Original Article

Characterization of cryptic rearrangements, deletion, complex variants of *PML, RARA* in acute promyelocytic leukemia

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Acute promyelocytic leukemia (APL) is characterized by a reciprocal translocation t(15;17)(q22;q21) leading to the disruption of Promyelocytic leukemia *(PML)* and Retionic Acid Receptor Alpha *(RARA)* followed by reciprocal *PML–RARA* fusion in 90% of the cases. Fluorescence *in situ* hybridization (FISH) has overcome the hurdles of unavailability of abnormal and/or lack of metaphase cells, and detection of cryptic, submicroscopic rearrangements. In the present study, besides diagnostic approach we sought to analyze these cases for identification and characterization of cryptic rearrangements, deletion variants and unknown *RARA* translocation variants by application of D-FISH and RARA break-apart probe strategy on interphase and metaphase cells in a large series of 200 cases of APL. Forty cases (20%) had atypical *PML–RARA* and/or *RARA* variants. D-FISH with PML/RARA probe helped identification of *RARA* insertion to *PML*. By application of D-FISH on metaphase cells, we documented that translocation of 15 to 17 leads to 17q deletion which results in loss of reciprocal fusion and/or residual *RARA* on der(17). Among the complex variants of t(15;17), *PML–RARA* fusion followed by residual *RARA* insertion closed to *PML–RARA* on der(15) was unique and unusual. FISH with break-apart RARA probe on metaphase cells was found to be a very efficient strategy to detect unknown *RARA* variant translocations like t(11;17)(q23;q21), t(11;17)(q13;q21) and t(2;17)(p21;q21). These findings proved that D-FISH and break-apart probe strategy has potential to detect primary as well as secondary additional aberrations of *PML, RARA* and other additional loci. The long-term clinical follow-up is essential to evaluate the clinical importance of these findings.

Key Words: *PML-RARA, RARA* variant, D-FISH, APL, 17q deletion

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Introduction

Acute promyelocytic leukemia (APL) is characterized by reciprocal translocation t(15;17)(q22;q21) leading to the disruption of *PML* and *RARA* followed by a reciprocal *PML–RARA* fusion in 90% of the cases.[1-7] Molecular cytogenetics like interphase fluorescence *in situ* hybridization (FISH) has overcome the hurdles of unavailability of abnormal and/or lack of metaphase cells, detection of cryptic, submicroscopic rearrangements due to insertion events, identification of complex variants as well as *RARA* translocation variants that has diagnostic utility in the diagnosis as well as management of disease.[6,8-12]

Besides diagnostic approach, we sought to analyze these cases for identification and characterization of cryptic rearrangements, deletion variants and unknown *RARA* translocation variants by application of D-FISH and RARA break-apart probe strategy on metaphase cells in APL at diagnosis.

Materials and Methods

The present study included 200 APL patients who belonged to the age group 1–70 years. Patients who

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were diagnosed by standard morphology criteria and immunophenotype criteria, between January 2005 and December 2010, were enrolled in this study. Molecular Cytogenetics-Fluorescence-*in-situ* hybridization (FISH) is part of the routine diagnostic criteria and also for monitoring treatment for evaluation of cytogenetic remission.

Cytogenetic preparation was carried out on bone marrow aspirate and occasionally on peripheral blood. Interphase and metaphase D-FISH were performed using LSI dual color dual fusion *PML/RARA* and dual color break-apart rearrangement RARA probe (Vysis Abbott molecular, Delkenheim, Germany) as per manufacturer's protocol and instructions. For every batch of probe, studies were carried out on 10 specimens which included peripheral blood from normal individuals and marrow aspirations from Bone Marrow Transplantation (BMT) donors to check the sensitivity of probe. We scored 500 interphase nuclei and 10–20 metaphase cells in each normal specimen. In patients, a minimum of 100–200 and 5–15 metaphase cells were scored for evaluation of FISH signals by two observers in a blinded fashion.

For PML/RARA probe, presence of two separated red (R) and two green (G) signals in non-overlapping distinct nuclei were considered as *PML–RARA* negative and 1R, 1G and 2 co-localized/fusion red/green signals were considered as *PML–RARA* dual fusion positive. For *RARA* break-apart probe, distinct, well-separated red and green signals in non-overlapping nuclei were considered as *RARA* gene break/split.

Deviation in signal pattern of *PML–RARA* in interphase cells was evaluated on metaphase cells whenever available. Deletion, complex variants and *RARA* translocation variants were additionally confirmed on inverted 4', 6-diamidino-2-phenylindole (DAPI) image. Whenever required, CEP probe/s were used to confirm suspected chromosome.

Results and Discussion

Molecular characterization and interpretation of *PML–RARA* fusion positive cases by D-FISH using PML/RARA probe is given in Table 1 and Figure 1.

D-FISH using LSI PML/RARA probe confirmed standard reciprocal *PML–RARA* in interphase and metaphase cells in 160 patients [Figure 1a]. The frequency for false-positive interphase nuclei in normal controls for 1R 1G 2F, 2R 1G 1F, 1R 2G 1F, 1R 1G 3F, and 2R 2G 1F was 0.2%, 0.4%, 0.4%, 0.2%, and 0.2%, respectively. The specimens with 1R 1G 2F, 2R 1G 1F, 1R 2G 1F, 1R 1G 3F, and 2R 2G 1F were considered positive if the value exceeded >1%. The specimens with 1R 1G 1F were considered positive if the value exceeded >5%. The *PML–RARA* positive or *RARA* split positive cells were in the range of 50–90%. In 12 out of 40 patients with variant *PML–RARA* pattern, proper metaphase cells were not available for characterization either due to lack

Table 1: Signal pattern and incidence of standard *PML–RARA*, cryptic/masked *PML–RARA*, deletion variants and *RARA* variant translocations by D-FISH with PML/RARA probe and RARA break-apart rearrangement probe, respectively

Signal pattern	Probe	Interpretation	No. cases (frequency)
1R 1G 2F	PML/RARA	Standard *PML–RARA* dual fusion	160 (80)
2R 1G 1F	PML/RARA	*RARA* translocation to 15q22 followed by *PML–RARA* fusion. Presence of residual *PML* on der(17) with absence of reciprocal *PML–RARA* due to loss of 17q deletion	12 (6)
1R 1G 1F	PML/RARA	Interstitial insertion of whole *RARA* to *PML* on der(15) and/or *RARA* translocation to 15 along with loss of 17q deletion	9 (4.5)
1R 2G 1F	PML/RARA	The nonreciprocal fusion pattern with presence of residual *RARA* probably suggests deletion of 15qter region	3 (1.5)
1R 1G 3F	PML/RARA	Standard *PML–RARA* and additional copies of *PML–RARA* due to i(17q) or duplication of der(17)	5 (2.5)
2R 2G 1F	PML/RARA	Translocation of *RARA* to *PML* on der(15) followed by an insertion of residual *RARA* closed to *PML–RARA* on der(15)/complex variant of 15;17 translocation with additional involvement of 3rd chromosome: t(15;17;?)	5 (2.5)
1R 1G 1 Y	RARA break apart	*RARA* variant translocations t(11;17)(q23;q21) (2 cases) t(11;17)(q13;q21) (2 cases) t(2;17)(p21;q21) (1 case)	6 (3)

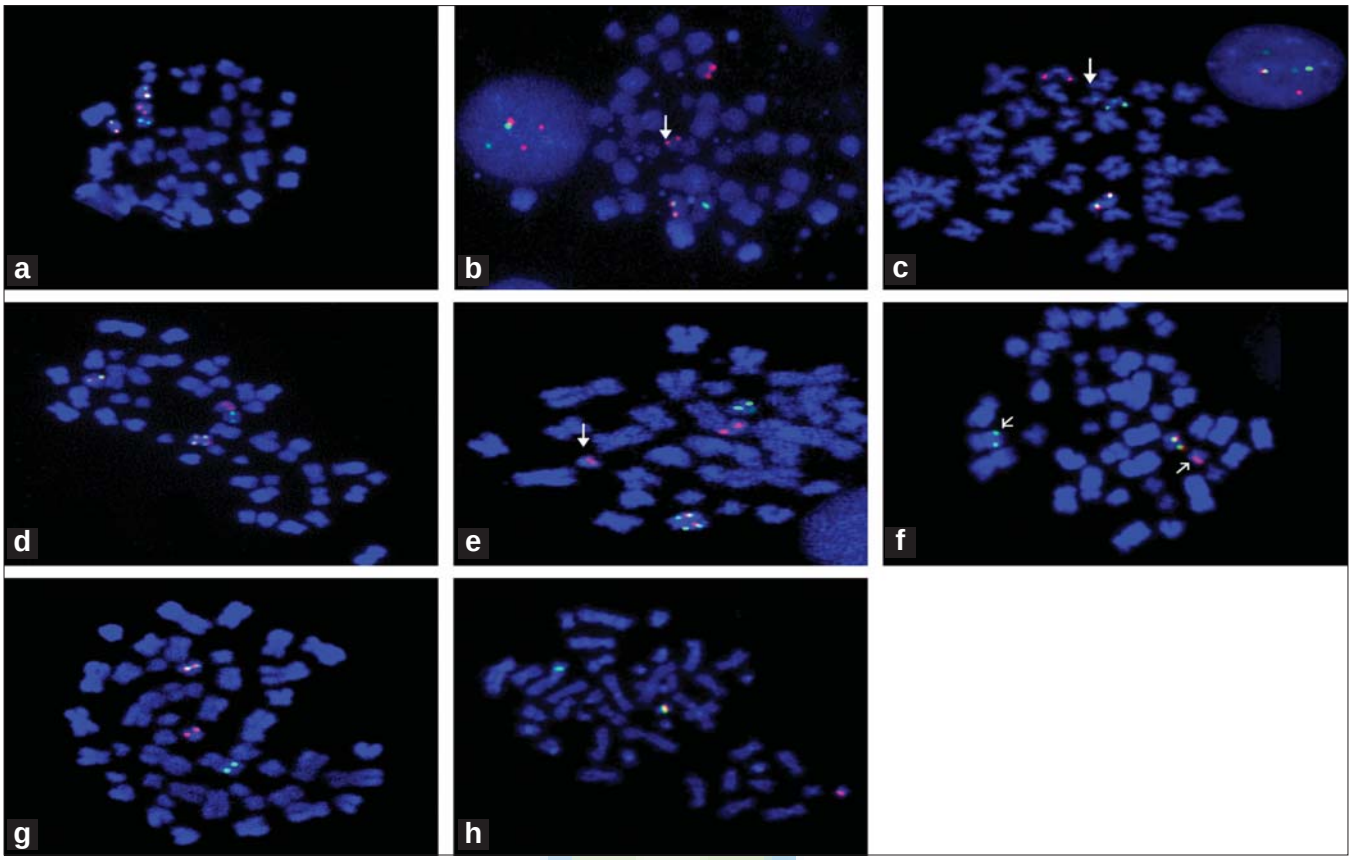

Figure 1: (a) D-FISH with LSI *PML–RARA* on metaphase cell shows normal *PML* allele (red signal), normal *RARA* allele (green signal) reciprocal *PML–RARA* fusion on der(15) (yellow signal) and der(17) (yellow signal). (b) LSI *PML–RARA* on metaphase cell shows *PML–RARA* fusion on der(15) (yellow signal) and residual PML on der(17) (red signal) (white arrow). (c) LSI PML–RARA on metaphase cell shows *PML–RARA* fusion on der(15) (yellow signal), also shows Aqua CEP 17 normal and der(17) (white arrow). (d) LSI *PML–RARA* on metaphase cell shows *PML–RARA* fusion on der(15) (yellow signal) and duplication of PML–RARA on i(17q) (yellow signals). (e) LSI PML–RARA on metaphase cell shows *PML–RARA* fusion on der (15) (yellow signal), residual *RARA* on der(15) (green signal) next to *PML–RARA* fusion and residual *PML* signal on der(17) (red signal) (white arrow). (f) Dual color RARA break-apart probe on metaphase cell shows normal *RARA* allele on 17 (yellow signal), residual *RARA* on der(17) (red signal) and residual *RARA* on der(11) at band 11q23 (green signal). (g) Dual color *RARA* break-apart probe on metaphase cell shows normal *RARA* allele on 17 (yellow signal), residual RARA on der17 (red signal) and residual *RARA* on der(11) at band 11q13 (green signal). (h) Dual color RARA break-apart probe on metaphase cell shows normal *RARA* allele on 17 (yellow signal), residual *RARA* on der(17) (red signal) and residual *RARA* on der(2) at band 2p21 (green signal)

of metaphase cells or presence of metaphase cells of poor morphology or presence of normal dividing cells.

In 9 out of 12 cases with 2R 1G 1F signal pattern, 17q21→qter region with residual *RARA* translocated to 15q22 followed by fusion *PML–RARA* (yellow signal). Presence of residual *PML* on der(17) was due to reciprocal translocation; however, derivative 17 showed lack of reciprocal fusion due to loss of 17q deletion which was confirmed by inverted DAPI image [Figure 1b, Table 1].

In six out of nine cases with 1R 1G 1F signal pattern in metaphase cells, there was a lack of reciprocal fusion on der(17). An inverted DAPI image revealed normal appearing chromosome 17 in four cases which indicated interstitial insertion of whole *RARA* into *PML* on der(15). This is consistent with masked *PML–RARA*, a common event known to occur in APL with t(15;17) negative cases by conventional karyotyping.[9,10,13,14] In three cases, der(17) appeared to be small [Figure 1c]. These findings and documented evidence of the presence of residual *PML* on der(17) in cases with 2R 1G 1F [Figure 1b] support the fact that loss of DNA at 17q21 region occurs in a small group

of APL patients. The poor morphology of metaphase cells probably masks the abnormal 17 by conventional cytogenetics as discussed by others.[7,15] The signal pattern 1R 2G 1F observed in three cases was detected on interphase cells due to unavailability of abnormal metaphase cells. The nonreciprocal fusion pattern with the presence of residual *RARA* probably suggests deletion of 15qter region. Loss of DNA material on 9q is very common in Chronic Myeloid Leukemia (CML) cases.[16,17] We have also detected nonreciprocal *BCR-ABL* with 9q deletion/res *ABL* deletion/res *BCR* deletion in a large CML series of 2000 cases (our unpublished data). Our vast experience in various hematological malignancies like Acute Lymphoblastic Leukemia (ALL)[18-20] in lymphoma[21] which support the notion that genomic deletions followed by translocations are common events in hematological malignancies.

Metaphase analysis of cases with 1R 1G 3F helped identification of duplication of *PML–RARA* on both arms of 17q as a result of i(17q) in three cases [Figure 1d]. Additional copies of *PML–RARA* due to i(17q) or duplication of der(17) are not uncommon events in APL.[7,22] An i(17q) with duplication of *PML–RARA* is similar to Ph duplication as a result of either i(ph) or two separate copies of Ph chromosome in Chronic Myeloid Leukemia – Accelerated Phase (CML-AP) or CML-BP.[6,23]

D-FISH strategy was found to be very efficient to detect complex variant translocation of *PML–RARA*. We identified six cases with 2R 2G 1F signal pattern. We could characterize one variant with *PML–RARA* on der(15) at locus 15q22 as result of translocation and additionally insertion of residual *RARA* at locus 15q15 next to *PML–RARA* and residual *RARA* on der(17) [Figure 1e]. The overall signal pattern on metaphase cell was interpreted as two sequential events: first t(15;17): *PML–RARA* on der(15), followed by an insertion of residual RARA on der(15). Such atypical *PML, RARA* rearrangement was very unusual, and not reported before to our knowledge. The complex variants involving 15, 17 and a third chromosome have been reported.[11,13,15]

Review of literature on deletion variants or cryptic masked/complex variants of *PML–RARA* revealed very few reports. Studies of Thomas *et al*.(11) in 52 cases of APL showed 6% incidence. Our large series data revealed 14% incidence of deletion/complex variants of *PML–RARA*.

Six cases with 2R 1G 2 dimG signal pattern revealed RARA translocation with partner chromosome other than 15 [Table 1]. Application of FISH with dual color RARA break-apart probe on interphase cells showed 1R 1G 1Y signal. The analysis of metaphase cells identified chromosome 11 as a partner chromosome in four cases. Inverted DAPI image helped in the identification of break point at band 11q23, a locus of *PLZF* in two cases [Figure 1f] and band 11q13, a locus of nuclear mitotic apparatus *(NUMA)* (two cases) [Figure 1g]. Among the variant *RARA* translocations, translocations involving 11q23 and 11q13 locus are frequent and found to show resistance to retinoid therapy.[24-26] Besides t(11;17(q13;q21) and t(11;17)(q23;q21), t(2;17)(p21;q21) was identified in our series [Figure 1h]. To our knowledge, t(2;17)(p21;q21) was not reported before in APL. The incidence of *RARA* variant translocations in our series of APL was 3% which is similar (1–2%) to those reported in other geographic areas.[5,7,25]

In conclusion, D-FISH strategy was found to be very efficient, sensitive and reliable approach in comparison with conventional cytogenetics in the diagnosis of APL. In the present large-scale study, it helped in the identification of cryptic rearrangements like insertion of *RARA* to *PML*. We also documented that translocation of 15 to 17 leads to 17q deletion which results in loss of reciprocal fusion and/or residual *RARA* on der(17). Apart from deletion variants, complex variant translocations of *PML–RARA* could also be detected by D-FISH interphase and metaphase approach. Among complex variants, t(15;17) followed by residual *RARA* insertion was unique and unusual. Combination of D-FISH and break-apart *RARA* probe proved its ability to detect unknown variant *RARA* translocations other than t(11;17) like t(11;17)(q23;q21), t(11;17)(q13;q21) and t(2;17)(p21;q21). The understanding of underlying biology will be able to focus upon the pathogenesis with respect to resistance to All-Trans-Retinoic-Acid (ATRA) therapy in APL. These findings proved that D-FISH and break-apart probe strategy have the potential to detect primary as well as secondary additional aberrations of *PML* and *RARA* and other additional loci. The long-term clinical follow-up is essential to evaluate the clinical importance of these findings. Also, additional large-scale studies are awaited to support the clinical and biological significance.

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