Removal Kit (Epicentre). The samples were prepared according to the Illumina protocol. The library was sequenced on an Illumina HiSeqTM 2000 platform. The SOAPfuse software was employed to discover the fusion transcripts.[7] As a result, we identified a novel gene fusion event between *HNRNPC* and *RARG*, leading to the formation of a couple of reciprocal transcripts, *HNRNPC-RARG* and *RARG-HNRNPC*. In *HNRNPC-RARG*, *HNRNPC* exon 3 was fused in-frame to RARG exon 4. Whereas in *RARG-HNRNPC*, *RARG* exon 9 was fused in-frame to *HNRNPC* exon 4 (Figure 2A-B).

Total RNA was also extracted from the same blood mononuclear cells and reverse transcribed into cDNA. PCR was performed, and the following primers were used to amplify *HNRNPC-RARG* mRNA: 5'-TGTTCATACGGGCTTTGCCTTCGT TCAG-3' for *HNRNPC* and 5'-CATGGCTTGTAGACCAGAGGAGGCGGAG-3' for RARG. The following primers were used to amplify RARG-HNRNPC mRNA: 5'- ACAAGCTGTAGGAGCCACTGCTGGAAGC-3' for RARG and 5'-CAAAAGAGGACCCGTACATCTCCGCTGC-3' for *HNRNPC*. After Sanger sequencing, the fusion transcript amplicons were compared with GenBank sequences using the BLAST program. The amplicon size of *HNRNPC-RARG* fusion was 186-bp (Figure 2C), as predicted. Sanger sequencing demonstrated that the amplicon sequence can be completely aligned to that of RNA-seq (Figures 2D). The *HNRNPC* 5'-region encodes an RNA recognition motif (RRM), and the segment from RARG encodes a DNA binding domain (DBD, Figure 2G). We also found a reciprocal chimeric transcript. A 213-bp *RARG-HNRNPC* fusion product was specifically amplified from the patient's cDNA (Figure 2E). Also Sanger sequencing confirmed the 100% sequence identity (Figure 2F). The *RARG* 5'-region encoding the ligand-binding domain was fused to the *HNRNPC* 3'-region, where a cluster of phosphorylation sites is located (Figure 2H). PCR with primers flanking the *HNRNPC* breakpoint was also performed, but an amplicon of chimera was not obtained.

HnRNP C is a ubiquitously expressed RNA-binding protein (RBP). RBP are believed to influence pre-mRNA metabolism such as splicing, polyadenylation, stability, transport, and translation mediated by internal ribosome entry site.[8,9] HnRNP C also plays an essential role in cell progression and the regulation of several DNA repair proteins.[10-12] *RARA, RARB*, and *RARG* are three RAR subtypes which share highly similar sequences and functions. A study on RAR knockouts observed that RARG overexpression enhanced multipotent hematopoietic stem cell (HSC) self-renewal and the loss of RARG promoted HSC differentiation. Thus, RARG seems to act as a major regulator maintaining the balance between HSC self-renewal and differentiation.[13]

Acute myeloid leukemias mimicking acute promyelo-

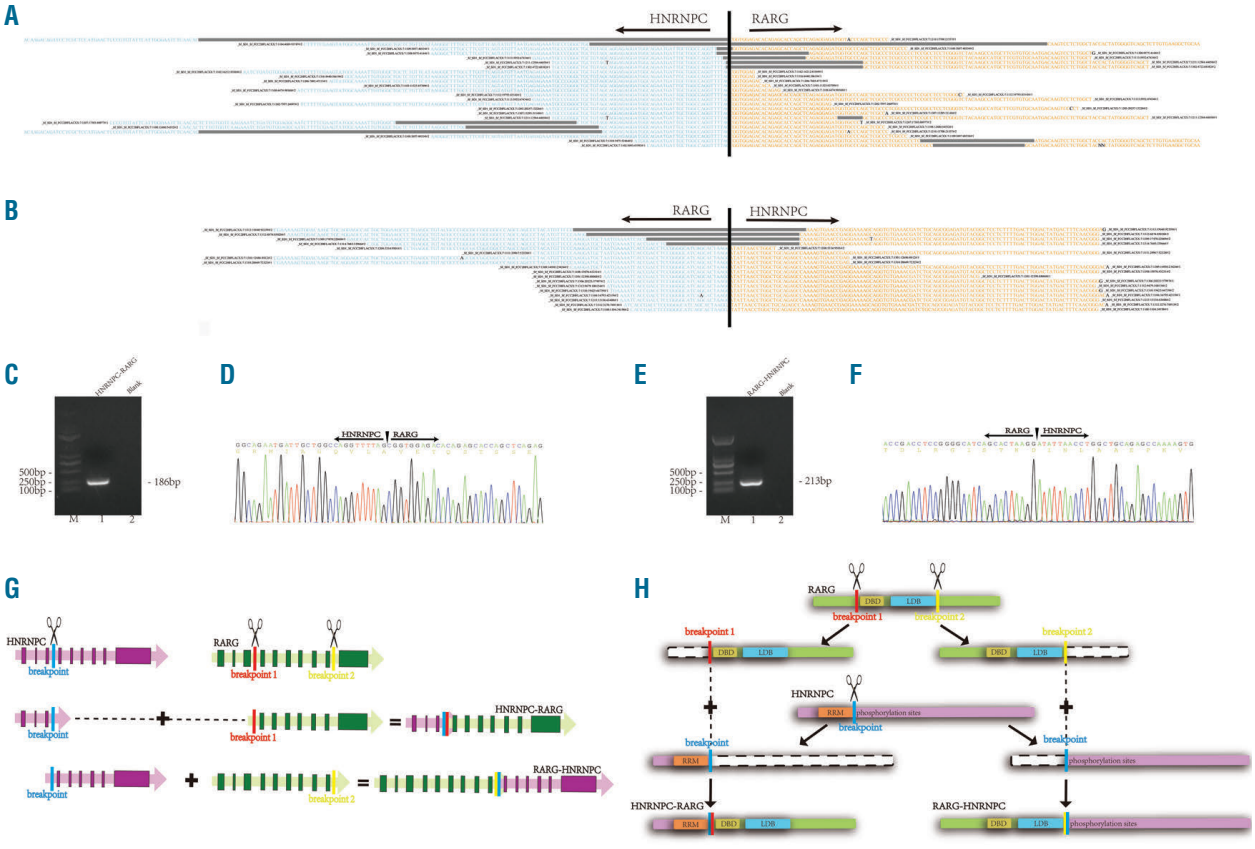

Figure 2. Detection of the reciprocal *HNRNPC/RARG* and *RARG/HNRNPC* fusion. (A) and (B) SOAPfuse obtained Span reads and junction reads of *HNRNPC/RARG* and *RARG/HNRNPC* respectively from RNA-sequencing data. (C) and (E) Gel picture of the amplified fragment. M: DNA marker; lane 1: PCR amplicons; lane 2: blank controls. (D) and (F) Sanger sequencing showing the junction of the *HNRNPC/RARG* and *RARG/HNRNPC* genes respectively. From the nucleotide sequence surrounding the fusion regions, amino acid sequences may be in frame. (G) Deduced schematic overview of the structures of reciprocal rearrangements, and the construction of the corresponding chimeric proteins is illustrated in figure (H). DBD: DNA binding domain; LBD: ligand binding domain; RRM: RNA recognition motif.

cytic leukemia, or acute promyelocytic-like leukemias (APLL), share the same morphology and immunocytochemistry features with typical APL, and little is known about the molecular mechanisms of APLL. In this study, we identified a novel fusion gene *HNRNPC-RARG* and its reciprocal in APLL. The sequences and function of the *RARG* and *RARA* are highly alike, and therefore can logically explain the similarity of the biological characteristics between the two entities. Three other fusion genes harboring *RARG* have been found in APLL. Such *et al*. reported a case of APLL carrying a *NUP98-RARG* fusion transcript. The patient had CR after standard chemotherapy and then received consolidation therapy and intensification by autologous stem-cell transplantation. He relapsed 2 years later, and re-induction therapy introduced ATRA besides chemotherapy. Although the patient achieved once again CR, ATRA treatment effect could not be evaluated *in vivo*. In fact, the follow-up studies have demonstrated the patient's leukemic cells were not sensitive to ATRA *in vitro*.[3,14] A novel *PML-RARG* fusion was identified in a patient of APLL thereafter, and two kinds of fusion transcripts were detected. While the administration of ATRA continued for nine days, response could not be evaluated.[4] Interestingly, cells carrying the artificial *PML-RARG* fusion protein responded well to retinoic acid *in vitro*.[15] Recently, three cases with recurrent CPSF6-RARG fusions which were composed of three isoforms according to *RARG* break points were reported, and the main functional domains of *RARG* were retained. Unfortunately, they showed resistance to treatment with ATRA or ATRA plus arsenic. Two patients died after failing induction therapy and only one patient achieved CR benefiting from combined chemotherapy.[5,6] Our patient also showed a resistance to ATRA at onset and at relapse. The patient received two cycles of combined chemotherapy before CR, and re-induction failed at relapse, all of which indicate drug resistance. Moreover, poor prognosis was observed likewise. Thus, it may be speculated that in comparison with *RARA*-rearranged APL, functions of *RARG* chimeric transcripts might be different. Combined chemotherapy should be administrated during induction therapy unhesitatingly rather than the alternate of ATRA or arsenic. Moreover, all the evidence above confirms that *RARG* rearrangements are not random but recurrent genetic abnormalities.

In conclusion, we present a novel *HNRNPC-RARG* fusion gene and its reciprocal in APLL, and suggest that at least a portion of APLL have *RARG* gene rearrangements. *RARG*-rearranged APLL may be a novel candidate subtype of APL.

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Funding: this work was supported in part by National Natural Science Foundation of China (NO.81101794, 91442204, 81125002). We thank Dr. Aixiang Jin from BGI (China) for his help with transciptome analysis. Also we thank the patient, Prof. Hu.

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doi:10.3324/haematol.2019.244715

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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