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RESEARCH ARTICLE

# Mutational landscape of patients with acute promyelocytic leukemia at diagnosis and relapse

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## Abstract

Despite the high probability of cure of patients with acute promyelocytic leukemia (APL), mechanisms of relapse are still largely unclear. Mutational profiling at diagnosis and/or relapse may help to identify APL patients needing frequent molecular monitoring and early treatment intervention. Using an NGS approach including a 31 myeloid gene-panel, we tested BM samples of 44 APLs at the time of diagnosis, and of 31 at relapse. Mutations in *PML* and *RARA* genes were studied using a customized-NGS-RNA panel. Patients relapsing after ATRA-chemotherapy rarely had additional mutations ($P$ = .009). In patients relapsing after ATRA/ATO, the *PML* gene was a preferential mutation target. We then evaluated the predictive value of mutations at APL diagnosis. A median of two mutations was detectable in 9/11 patients who later relapsed, vs one mutation in 21/33 patients who remained in CCR ($P$ = .0032). This corresponded to a significantly lower risk *of relapse* in patients with one or less mutations (HR 0.046; 95% CI 0.011-0.197; $P$ < .0001). NGS-analysis at the time of APL diagnosis may inform treatment decisions, including alternative treatments for cases with an unfavorable mutation profile.

## 1 | INTRODUCTION

Acute Promyelocytic Leukemia (APL) is characterized by the t(15;17) translocation, which generates the disease-unique *PML/RARA* oncoprotein. Until the early 90s, APL was considered the most aggressive and rapidly fatal form of AML. Over the past 25 years, important advances have been made in the understanding of APL biology and in developing new treatments. Together, these have resulted in extraordinary improvements in outcome, such that the majority of patients are nowadays curable with targeted therapy combining all-trans retinoic acid (ATRA) and arsenic trioxide (ATO).[1,2]

Licia Iaccarino and Tiziana Ottone contributed equally to this study.

Although the translocation involving *PML/RARA* is a constant event in APL, the long latency period and the variable penetrance required for leukemia development in transgenic mice,[2] suggest that the oncoprotein is not *per se* sufficient to induce leukemogenesis. Thus, it is likely that secondary genetic abnormalities might contribute to APL pathogenesis. Furthermore, the genetic patterns associated to the infrequent development of ATRA and/or ATO resistance in APL are poorly understood.

To the best of our knowledge, only few studies have analyzed the genetic features of APL using next generation sequencing (NGS)-based approaches.[3-6] A comprehensive mutational analysis of paired diagnostic/relapse samples from APL patients reported a mutational profile significantly different from that observed in other AML

  

subtypes. Such a profile was characterized by recurrent alterations of *FLT3*, *WT1*, *NRAS*, *KRAS*, *ARID1B* and *ARID1A* genes, both at the time of diagnosis and at relapse.[5,6] Mutations in addition to the *PML/RARA* fusion gene were reported in approximately 70% of patients analyzed at diagnosis, while changes in the molecular profile at the time of relapse were detected in >70% of cases.[6]

Recent investigations, including one from our group,[7] suggested that point mutations occurring in the two moieties of the *PML/RARA* hybrid may play a role in resistance to targeted therapy with ATRA and ATO. In keeping with this hypothesis, mutations of the PML-B2 domain of PML-RARA have been shown to prevent ATO binding. This inhibits degradation of the oncoprotein, thus hindering oligomerization into nuclear bodies.[8] On the other hand, mutations in the RARA-Ligand Binding Domain (LBD) are known to reduce the affinity of ATRA binding *in vitro*, and result in a differentiation block following treatment with ATRA.[9,10] Finally, mutations in the un-rearranged *PML* allele have been proposed as additional mechanisms potentially associated with ATO resistance.[7,11]

We hypothesized that improved understanding of the molecular mechanisms involved in APL relapse and resistance may be important for the early identification of patients in need of salvage pre-emptive therapy. Here, we report the results of a comprehensive molecular study conducted in APL patients relapsing after ATRA and chemotherapy, or ATRA and ATO.

## 2 | METHODS

### 2.1 | Samples collection, genomic DNA and RNA extraction

Relapse bone marrow (BM) samples from patients with *de novo* or therapy-related APL, as well as samples collected at time of diagnosis and complete remission, were available in our biobank at the Institute of Hematology of the University Tor Vergata of Rome. The center has long since served as a reference institution for sample centralization for diagnostic and follow-up monitoring of APL patients. According to the declaration of Helsinki, all patients gave informed consent for the present study, which has been approved by the Institutional Review Board of the Policlinico Tor Vergata of Rome. We collected 31 genomic DNA samples at the time of APL relapse after ATRA-chemotherapy (CHT) (n = 19) and ATRA-ATO treatment (n = 12) (Figure S1). Clinical and biological features of APL patients analyzed at relapse were reported in Table S1. In particular, ATRA-ATO was used as second-line treatment in 11 cases, and as first-line treatment in one case (Figure S1). Within the group of 31 relapsed patients, four had experienced multiple relapses after an ATRA-ATO regimen, and DNA from several samples (median 3, range 2-5) were available. Moreover, for 11 patients who relapsed at a median time of 12 months (range 5-21 months), after ATRA-CHT (n = 9) and ATRA-ATO (n = 2), paired DNA collected at time of APL diagnosis were available.

Diagnostic BM samples were also available in 33 APL patients who remained in continuous complete remission (CCR), for a median time of 60 months (range 20-124 months) after ATRA-CHT (n = 26) or ATRA-ATO (n = 7). Seventeen of 44 patients analyzed at diagnosis, were classified as high-risk (39%), and 27 (61%) as standard-risk APL, according to Sanz score.[12] Clinical and biological features of APL patients analyzed at diagnosis were shown in Table S1.

RNA samples were also available from 30 *de novo* APL cases collected at the time of relapse (occurring at a median time of 13.5 months from diagnosis, range 3-75). Of these, 15 patients had relapsed after ATRA-CHT and 15 patients after ATRA-ATO (Figure S1).

Genomic DNA was extracted from cell pellets using Qiagen kit protocol (Qiagen, Hilden, Germany) and quantified using a Qubit Fluorometer (Life Technologies, Carlsbad, CA, USA). A schematic illustration of the type of samples in relation to treatments and time points is illustrated in the Figure S1.

Total RNA was extracted from Ficoll-Hypaque isolated BM mononuclear cells using standard procedures[13] and reverse-transcribed using random hexamers as primers.

### 2.2 | Molecular genetic analyses and NGS

After the genetic confirmation of APL,[14] quantitative assessment of *PML/RARA* copy number was carried out at pre-defined time points during routine follow-up, using methods reported elsewhere.[15] The *FLT3* mutational status (ITD and TKD) was evaluated by PCR amplification, followed by capillary electrophoresis using ABI 3130 as reported.[16]

To investigate the genetic features of disease progression in APL, we evaluated the frequency of additional mutations using a 31-gene panel targeting *ASXL1*, *CBL*, *CSF3R*, *CSNK1A1*, *DNMT3A*, *ETNK1*, *ETV6*, *EZH2*, *FLT3*-TKD, *GATA1*, *GATA2*, *JAK2*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MYC*, *NPM1*, *NRAS*, *PHF6*, *PIGA*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1*, *WT1* and *ZRSR2*. The library was generated using the TruSeq Custom Amplicon protocol (Illumina, San Diego, CA) and sequenced using the Illumina platform (minimum coverage 400X). The NGS panel was performed in 31 APL samples at relapse, *including four patients* with *multiple relapse*. Moreover, the NGS mutational profile was carried out on 44 APL samples at diagnosis (33 remaining in CCR and 11 who later relapsed, Figure S1). Based on identified gene alterations, we investigated in selected cases the mutation dynamics by backtracking earlier samples with NGS using customized amplicons, which target the mutated positions. For these cases, we also collected remission samples where available. The FASTQ files were further processed using the Sequence Pilot software version 4.1.1 (JSI Medical Systems, Ettenheim, Germany) for alignment and variant calling. The validity of the somatic mutations was checked on the publicly accessible Catalogue Of Somatic Mutations In Cancer (COSMIC) v69 database. Functional interpretation was performed using SIFT 1.03, PolyPhen 2.0 and MutationTaster 1.0 algorithms.[17] Single-nucleotide polymorphisms (SNPs) were annotated according to the National Center for Biotechnology Information Single Nucleotide Polymorphism Database (NCBI dbSNP). The detection limit for small nuclear variants was 3% variant allele frequency (VAF).

### 2.3 | Targeted RNA sequencing assay

The NGS assay for *PML* and the *PML/RARA* fusion transcript was designed from cDNA templates of APL patients.[7] It was carried out

on 30 relapse APL samples, using the Illumina platform (Illumina, San Diego, CA) (sensitivity of 1%) (Figure S2). Based on NGS results, it was done on selected paired RNA diagnostic samples. A dilution test was performed to assess the specificity of the method. The RNA from one APL sample, with a high and well-defined *RARA* mutation load identified by NGS (VAF = 98%), was diluted with wild type RNA at 1:2 and 1:10 ratios. This simulated low-percentage mutation loads (Figure S3A). These experiments showed a high correlation between the calculated mutation load and the sequencing result. To test for sensitivity, we performed independent technical replicates (n = 5) of patient samples with mutation levels below 20%. Replicates were performed from different cDNA with independent PCR amplifications and sequencing reactions, which showed high sensitivity and reproducibly of the assay even at low VAF (3%) (Figure S3B).

The *PML/RARA* fusion transcript was amplified (first PCR), and sequencing amplicons of the *PML*-B2 and *RARA*-LBD were generated (second PCR) with FastStart High Fidelity PCR System (Roche Applied Science, Penzberg, Germany). The PCR products were analyzed on the Bioanalyzer (Agilet Technologies, Santa Clara, CA, USA). Products were purified by Agencourt AMPureXP beads (Beckman Coulter, Krefeld, Germany) and equally pooled. Sequencing was performed by the Illumina platform. The amplicon design is reported in Figure S2. In case of unreported mutations, the NGS analyses were performed in duplicate, to exclude false positive results from technical artifacts. Clinical and biological features of APL patients analyzed were reported in Table S1.

### 2.4 | Statistical analysis

Dichotomous variables were compared between different groups using the Wilcoxon-Mann-Whitney test, and continuous variables were compared by the Student's *t*-test and log rank (Mantel-Cox's) test. Multivariate linear regression analyses were performed to assess the role of different variables on patients' outcome. Role of mutations number as relapse risk predictor was assessed using Cox proportional hazards regression analysis. Results were considered significant at $P < .05$. Statistical analyses were performed using the SPSS version 19.0 (IBM Corporation, Armonk, NY, USA); the reported *P*-values are two-sided.

## 3 | RESULTS

### 3.1 | Mutational landscape of APL at relapse and at initial diagnosis

Using a NGS panel including 31 genes, we looked for mutations in myeloid-specific genes, other than *PML* and *RARA*. At time of relapse after ATRA-CHT, 9 of 19 (47%) patients had a median of 0 mutation *per* patient (range, 1-2), with *WT1* (3 of 19, 16%) and *FLT3* (3 of 19, 16%) as the most frequent alterations (Figure 1). By contrast, in patients who relapsed after ATRA-ATO, a median of 2 mutations *per* patient (range, 0-5) was detected in 9/12 cases (75%) ($P = .009$ Mann-Whitney test). The most common mutations were in *ETV6* (2 of 12, 16.6%), *DNMT3A* (2 of 12, 16.6%) and *TET2* (3 of 12, 16.6%) genes. Nearly all mutations were heterozygous, with a median VAF of 15% (range: 3-51% VAF). Of note, in one patient we found a *DNMT3A*-mutated subclone at VAF 7% at the time of CCR and at relapse, but not at diagnosis, probably related to clonal hematopoiesis of undetermined significance (CHIP).

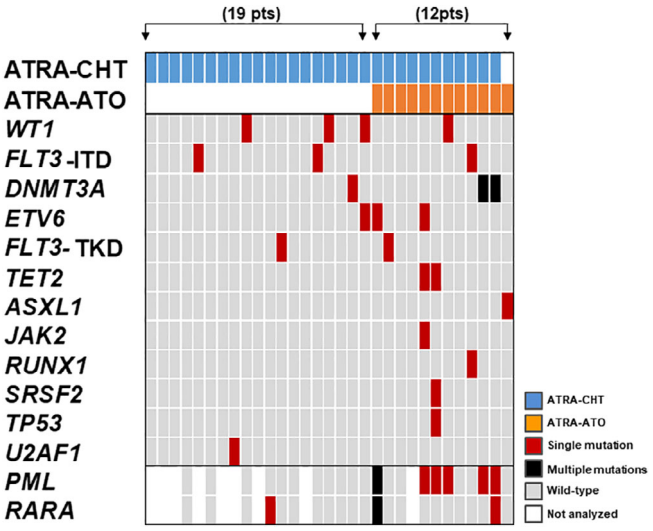


**FIGURE 1** Mutational profile of samples from 31 APL patients analyzed at relapse. The NGS mutational profile of samples collected at relapse after ATRA-CHT (left side) (n = 19) and ATRA-ATO (right side) (n = 12) treatments is shown. Individual patient samples are displayed as columns

At the time of *APL diagnosis*, we found a median of two additional mutations *per* patient (range, 0-3), in 11 patients who later relapsed (Figure 2A). By contrast, the number of mutations was significantly lower (median of one mutation *per* patient, range, 0-2) in the diagnostic samples in 21 of 33 APL patients, who remained in CCR ($P = .0032$ Mann-Whitney test) (Figure 2A). Interestingly, *NRAS* and *RUNX1* mutations were found only in patients who later relapsed, with *FLT3* (ITD/TKD: 5 of 11 patients, 45%) and *NRAS* (3 of 11 patients, 27%) as the most frequently mutated genes. In addition, at diagnosis, there were no differences in the number of mutations when grouping patients according to the Sanz risk score (standard risk, n = 27 vs high-risk, n = 17, Figure 2A). The *FLT3*-ITD mutations were present in most high-risk patients (13 of 17, 76.5%, vs 2 of 27 standard risk patients, 7.5%, $P = .0001$), but were not significantly associated wtih the risk of relapse. The univariate analysis using the log-rank (Mantel-Cox) test showed that patients who harbor ≥2 mutations at diagnosis, have significantly higher cumulative incidence of relapse (CIR) ($P < .0001$) than patients with <1 mutations (Figure 2B). The analysis including type of treatment (ATRA-CHT vs ATRA-ATO) and Sanz score, showed that the number of mutations was an independent relapse predictor (hazard ratio, HR 0.046; 95% CI 0.011-0.197; $P < .0001$).

### 3.2 | Analysis of PML and PML/RARA by targeted RNA sequencing assay

We analyzed mutations in *PML* and *RARA* RNAs, in particular the *PML*-B2 domain and the LBD of *RARA*. This was using NGS with a median coverage of 33 886X (range, 1660-47 468), and 26 051X

(range, 12 188-47 799) for *PML* and *RARA*, respectively. This corresponds to a sensitivity of 1%.

We detected mutations within *PML* and *RARA* genes in eight of 30 (27%) relapsed APL patients (Figure 3A and B). In particular, there were two patients, who relapsed after ATRA-CHT with mutations in the *RARA*-LBD only. Conversely, we found several mutations in both *PML* and *RARA* genes in six patients. They relapsed after both ATRA-CHT and/or ATRA-ATO, with *PML* A216V as the predominant mutation (Figure 3A and B). The A216T/A216V mutation site in the *PML* gene, and R272Q, T285I and S287 L in *RARA* have been previously

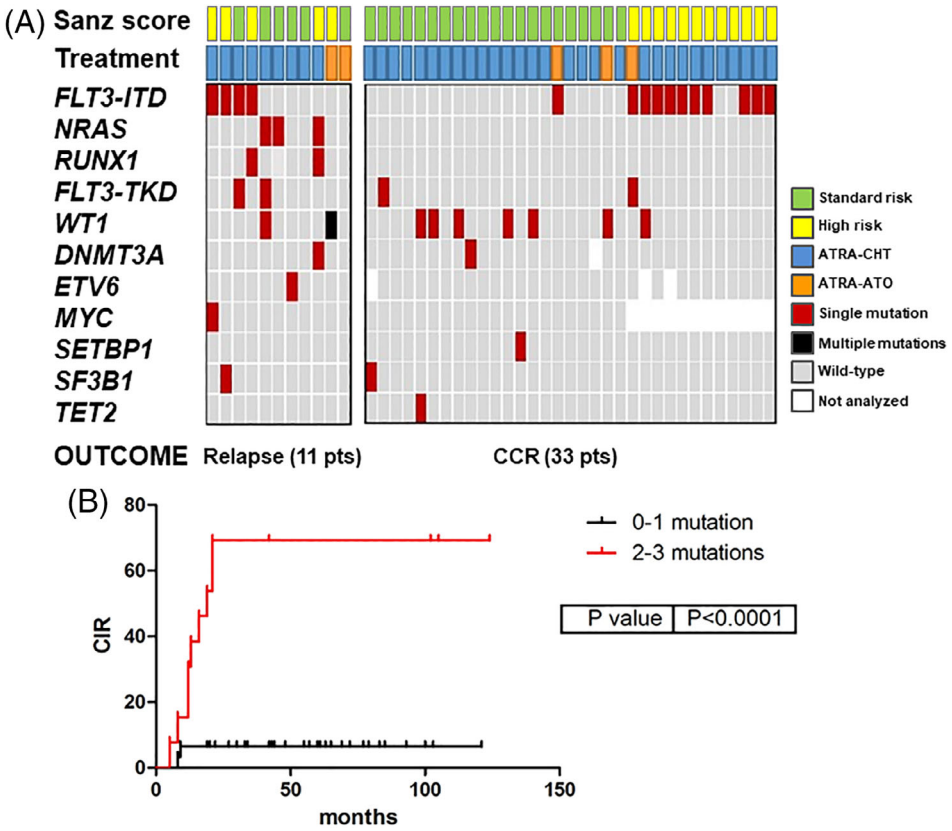


**FIGURE 2** Mutation pattern of 44 APL patients analyzed at diagnosis by NGS. A, Analysis of diagnostic samples of patients who relapsed (left side) (n = 11) and patients who achieved CCR (right side) (n = 33). Individual patient samples are displayed as columns. B, Cumulative incidence of relapse (CIR) according to the number of additional mutations present at diagnosis in 44 APL patients. Univariate analysis was performed using the log-rank (Mantel-Cox) test

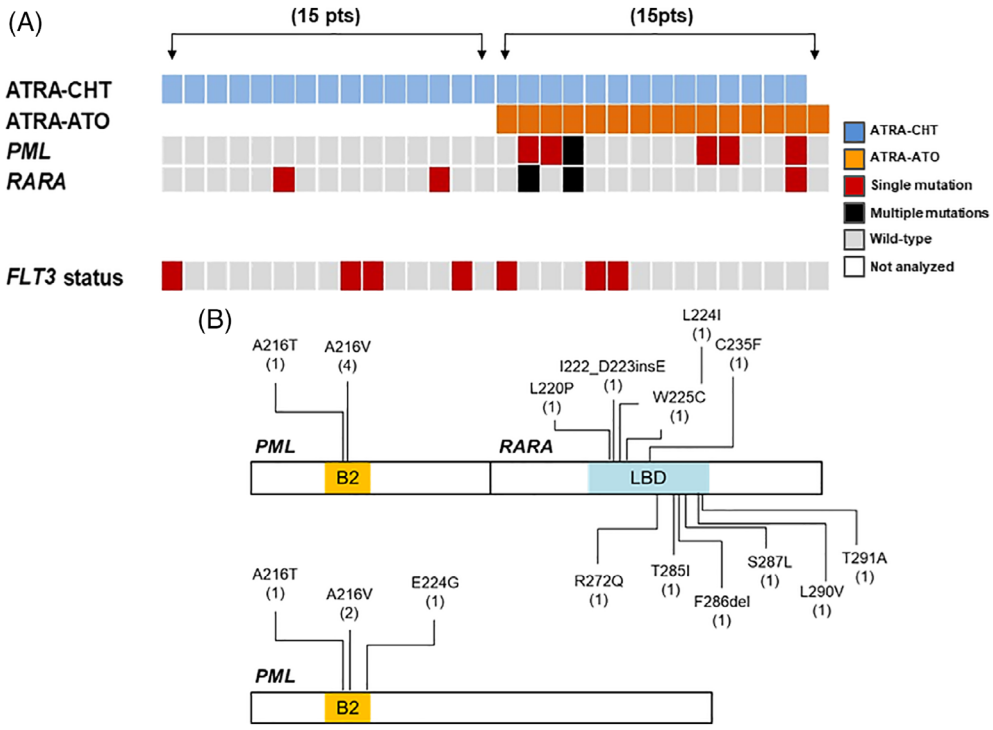


**FIGURE 3** *PML* and *RARA* target sequencing analysis in 30 APL patients at relapse Mutations in *PML/RARA*, and the unrearranged *PML* allele were studied at relapse in 30 APL patients A. The *FLT3* mutation status is also shown. Individual patient samples are displayed as columns. B, Mutation type in the *PML*-B2 (yellow) and *RARA* ligand binding domain (LBD) (light blue) are shown. The VAFs for each mutations are shown in brackets [Color figure can be viewed at wileyonlinelibrary.com]

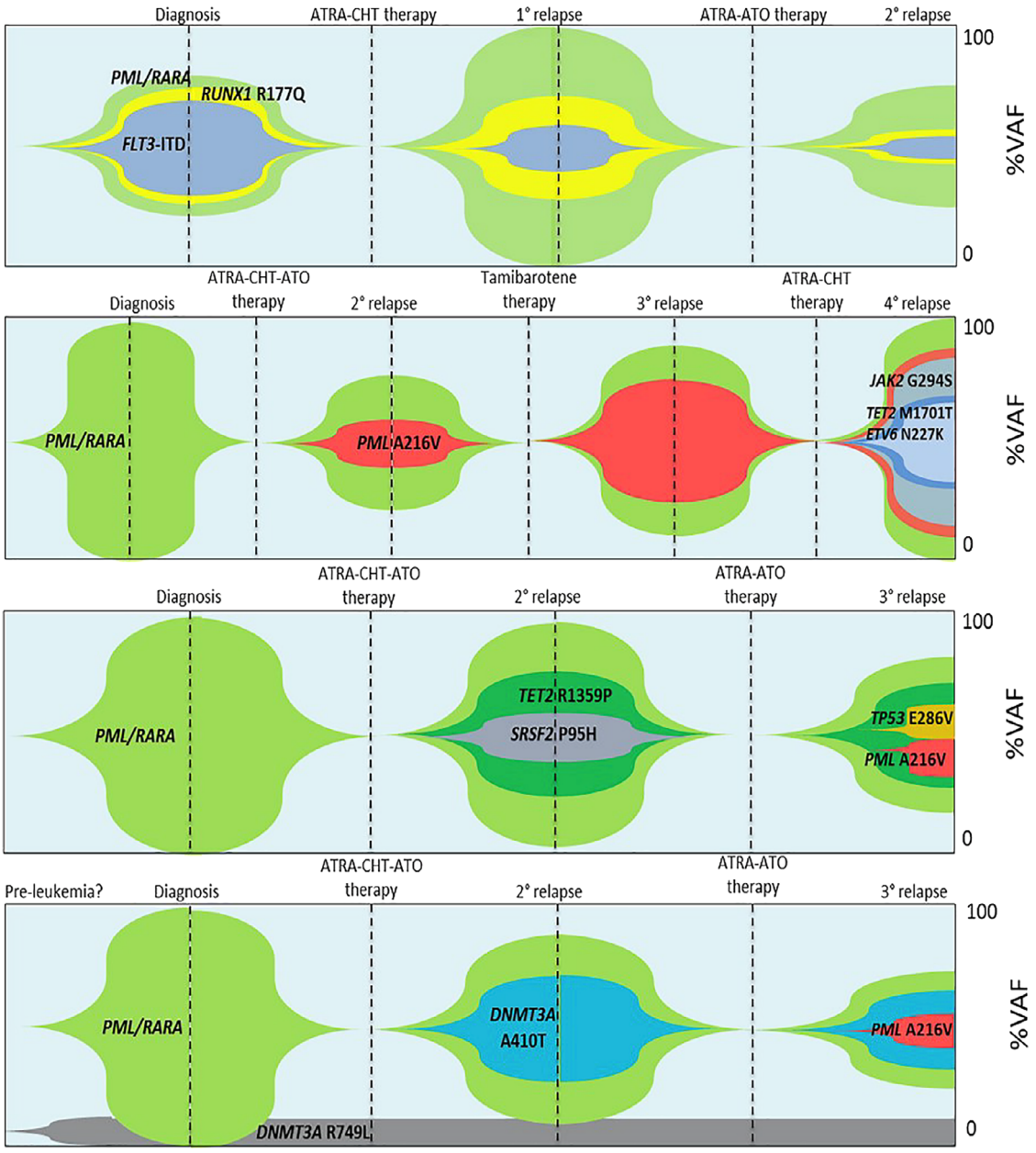


**FIGURE 4** Models of clonal evolution in relapsed APL. The figure shows disease course, treatment and the mutation load (y-axis) in 4 patients who experienced multiple relapses after ATRA/CHT and ATRA/ATO. Emerging clones are displayed with different colors [Color figure can be viewed at wileyonlinelibrary.com]

reported.[7,8] In the present study we describe *PML* E224G and six novel missense mutations in *RARA* (L220P, L224I, W225C, C235F, L290 V, T291A). Target sequencing, performed on paired diagnostic RNA samples in five of eight mutated patients at relapse, did not reveal mutations in *PML* and *RARA* genes at the time of initial diagnosis. Interestingly, two cases with refractory t-APL showed several mutations, reflecting the aggressive disease course. In particular, in one patient we found two mutations in the *PML* moiety and five mutations in the *RARA* moiety of *PML/RARA*, in concomitance with three mutations within the unrearranged *PML* allele. Genetic analysis of *FLT3* gene in this cohort of 30 APL samples showed a high prevalence of FLT3 mutations at diagnosis (*FLT3*-ITD n = 5 and *FLT3*-TKD n = 2), mutually exclusive with *PML* and *RARA* mutations (Figure 3A).

We then studied the pattern of clonal progression in four patients who experienced multiple relapses, and compared the mutational profile at the time of morphological relapse to that of the diagnostic sample (Figure 4). In three patients, the sample collected at the time of relapse was characterized by additional mutations. They were not only in the *PML* gene, but also in other myeloid-specific genes, while the mutation pattern remained stable in one patient.

## 4 | DISCUSSION

The comprehensive molecular analysis data of relapsed APLs remains limited. Our sensitive mutational analysis allowed the detection of a

high prevalence of *PML* and *RARA* mutations in 27% of our patients, including 30 relapsed APLs. That is concordant with other studies, which described *PML* and *RARA* mutations in up to 47% of APL.[3,7,8,18,19] All mutations described were localized in critical domains of *PML* and *RARA*, known to be associated to ATO and ATRA binding. As reported by several studies, the majority of *PML* mutations were clustered at the A216 amino acid residue of *PML*. This is a hotspot known to confer resistance to ATO, by preventing irreversible drug binding.[18,20] Moreover, we found *PML* mutations in *PML/RARA* in three out of five cases, in the un-rearranged *PML* allele in one case, and in both the rearranged and the un-rearranged alleles in one further case. Mutations in both *PML* alleles have been described as additional mechanism that may reinforce ATO resistance.[7,11] All five patients who harbored *PML* mutations showed clinical resistance to ATO-based therapy, and had progressive disease. In this setting, using a sensitive ddPCR technique we were able to detect the *PML*-A216V at very low copy numbers in patients relapsing after ATO-ATRA.[21]

As of the *RARA* gene, mutations were analyzed only in the *PML/RARA* fusion transcript. It would be interesting also to analyze the un-rearranged *RARA* allele to better understand whether these alterations are pre-leukemic mutations. It is alsoto know whether they have been acquired after the occurrence of the *PML/RARA* translocation. The *PML* and *RARA* mutations were not detectable at initial diagnosis. It is conceivable that they may have been present at very low levels, under the limit of detection by the NGS assay, and were later on selected under treatment pressure. In addition, the mutual exclusivity with *FLT3* mutations, suggests that the different mutations may have distinct roles in disease pathogenesis and progression. As of *FLT3*-ITD mutations, they were almost exclusive of high-risk APL patients, but did not correlate with the probability of relapse.

Since the frequency of the *PML* and *RARA* mutations is relatively low, they might not be the only clinically relevant cause of subsequent APL relapses. Thus, we analyzed by NGS a 31-gene panel in 31 relapsed APL patients, including four cases with multiple relapses. Our data suggest that molecular patterns detectable at diagnosis may predict treatment response. Comparing the initial mutational status of patients who relapsed during the course of disease (n = 11) with those in CCR (n = 33), we found differences in concomitant mutations, with *NRAS* and *RUNX1* mutated only in the first group of patients, suggesting their possible role as predictive markers of relapse. Of note, also the number of concomitant mutations *per* patient was significantly higher in the group who later relapsed ($P < .0001$). However, validation studies in prospectively analyzed APL cohorts are needed to confirm the predictive value of our hypothesis. All APL with multiple relapses after ATRA-ATO displayed a significantly higher number of mutations ($P = .04$), indicating an accumulation of genetic alterations during disease progression. In particular, we found mutations associated with clonal hematopoietic expansion, like *ASXL1*, *DNMT3A*, *JAK2*, *SRSF2*, *TET2* and *TP53*. However, a functional in vitro assay is needed to confirm their role in APL pathogenesis.

The identified mutational patterns suggested different models of disease progression. In some patients, relapses may originate from the driver clone present at diagnosis, with an unclear mechanism. In contrast, in at least three of our patients, relapses probably emerged from ATO-resistant subclones. Particularly, *PML*-mutated subclones seem to arise under selective pressure of ATO treatment. During clonal selection, these subclones may acquire mutations in genes, which confer advantages in self-renewal and proliferation, resulting in clonal expansion of the affected cells. These two mechanisms of disease relapse may explain why *PML* and *RARA* mutations were mutually exclusive, with *FLT3* mutations in our cohort of APL patients. Interestingly, in one patient a *DNMT3A* mutation was also detectable in a *PML/RARA*-negative sample. This was at the time of CCR, and was probably suppressed by the *PML/RARA*-positive dominant clone at diagnosis, possibly indicating CHIP (Figure 1). Further investigations using more sensitive methods, like mutation-specific ddPCR, would be useful to better understand the clonal evolution in cases harboring several mutations acquired during disease progression. In addition, experiments by single-cell sequencing would be interesting to investigate the role of CHIP in APL.

In conclusion, early detection of *PML*-mutated subclones may help to promptly identify ATO resistant APL, in order to adopt alternative treatment strategies to overcome the accumulation of other gene mutations. Unraveling the molecular pathways involved in APL resistance may also help to identify new molecular targets and allow the design of novel therapies (possibly combinatorial) including arsenic trioxide.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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