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# Identification of PML/RARα fusion gene transcripts that showed no t(15;17) with conventional karyotyping and fluorescent in situ hybridization

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**ABSTRACT.** Acute promyelocytic leukemia (APL) is characterized by a reciprocal translocation, t(15;17)(q22;q11-21), resulting in the fusion of the promyelocytic leukemia (*PML*) and retinoic acid receptor alpha (*RARα*) genes. Using conventional cytogenetic methods, these translocations are normally detected in about 70-90% of patients; most negative results are due to technical problems or cryptic variants. These masked *PML/RARα* fusions can be identified by molecular analyses, such as reverse transcriptase-polymerase chain reaction (RT-PCR) or fluorescence *in situ* hybridization (FISH). Approximately 5 to 10% of all APL cases reported do not show *PML/RARα* fusion transcripts, even with dual-colored FISH. We report three of 40 diagnosed APL cases that showed morphological, cytochemical, and immunophenotypic features of hypergranular APL, but did not show a *PML/RARα* fusion signal or any of its variants, on FISH. All cases were identified by RT-PCR, which

 

was further confirmed by cDNA sequencing. Conventional karyotyping showed other clonal aberrations in these cases, but failed to show t(15;17) or any other variants or complex translocations.

**Key words:** Acute promyelocytic leukemia; Cryptic insertions; Promyelocytic leukemia/retinoic acid receptor alpha; Reverse transcription-polymerase chain reaction; Fluorescence *in situ* hybridization; Sequencing analysis

## INTRODUCTION

The genetic hallmark of acute promyelocytic leukemia (APL) is the balanced reciprocal translocation t(15;17)(q22;q11-21) leading to a fusion of the promyelocytic leukemia (*PML*) gene on chromosome 15 and the retinoic acid receptor-α (*RARα*) gene on chromosome 17. t(15;17)(q22;q11-21) is seen in 85-90% of APL cases, leading to the formation of two reciprocal fusion genes, *PML/RARα* on the der(15) and *RARα*-PML on the der(17) chromosome (Rowley et al.,1977). These changes are believed to impair myeloid differentiation and inhibit apoptosis, contributing to leukemic transformation (Biondi et al., 1991; Allford et al., 1999).

A number of different variant translocations have been characterized in the remaining cases of APL. There are also APL patients with cytogenetically normal 15 and 17 chromosomes. In these cases masked or cryptic t(15;17) was shown to be generated by submicroscopic insertions of *PML* or *RARα* or more complex rearrangements (Allford et al., 1999; Lo Coco et al., 1999).

Combining molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR), fluorescence *in situ* hybridization (FISH), or sequencing has been a useful tool in identifying these abnormalities. We report three *de novo* APL cases, which were positive by RT-PCR but negative by dual-colored FISH and conventional karyotyping.

## CASE HISTORY

### Case 1

A 26-year-old female presented fever, pallor and bleeding gums. At presentation, total leukocyte count (TLC) was 0.6 x $10^9$/L, platelet count was 155 x $10^9$/L and hemoglobin was 7.7 g/dL. Coagulation profile showed prothrombin time (PT) of 15.3 s, activated partial thromboplastin time (APTT) of 32.1 s, and international normalized ratio of 1.24. Bone marrow morphology showed 86% abnormal hypergranular promyelocytes with few faggot cells and 2% blasts. Immunophenotyping by flow cytometry revealed positivity for CD13, CD33 and CD117 and negativity for HLA-DR and CD34. RT-PCR showed the short isoform (S form) of *PML/RARα*. FISH was negative for t(15;17) and for any of the other variants. Conventional karyotyping showed del(5q) in 6% of interphases using the telomeric region of 5p, but did not show t(15,17) or any other variants. The patient was placed on all-trans-retinoic acid (ATRA) induction therapy. The patient was in molecular remission postinduction. The patient received 3 doses of daunomycin followed by which the patient was placed on ATRA maintenance (Head et al., 1995; Adès et al., 2006). The patient is in molecular remission with a follow-up of 8 months. She has completed two maintenance cycles of ATRA.

## Case 2

A 33-year-old female presented chief complaints of fever and fatigue. At presentation, TLC was 1.39 x $10^9$/L; platelet count was 9.8 x $10^9$/L and hemoglobin was 7.1 g/dL. Coagulation studies were normal. Bone marrow morphology showed 90% abnormal hypergranular promyelocytes and occasional presence of faggot cells. Immunophenotyping by flow cytometry revealed positivity for CD13, CD33 and CD117 and negativity for HLA-DR and CD34. RT-PCR showed the short isoform (S form) of *PML/RARα*. FISH was negative for t(15;17) or any of the other variants. No other chromosomal abnormality was detected by conventional karyotyping. This patient was placed on arsenic induction therapy (Mathews et al., 2006). The patient was in molecular remission postinduction. Thereafter, the patient was placed on maintenance cycle with arsenic. To date, she has completed five cycles of maintenance, and remains in molecular remission with a follow-up of 12 months.

## Case 3

A 46-year-old male presented a 3-week history of fatigue and intermittent fever. A complete hemogram showed a TLC of 63.8 x $10^9$/L; platelet count of 146 x $10^9$/L and hemoglobin of 6.6 g/dL. The coagulation profile showed a PT of 16.4 s (normal range from 10.8 to 14.6 s), APTT of 28.8 s (normal range 23-35 s) and INR value of 1.34 (normal ratio from 0.8 to 1.2). The bone marrow aspirate showed 71% abnormal hypergranular promyelocytes, with no faggot cells. These abnormal promyelocytes were strongly positive for myeloperoxidase staining. Immunophenotyping by flow cytometry revealed positivity for CD13, CD33 and negativity for CD117, HLA-DR and CD34. RT-PCR and FISH were performed for molecular confirmation of APL. RT-PCR showed the long isoform (L form) of *PML/RARα*. Dual-colored *RARα* break apart probes were used for diagnosis by FISH. However, FISH failed to show any of the APL variants. Conventional karyotyping showed other abnormal chromosomal aberrations such as del(19p13), del(12q24.1) and del5 but failed to show any of the translocations associated with APL. The patient was initially started on ARA-C and daunomycin, but later, placed on oral ATRA. The patient responded well to ATRA. This patient continues to be in molecular remission by RT-PCR with a follow-up of 8 months.

# MATERIAL AND METHODS

## Reverse transcription-polymerase chain reaction

Total RNA was extracted from bone marrow samples using standard techniques. cDNA was prepared using a commercially available kit (Fermentas) along with Random Hexamer primer. To develop the sensitivity and specificity for *PML/RARα*, HOT start RT-PCR was performed (Tobal and Liu Yin, 1998) using a specific primer for *PML/RARα*, with the sequence of forward: 5'ACC GAT GGC TTC GAC GAG TTC 3' and reverse: 5'AGC CCT TGC AGC CCT CAC AG 3'. PCR conditions were as follows: an initial denaturation at 95°C for 15 min, followed by 5 cycles with each cycle consisting of denaturation at 96°C for 1 min, annealing at 55°C for 15 min and extension at 72°C for 2 min. At the end of 5 cycles, a final extension at 72°C for 2 min. This was followed by 30 cycles, each cycle consisting of denaturation at 94°C for 35 s, annealing at 55°C for 1 min and extension at 72°C for 3 min. At the end of 30 cycles,

a final extension at 72°C for 10 min.

PCR product was electrophoresed on 2% agarose gels and the bands were analyzed. The L form showed 3 bands at 291, 550 and 691 bp, while the S form showed a single band at 220 bp (Figure 1). β-actin mRNA was used as an internal control.

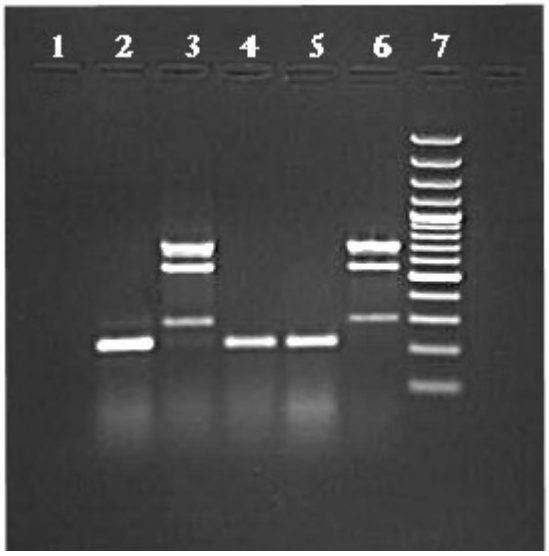


**Figure 1.** Ethidium bromide-stained agarose gel showing acute promyelocytic leukemia isoforms. Lane 1: Negative control; lane 2: positive control 'S' form; lane 3: positive control 'L' form; lane 4: case 1 - 'S' form; lane 5: case 2 - 'S' form; lane 6: case 3 - 'L' form; lane 7: 100-bp DNA ladder.

## Sequencing

The amplified RT-PCR product was purified and sent for cDNA sequencing to confirm the presence of the *PML/RARα* hybrid transcripts. The primer sequences that were used were the same as above. The product sequence showed *PML/RARα* transcript sequence. This confirmed our RT-PCR results of a *PML/RARα* hybrid transcript.

## Conventional cytogenetics

For conventional cytogenetic studies, bone marrow specimens were processed by direct as well as a 24-h culture method. In each sample, 20 metaphases were karyotyped.

Processing of sample for karyotyping followed the method used by Tijo and Whang (1965) and Seabright (1971) with modifications. One milliliter of bone marrow aspirate was collected in 5 mL complete RPMI medium with colcemid (mitotic inhibitor) and incubated at 37°C with 5% $CO_2$ for 30 min to 2 h. The cells were pelleted and treated with hypotonic KCl solution at 37°C for 30 min. Leukocytes were then fixed with fresh chilled fixative. The leuko-

cyte pellet was washed 2-3 times with fresh fixative until a clear creamy pearly leukocyte pellet was seen. The fixed sample was then used for conventional cytogenetics. Freshly prepared slides were then processed for G-band staining. For GTG banding, the slides were incubated in Sorenson's buffer at 60°C and treated with trypsin-EDTA. Slides were rinsed in cold saline and then stained with Giemsa.

G-banded slides were screened using a bright field microscope. Fifty- to twenty-well banded metaphases per specimen were captured on computer by a digital CCD camera with the help of a karyotyping software. The chromosome aberrations were defined according to Pinkel et al. (1986) and to the International System for Human Cytogenetic Nomenclature (ISCN, 1995).

## Fluorescence *in situ* hybridization

FISH was carried out using the direct fluorescence method on fixed cytogenetic specimens of bone marrow aspirate samples following protocols supplied by Vysis Inc./Oncor, with some modifications (Pinkel et al., 1986; Amare et al., 2001).

After dehydrating the slides in graded ethanol series (70, 85, and 100%), slides were co-denatured along with the FISH probe in a Hybrid machine for 5 min at 73°C and then kept overnight for hybridization at 37°C. Stringent washes of slides were done in 0.4X SSCN (NP 40-0.3%) at 73°C for 2 min followed by 2X SSCN (NP 40-0.1%) at room temperature for 1 min. DAPI/Antifade was applied as an Antifade counter stain on the marked area and covered with a cover slip, which was sealed with fingernail polish. Signals were observed under epi-fluorescent microscopy using a dual/triple band pass fluorescent microscope. In every specimen, a total of 500 interphase cells and 10-20 metaphase cells were analyzed. Dual-colored *RARα* break apart probes were used.

## Fluorescent *in situ* hybridization results

Normally in APL with t(15,17) or any of the variants, 1 dual-color orange/green signal (normal *RARα* allele), 1 orange and 1 green signal, which indicates break and separation of 5' and 3' sequences of *RARα* gene allele, are seen. However, in these three patients, interphase cells revealed 2 orange signals (normal *PML* allele) and 2 green signals (normal *RARα* signal) indicative of no evidence of *PML/RARα* fusion.

## DISCUSSION

The pathophysiological mechanisms of APL differ substantially from those of other myeloid leukemias, and APL is, therefore, considered to be a specific leukemia unlike other acute myeloid leukemias. Fusion transcripts of *PML/RARα* derived from t(15;17) play an important role in leukemogenesis in APL (Stone and Mayer, 1990).

Several studies have shown that t(15;17) is not detected by conventional cytogenetic analysis in 10% of APL cases; however, the majority of cases showing morphological APL, but lacking t(15;17), remain associated with the formation of the *PML/RARα* fusion gene (Stone and Mayer, 1990; Hiorns et al., 1994; Zaccaria et al., 2002; Oh et al., 2003; de Botton et al., 2004; Owatari et al., 2007). The main reason is either the presence of cryptic insertion events or complex rearrangements leading to *PML/RARα* fusion (Hiorns et al., 1994). FISH analysis and RT-

PCR would be valuable for determining which of these two mechanisms leads to a *PML/RARα* hybrid transcript in a cytogenetically negative APL patient (Hiorns et al., 1994; de Botton et al., 2004). Several studies have reported additional chromosomal abnormalities with or without the presence of t(15;17). For example, trisomy 8 is the most frequent secondary abnormality, and other abnormalities involving chromosomes 9, 17, 7, 21, 16, and 12 have been described (de Botton et al., 2004; Owatari et al., 2007). In addition, t(2;17) is another sole abnormality found in the absence of t(15;17) (Zaccaria et al., 2002; Oh et al., 2003; Owatari et al., 2007).

These RT-PCR positive cases for hybrid *PML/RARα* transcript define a new cytogenetic subgroup of APL. This subgroup is composed of patients who are t(15;17) negative but *PML/RARα* positive and is analogous to the Philadelphia chromosome negative CML patients who nevertheless are *BCR/ABL* positive (Hiorns et al., 1994). In our study, except for deletions, no other chromosomal abnormalities were found. This suggests that there may be a small cryptic insertion or masked translocation of t(15;17), which has not been detectable by FISH because of technical limitations. The routinely used dual-colored break apart probes that are used in FISH are not sensitive enough to hybridize with such small cryptic insertions and therefore do not produce a signal. However, these small cryptic insertions can be amplified and detected by RT-PCR. In one of the studies conducted by the European Working Group (Grimwade et al., 2000), 7 of 60 patients lacking the classical t(15,17) were analyzed using different specific probes such as ICRF *PML* and *RARα* cosmid probes. In these 7 ins(15;17) cases, the Oncor probe set was used in parallel either with ICRF *PML* 15.5 and *RARα*121 cosmid (4 cases) or Vysis probes (3 cases), giving identical results, suggesting that the Oncor *RARα* probe is not only centromeric, but also spans the 17q breakpoint. All these ins(15;17) patients had apparently normal chromosomes 15 and 17 by conventional cytogenetic analysis and by FISH using whole chromosomal painting probes and were thus cryptic. In such cases, where there is a small insertion of the hybrid transcript of *PML/RARα*, the RT-PCR technique was found to be more advantageous (Grimwade et al., 2000).

Thus, our study highlights the importance of RT-PCR at baseline, which can detect this small subset of t(15;17) APL negative cases, with cryptic or masked insertions. RT-PCR is a very sensitive technique and can detect as few as 1 leukemic cell in $10^4$ cells. PCR using the HOT START protocol is more specific and efficient than standard RT-PCR, especially where multiple band size is to be detected, as in the case of the L isoform. HOT START is also important in samples with low target copy numbers because it minimizes any non-specific annealing of primers to non-target DNA sequences and reduces primer oligomerization. Both RT-PCR and FISH should be performed at baseline for better understanding of the pathogenesis of the disease and for optimal management of APL patients. These patients lacking the t(15;17) are predicted to show a beneficial response to ATRA or arsenic, and share the favorable prognosis of those with the classic t(15;17). The three patients in our study also responded well to treatment (two patients with ATRA and daunomycin and one patient with arsenic) and are in complete molecular remission with a median follow-up of 8 months.

In many respects, RT-PCR screening of cases of suspected APL affords a number of advantages: providing a rapid diagnostic test, distinguishing *PML* breakpoint patterns, and defining targets for residual disease monitoring, which has been shown to provide independent prognostic information. Also, it will be an added advantage, if whole chromosomal painting and cosmid probes are used to detect the interstitial insertions in identifying this group subset.

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