

## RESEARCH ARTICLE

# PHA-Induced Peripheral Blood Cytogenetics and Molecular Analysis: a Valid Diagnostic and Follow-up Modality for Acute Promyelocytic Leukemia Patients Treated with ATRA and/or Arsenic Tri-oxide

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### Abstract

**Background:** Acute promyelocytic leukemia (APML) is characterized by the reciprocal translocation t(15;17)(q22;q12) resulting in the PML-RAR $\alpha$  fusion gene. A dual diagnostic and follow up approach was applied including cytogenetic demonstration of the t(15;17) translocation and detection of PML-RAR $\alpha$  chimeric transcripts by molecular means. **Purpose:** Conventional cytogenetics involving bone marrow is beset with high probability of poor metaphase index and was substituted with phytohemagglutinin (PHA)-induced peripheral blood culture based cytogenetic analysis as a diagnostic & follow up modality in APML patients of Kashmir (North India). Both qualitative (RT-PCR) and quantitative (Q-PCR) tests were simultaneously carried out to authenticate the modified cytogenetics. **Materials and Methods:** Patient samples were subjected to the said techniques to establish their baseline as well as follow-up status. **Results:** Initial cytogenetics revealed 30 patients (81%) positive for t(15;17) whereas 7 (19%) had either cryptic translocation or were negative for t(15;17). Two cases had chromosome 16q deletion and no hallmark translocation t(15;17). Q-PCR status for PML-RAR $\alpha$  was found to be positive for all patients. All the APML patients were reassessed at the end of consolidation phase and during maintenance phase of chemotherapy where 6 patients had molecular relapse, wherein 4 also demonstrated cytogenetic relapse. **Conclusions:** It was found that PHA-induced peripheral blood cytogenetics along with molecular analysis could prove a reliable modality in the diagnosis and assessment of follow up response of APML patients.

**Keywords:** APML - cytogenetic response - ATRA - arsenic tri-oxide - peripheral blood - karyotyping

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### Introduction

Acute promyelocytic leukemia (APML) is a distinct subtype of acute myeloid leukemia. Morphologically, it is identified as AML-M3 by the French-American-British (FAB) classification. Cytogenetically, APML is characterized by a balanced reciprocal translocation between chromosomes 15 and 17, which results in the fusion between the promyelocytic leukemia (PML) gene and retinoic acid receptor (RAR). Variant chromosomal translocations e.g., t(11;17), t(5;17) can be detected in no more than 5% of APML patients.

The real incidence of APML is not known. The percentage of APML could be higher in children than in adults (So et al., 2011). The incidence is somewhat around 5% to 13% reported by many large clinical trials and single institutions in the US. Also of note, this incidence is similar to Latin American background patients (Douer

et al., 2003).

The genetic hallmark of APML is the balanced reciprocal translocation t(15;17)(q22;q21-12) leading to the fusion of the promyelocytic (PML) gene on chromosome 15 and the retinoic acid receptor- $\alpha$  (RAR $\alpha$ ) gene on chromosome 17. Depending on the location of breakpoints within the PML site, the PML/RAR $\alpha$  transcript subtypes bcr1, bcr2 and bcr3 may be formed. Of these, bcr1 and bcr2 are of similar size and together referred to as long (L) isoform, bcr2 as variable (V) and bcr3 as short (S) isoform. The PML/RAR $\alpha$  fusion is detectable by fluorescence in situ hybridization (FISH) or reverse-transcriptase polymerase chain reaction (RT-PCR) in >95% of morphologically defined APMLs, while in the remaining cases several variant rearrangements have been described that constantly involve RAR $\alpha$  and partner genes other than PML (Grimwade, 1999).

RAR $\alpha$  is a member of the RA nuclear receptor

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family that acts as ligand-inducible transcription factor by binding to specific response elements (RARE) at the promoter region of target genes. In the absence of ligand, RAR $\alpha$  forms heterodimers with the retinoid X receptor (RXR) and recruits a co-repressor complex containing histone deacetylase (HDAC) activities that induces chromatin condensation and transcriptional repression. Physiological concentrations of RA (it as  $1 \times 10^{-9}$  M raised to power 9 M) are able to release the nuclear co-repressors complex from the RAR-RXR and recruit co-activators with histone acetyltransferase activities (HAT). This results in hyperacetylation of histones at RARE sites, chromatin remodeling and transcriptional activation of RAR $\alpha$ -target genes (Dilworth and Chambon, 2000; Glass and Rosenfeld, 2000).

PML belongs to a family of proteins containing a distinctive C3HC4 zinc-binding domain referred to as RING finger. Like other members of this family, including BRCA1, PML has been implicated in tumor suppression and control of genomic stability (Zhong S, et al., 2000). In the nucleus, PML is detected in multiprotein complexes termed PML nuclear bodies, where it co-localizes with other proteins such as p53, pRb, Daxx, and CBP (Jensen K, et al., 2001). As a consequence of the PML/RAR $\alpha$  fusion, in APLM cells, PML is disrupted and the protein is de-localized into microspeckled nuclear particles. This variation in subcellular distribution of PML in APLMs bearing the PML/RAR $\alpha$  rearrangement is clinically relevant as it allows rapid diagnosis through immunostaining techniques.

The PML-RAR $\alpha$  protein functions as an aberrant retinoid receptor with altered DNA-binding properties as compared to wild type RAR $\alpha$ , and it acts as repressor of RA signaling. PML-RAR $\alpha$  can heterodimerize with the RXR, with PML and with another PML-RAR $\alpha$  chimeric protein forming a homodimer. Distinct from wild-type RAR $\alpha$ , the repression induced by un-liganded PML-RAR $\alpha$  is releasable only by pharmacological doses of ATRA ( $10^{-6}$  M). This is explained by the fact that compared to wild-type RAR $\alpha$ , the PML-RAR $\alpha$  hybrid binds the histone-deacetylase (HDAC)-recruiting co-repressor complex with higher affinity (Pandolfi PP, 2001). In addition to recruiting HDAC activity, PML/RAR $\alpha$  is also able to bind the DNA methylating enzymes Dnmt1 and Dnmt3a, leading to the methylation of RA target promoters in APLM blasts (Di Croce L, et al., 2009; Villa R, et al., 2006). In summary, PML/RAR $\alpha$  acts through various mechanisms as a constitutive and potent transcriptional repressor of RAR $\alpha$ -target genes (Carbone R, et al., 2006).

AML's a rare among the considerably incident AML leukemia's and ranks as the fifth among commonly occurring cancers in the Kashmir valley (North India) (Pandith and Siddiqi, 2012). We for the first time aimed at evaluating the efficacy of the peripheral blood cytogenetic technique along with molecular analysis in detecting the disease and monitoring the response in APLM patients treated with conventional chemotherapy. Base line peripheral blood cytogenetics along with qualitative PCR was performed to look for the type of the transcript followed by real time PCR quantification of the transcript

involved.

## Materials and Methods

We analyzed the course of all newly diagnosed and relapsed patients with APLM treated consecutively on protocols (ICAPL-2006 and AS<sub>2</sub>O<sub>3</sub>) in the department of Clinical Hematology and Medical Oncology at Sher-i-Kashmir Institute of Medical Sciences, (SKIMS) JandK (North India) and included in the study after a thorough approval from the 'Institute Ethics Committee' (IEC) of SKIMS and subjected to prospective evaluation of their response to conventional chemotherapy (ICAPL-2006 and AS<sub>2</sub>O<sub>3</sub>) at the Department of Immunology and Molecular Medicine, SKIMS from 2013 to 2015. All patients had the baseline bone marrow analysis done and were accordingly categorized against the morphologic criteria for the diagnosis of APLM [AML-M3] or M3-variant) according to the French-American-British classification system (Bennett JM, et al., 1985). The diagnosis was confirmed by presence of t (15:17) on peripheral blood cytogenetic studies as well as detection of PML-RAR $\alpha$  translocation by RT-PCR and Q-PCR.

Heparinized peripheral blood for cytogenetics and EDTA blood samples for RT-PCR and Q-PCR were collected after informed consent, from 37 newly diagnosed and relapsed patient. Median age of the patients was 31 years (range 06 to 92 years). We examined samples before patients began treatment, after they achieved clinical complete remission by standard criteria, and periodically thereafter.

### Cytogenetic analysis

Chromosomal analysis of the patients was done at baseline and different stages of treatment as per the ICAPL-2006 and AS<sub>2</sub>O<sub>3</sub> protocol. Cytogenetic analysis was carried out as per the technique followed in our previous work by Azad et.al (Niyaz A Azad, et al., 2015), wherein 40ul of PHA (Gibco) was added to every 5ml of RPMI-1640 culture media (Cell Clone) containing 10% fetal calf serum (Gibco) before adding about 0.5 ml of peripheral blood to it. In addition, a simultaneous PHA-free culture corresponding to each sample was also carried out for comparative purpose. Cultures were incubated for 48-72 hours followed by further 1 hour incubation after adding 50 ul of Colchicine (Loba Chemie-1 mg/10 ml) and 100ul of Ethidium bromide (1mg/10 ml) to each vial. Cultures were subsequently put to hypotonic shock with 0.075M potassium chloride and then fixed in Carnoy's fixative [methanol-acetic acid (3:1)]. Slides were prepared and GTG banding was performed as described by Seabright (Seabright M, 1971) Computerized work station- 'Cytovision 3.93' was used to identify and arrange chromosomes as per the International System for Human Cytogenetic Nomenclature (ISCN) (Mitelman F, 1995). At least 20 karyotypes were prepared and analyzed for each sample.

### RNA Extraction

Total RNA from Ficoll-Hypaque separated mononuclear cells from 5-7ml of peripheral blood was extracted using Trizol (Amresco). The extracted RNA

was checked for purity and integrity by DEPC-treated agarose gel electrophoresis. Concentration of RNA was measured spectrophotometrically and the ratio of optical density at 260 and 280 nm checked. A ratio of 1.80 to 2.00 was accepted as good quality RNA.

#### cDNA Synthesis

The reverse transcriptase reaction was performed using the Maxima cDNA synthesis kit (Thermo Scientific) containing Enzyme Mix (M-MuLV RT Enzyme and Ribolock Rnase Inhibitor), 5x Reaction Mix (reaction buffer, dNTP's, oligo(dt)18 and random hexamer primers and Nuclease free water).

Two micrograms of total cellular RNA was reverse transcribed to cDNA by an incubation of 10 minutes at 25°C, 15 minutes at 50°C, and 5 minutes at 85°C in a total volume of 20ul that contained 4ul of 5X Reaction Mix, 2ul of Maxima Enzyme Mix and 12ul of nuclease free water.

#### RT-PCR (Qualitative)

The amplification method as described by (van Dongen et al., 1999) was followed for qualitative RT-PCR studies. Multiplex PCR reaction was carried out in a 25ul reaction volume using reverse and forward primers sets for bcr1, bcr2 and bcr3 transcripts. PML-A1 5'-CAGTGTACGCCTTCTCCATCA-3', PML-A2 5'-CTGCTGGAGGCTGTGGAC-3', RARA-B 5'-GCTTGTAGATGCGGGGTAGA-3' and one self designed primer PML-RP 5'-CTGACTGTACCACAGCCATAGG-3'. Reaction times consisted of an initial denaturation of 94°C for 5 minutes, annealing at 54°C for 30 secs, and elongation at 72°C for 7 minutes, followed by 35 additional cycles (30 secs at 94 as centigrade, 30 secs at 54°C, and 30 secs at 72°C). 6-8ul of the PCR product was size-fractionated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under a UV transilluminator (Flourchem, HD2-Cell Biosciences) at 365 nm.

In case of PML/RAR $\alpha$ , due to alternative splicing of the breakpoint cluster regions (BCR) on PML locus of chromosome 15, PCR products of various sizes ranging from 345 to 381 bp were found with RARA-B primer which is found to be a "universal primer" for all the three forms of translocations seen in APLM (short isoform: intron 3-bcr 3; long variant isoform: exon 6-BCR 2; and long isoform: intron 6-BCR 1). A single product of either 381 or 345 bp was observed with PML-A1 primer that corresponded to the translocation breakpoints intron 6-BCR 1 or exon 6-Bcr2 and 376 bp products was observed with PML-A2 primer corresponding to BCR 3 whose breakpoint is located in intron 3. Primers PML-A2, RARA-B and self designed PML-RP primer set displayed an additional PCR product of 450 bp which served as an internal control and ensured good quality of cDNA used. Accordingly, the results of PCR for PML/RAR $\alpha$  were interpreted as long (l), long variant (v) isoform or short (s) isoform.

#### Q-PCR (Quantitative)

Extracted RNA was normalized to the concentration of approximately 500ng and subjected to the integrated

## Results

The APLM patients included 24 males (64.8%) and 13 females (35.1%) ranging from age 6 years to 95 years. 23 patients (62.1%) belonged to age group <30 yrs and the rest of the 14 patients (37.8%) were above 30 yrs of age. Of the 37 patients analyzed 10 patients (27.0%) belonged to urban area and the remaining 27 patients (72.9%) to rural areas (Table 1).

Patients were risk stratified into low risk, high risk and intermediate risk on the basis of TLC and platelet counts at presentation. 9 of the 37 (24.3%) patients presented with leucocytosis (>10x10<sup>9</sup>/L) and high platelet counts ( $\geq$  40x10<sup>9</sup>/cumm), whereas the remaining 28 patients (75.6%) had leucopenia ( $\leq$ 10x10<sup>9</sup>/L) with low platelet counts ( $\leq$ 40x10<sup>9</sup>/cumm) (Table 1).

Bone marrow examination of the patients revealed 07 patients (18.9%) with less than 60% promyelocytes and

**Table 1. Clinico-Pathological Parameters of APLM Patients and their Cytogenetic and Molecular Profile**

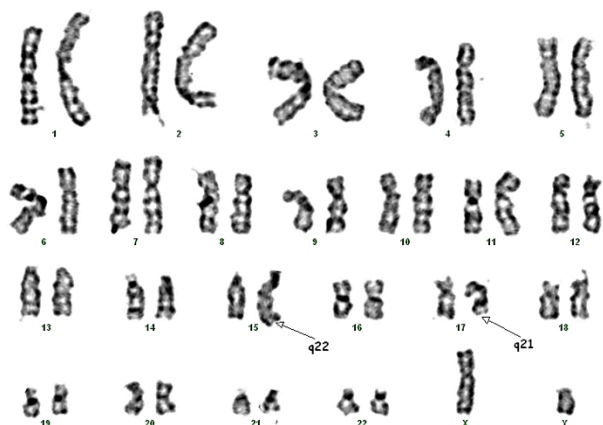
	Particulars	Number	Percentage %
Gender	Male	24	64.8
	Female	13	35.1
Age	$\leq$ 30	23	62.1
	$\geq$ 30	14	37.8
Dwelling	Urban	10	27
	Rural	27	72.9
Expired	Male	5	13.5
	Female	3	8.1
TLC (Baseline)	>10x10 <sup>9</sup> /L	9	24.3
	<10x10 <sup>9</sup> /L	28	75.6
Platelets	>40x10 <sup>9</sup> /cumm	28	75.6
	$\geq$ 40x10 <sup>9</sup> /cumm	9	24.3
Promyelocytes	<60%	7	18.9
	$\geq$ 60%	30	81
q-PCR value (Baseline)	$\geq$ 60%	13	35.1
	<60%	21	56.7
Karyotyping (Baseline)	PML-RAR $\alpha$ Positive	30	81
	PML-RAR $\alpha$ Negative	7	19
Transcript type Qualitative PCR	Bcr1	22	59.4
	Bcr2	3	8.1
	Bcr3	12	32.4
Therapy		27	72.9
	Arsenic (AS <sub>2</sub> O <sub>3</sub> )	10	27.1

**Table 2. Cytogenetic and Molecular Responses of APLM patients at Different Stages of Treatment**

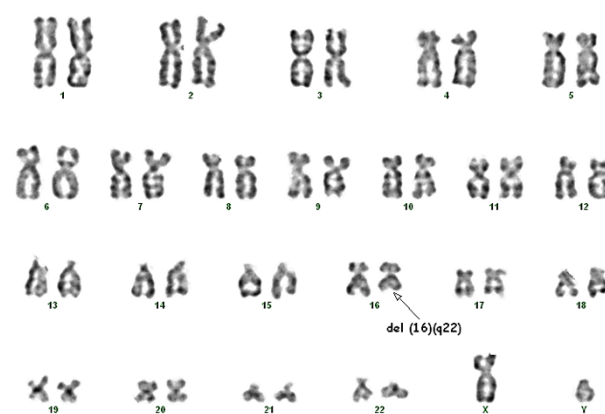
Particulars	Number	Cytogenetic Response (Karyotyping)		Molecular Response (q-PCR)			
		Remission	Relapse	Remission	Relapse		
Gender	Male	18	16	2	14	4	
	Female	11	9	2	9	2	
Age	<30	19	15	4	13	6	
	>30	10	10	0	10	0	
Dwelling	Urban	7	6	1	5	2	
	Rural	22	19	3	18	4	
CONSOLIDATION	TLC	High	0	0	0	0	
		Low	29	29	0	29	0
	PML-RARα q-PCR value (Q-pcr)	0-20%	29	29	0	29	0
		20-40%	0	0	0	0	0
		40-60%	0	0	0	0	0
	Transcript type (Qualitative PCR)	Bcr1	18	--	18	--	
		Bcr2	3	--	3	--	
		Bcr3	8	--	8	--	
	TLC	High	6	2	4	0	6
		Low	23	23	0	23	0
MAINTENANCE	PML-RARα q-PCR value (Q-pcr)	0-20 %	25	1	23	2	
		20-40%	0	1	0	1	
		40-60%	0	2	0	2	
	Transcript type (Qualitative PCR)	Bcr1	17	1	16	2	
		Bcr2	2	1	2	1	
		Bcr3	6	2	5	3	

**Table 3. Comparative Cytogenetic and Molecular Responses in APLM patients**

Plan	Response	age %	Relapse	age %	P value
Cytogenetics	25	86.2	04	13.8	0.7
Q-pcr	23	79.3	06	20.7	
Rt-pcr Qualitative					
bcr-1	16	55.1	02	11.1	0.2
bcr-2	02	6.9	01	33.4	
bcr-3	05	17.2	03	37.5	



**Figure 1. Representative Karyotype of the t(15;17) complement from PHA induced peripheral blood culture**



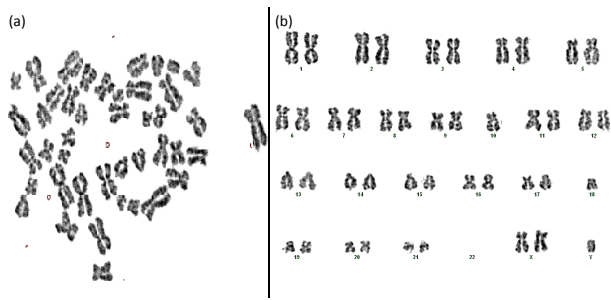
**Figure 2. Representative Karyotype showing chromosome 16q deletion in APLM patient**

30 patients (81%) with promyelocyte count greater than 60% (Table 1).

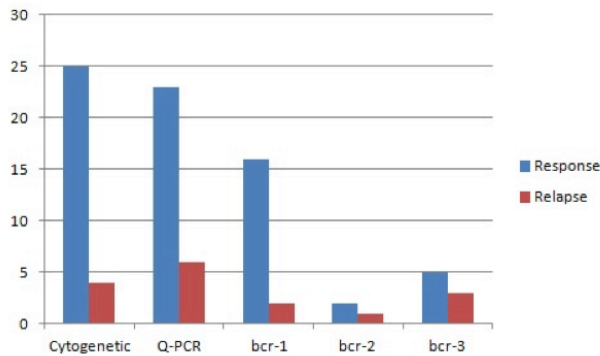
Initial PHA-stimulated peripheral blood karyotyping revealed 30 patients (81%) positive for t(15;17) whereas 7 patients (19%) had either cryptic translocation or were negative for t(15;17) (Figure 1). Incidentally, 02 such cases were detected as variants with chromosome 16q deletion and no hallmark translocation t(15;17) (Figure

2). The PHA-unstimulated cultures were not as reliable as the PHA-stimulated ones and were unable to report the variant karyotypes, which were clearly detected by the PHA-stimulated ones (Figure 3). The Q-PCR status for PML-RARα was found to be ≤ 60% for 21 patients (56.7%) while as it was >60% for 13 patients (35.1%) (Table 1). 3 patients had bcr-2 transcripts as detected by qualitative RT-PCR and could not be quantified because





**Figure 3. Representative metaphase (A) and its Karyotype (B) from unstimulated peripheral blood sample of APLM patient**



**Figure 4. Prognostic significance of Cytogenetic, Molecular (Q-PCR) and Transcript types (RT-PCR) in APLM patients**

most of the kits available quantify only bcr-1 and bcr-3 fusion transcripts.

Of the 37 patients at presentation, 22 patients (59.4%) had bcr-1, 03 patients (8.1%) bcr-2 and 12 patients (32.4%) were observed to harbor bcr-3 transcript (Table 1).

27 patients (72.9%) were subjected to treatment with ATRA based protocols whereas remaining 10 (27.1%), which included 6 relapsed and 4 newly diagnosed patients, were treated with arsenic tri-oxide ( $As_2O_3$ ) based protocols exclusively. During the course of study 08 patients (21.6%) expired including 05 males (13.5%) and 03 females (8.1%). Among these 06 patients died during induction phase of the treatment while as 02 patients relapsed and expired at the maintenance stage of chemotherapy (Table 1).

We followed 29 patients treated with either ATRA (in addition to conventional chemotherapy) (ICAPL-2006) or with  $As_2O_3$ . All patients achieved hematological remission (Differentiation) within first 35 days whereas molecular remission was achieved at different stages of consolidation phase of chemotherapy as revealed by normal TLC and platelet counts, negative Q-PCR, RT-PCR status for PML-RAR $\alpha$  fusion gene and also the absence of t(15;17) cytogenetically (Table 2).

Of the 29 patients, 06 patients presented with high TLC, 4 of them during maintenance and 2 after completion of maintenance phase of chemotherapy. All 6 patients had molecular relapse as indicated by their RT-PCR and Q-PCR values for PML-RAR $\alpha$  fusion gene which was less than 20% for 2 patients, 20 to 40% for one patient and 40 to 60% for other two patients. 1 patient being bcr-

2 positive could not be quantified. 02 of the 06 patients with molecular relapse had bcr-1 fusion transcript, one patient had bcr-2 fusion transcript, whereas bcr-3 transcript was detected in 03 patients. The molecular follow-up corresponded/concorded completely with the PHA-stimulated peripheral blood cultures and not with PHA-unstimulated ones, where 4 of the 6 relapsed patients had cytogenetic relapse in addition to molecular relapse (Table 2) (Figure 4).

## Discussion

The t(15;17) is the diagnostic hallmark of APLM and initially had been considered to be pre-sent in all patients with this condition (Warrell et al., 1993). However, it is now clear from that a sizeable minority of APLM patients actually lack this chromosomal aberration (David Grimwade et al., 2000). Three diagnostic approaches namely cytogenetics, quantitative reverse transcriptase-polymerase chain reaction (Q-PCR) and FISH analysis efficiently help in the diagnosis, management and treatment of APLM. In APLM a specific disease marker, i.e., the PCR amplifiable PML/RAR $\alpha$  fusion gene, allows molecular assessment of response to treatment and minimal residual disease (MRD) evaluation in 100% of patients (Cassnat B, et al., 2000). Cytogenetics detects the t(15;17) in the majority of patients, identifies secondary cytogenetic changes, and has revealed novel translocations in APLM, prompting subsequent molecular characterization of their respective breakpoint regions. Thus, evaluation of t(15;17) in APLM by conventional karyotyping is still considered as a standard tool for base line detection and for monitoring the targeted therapy.

Although the conventional cytogenetic studies on bone marrow have been viewed as the standard diagnostic and follow-up method for patients on chemotherapy, the technique continues to be confronted with the issue of quality of the signal metaphases produced, which are compact and dysmorphic, thereby making them not amenable to prominent G-banding for easy and convenient analysis (Seabright, 1971; Bennett et al., 1985). In a previous study conducted on patients with chronic myeloid leukemia, use of PHA and that too on the peripheral blood showed good promise of serving as a reliable alternative to the conventional PHA-free cytogenetics as there was a marked improvement in terms of the quality of the metaphases generated with PHA-induced cultures of peripheral blood (Niyaz A Azad, et al., 2015). In line with this assessment, we aimed to evaluate the application of Phytohemagglutinin (PHA)-induced peripheral blood culture based cytogenetic analysis (Karyotyping) in base line diagnosis and monitoring chemotherapy response of the APLM patients of Kashmir region (North India). Patients were also assessed by Q-PCR and RT-PCR at base line and follow up to correlate with cytogenetic evaluation. In this study we replicated the same procedure but the samples were peripheral blood from APLM patients

In our cytogenetic report, additional chromosomal alterations were observed in 19% of the APLM patients in tandem with the marker translocation t(15;17). The

additional chromosomal abnormalities found in our study included chromosome 16 (q) deletion, 16q del with loss of Y chromosome, more complex karyotypes were found in 17.1% of the APL patients. It is well documented that the incidence of chromosome abnormalities in addition to t(15;17) in APL has been reported before to be around 25-40% in primary APL patients and even higher in relapse (De Botton et al., 2000). 66% of (4 of 6) the APL patients with relapse had complex karyotype with presence of some unidentified marker signatures in addition to marker translocation t(15;17). Several studies have reported a series of alternative chromosomal aberrations including t(11;17) (Chen et al., 1993; Chen et al., 1993; Wells et al., 1997), t(5;17) (Redner et al., 1996), and del(17) (Arnould et al., 1999) whereby RAR $\alpha$  is fused to the PLZF, NPM, NuMA, and STAT5b genes, respectively. However, we did not find any such translocations in our series of APL cases.

In addition to the diagnostic work-up of APL by conventional or fluorescent in situ hybridization (FISH) cytogenetic (Iqbal S, et al., 2000), molecular detection of PML-RARA fusion gene by quantitative RT-PCR helps in base line detection and monitoring minimal residual disease (Wang AM, et al., 1989; Cassnat B, et al., 2000). We used Q-PCR assay for detection of the PML/RAR-a rearrangement with high sensitivity. The assay was performed on APL cells obtained from 37 patients whose APL diagnosis was made by conventional clinical criteria as well as peripheral blood cytogenetics. The results reveal that this Q-PCR assay for the PML/RAR $\alpha$  fusion mRNA is more sensitive. By Q-PCR for PML-RAR $\alpha$ , we found all the patients positive for PML-RAR $\alpha$  except 03 patients whose minimal residual disease (MRD) could not be ascertained as they were found to harbor bcr-2 transcripts by RT-PCR. This is because most of the kits available quantify only bcr-1 and bcr-3 fusion transcripts. The performance of this assay was observed to be reliable in diagnosis and later follow up the APL patients.

Of the total 37 patients enrolled initially in this study, 08 patients expired during the course of study, out of which 06 patients died during induction phase of the treatment and 02 patients relapsed and expired at the maintenance stage of chemotherapy. Out of the remaining 29 patients, 19 were treated with ATRA based protocols and 10 patients (6 relapsed and 4 newly diagnosed patients) were treated with arsenic tri-oxide (As<sub>2</sub>O<sub>3</sub>) based protocols (Shen et al., 1997; Ghavamzadeh et al., 2006; 2010). There was 100% remission at both cytogenetic and molecular level in consolidation phase confirming ATRA based protocols as effective means to produce complete remission and long-term cures, although in some cases, remissions do not appear to be long term which was evident in our study as well as the studies conducted earlier (Huang et al., 1988; Castaigne et al., 1990; Chomienne C, et al., 1990; Warrell et al., 1991). All the APL patients were reassessed during maintenance phase where 6 patients showed relapse as evident by positive RT-PCR in all the 6 patients and 4 showed cytogenetic relapse as well. All the 6 relapsed patients were treated with Arsenic tri-oxide (As<sub>2</sub>O<sub>3</sub>) exclusively (Shen et al., 1997). Two patients died during this phase which had a complex Karyotype with

6q deletion in addition to marker translocation t(15;17). In our study molecular analysis (Q-PCR + RT-PCR) was found more sensitive while following the disease status of the patients as it picked up six patients as compared to 4 by cytogenetics which were later found to have the relapse upon clinical correlation.

We evaluated the transcripts types of all the 37 APL patients by qualitative RT-PCR, where bcr-1 frequency was found to be highest in 22 (59.4%) followed by bcr-3 transcript in 12 patients (32.4%) and 03 patients were bcr-2. It is known that breakpoint on chromosome 17 invariably disrupts the RARA gene in the second intron, but rearrangement of the PML gene may occur at 2 major breakpoints, one within PML intron 3 (bcr-3) yielding the short fusion mRNA transcript that joins PML exon 3 and RAR-a exon 3. Another breakpoint occurs within PML intron 6 (bcr-1) that consistently produce the long transcript from the joining of PML exon 6 to RAR-a exon 3. Additionally, breakpoints within PML exon 6 (bcr-2) are associated with a variable length transcript in a small number of patients (Miller WH Jr, et al., 1992; Borrow J, et al., 1992; Pandolfi PP, et al., 1992; Biondi A, et al., 1992). The influence of breakpoint site on patient outcome remains controversial. Early reports suggested that patients who express the short isoform have an increased incidence of clinical relapse and shorter survival compared to patients with the long isoform (Huang et al., 1993; Vahdat et al., 1994). Others have reported no difference in disease-free survival between patients with the long or short isoforms after adjusting for the association of the short form with a higher leukocyte count at presentation (Gallagher et al., 1997). Our study found that 37.5% APL patients showed relapse with a short transcript type (BCR 3) and had unfavorable prognosis as compared to patients with a long transcript type (BCR1-2) who had relapse in only 11% patients (Table 3). Similar scenario was depicted by Huang et al. (1993) who have reported that patients with a BCR 3 treated with ATRA alone have a more unfavorable prognostic outcome than patients with bcr 1-2. One more study conducted by Vahdat et al. has depicted the similar picture regarding the prognosis of the APL patients on the basis of presence of the transcripts (Vahdat et al., 1994).

Further investigations in larger series are needed in order to establish whether different PML breakpoints are associated with variable diagnostic features and/or prognosis. Finally, the clinical significance of additional karyotypic abnormalities sometimes found in APL has not been evaluated.

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