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Molecular landscape of acute promyelocytic leukemia at diagnosis and relapse

Acute promyelocytic leukemia (APL) occurs in about 5-30% of adult *de novo* AML and is characterized by the presence of the chromosomal translocation t(15;17)(q24;q21). The resulting *PML-RARA* chimeric gene involves the *retinoic acid receptor alpha-(RARA)* gene on chromosome 17 and the *PML* gene, a putative transcription factor, on chromosome 15. The resulting PML-RARA chimeric protein is crucial to the pathogenesis of APL as it is thought to contribute two oncogenic hits in one: the block of differentiation and the aberrant self-renewal of APL cells.[1]

Since the introduction of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) for the treatment of APL, the overall survival rate has improved dramatically.[2,3] However, relapse/refractory patients showing resistance to ATRA and/or ATO are still recognized as a clinically significant problem. It is likely that concurrent genetic aberrations at initial diagnosis predispose patients to relapse or that additional genetic lesions acquired during the course of APL lead to treatment resistance.[4] Genetic mutations resulting in amino acid substitution in the RARA ligand binding domain (LBD)[5-7] and the PML-B2 domain of PML-RARA, respectively, have been reported as molecular mechanisms underlying resistance to ATRA and ATO. In the presence of LBD mutation, binding of ATRA to LBD is generally impaired, and ligand-dependent co-repressor dissociation and degradation of PML-RARA by the proteasome pathway, leading to cell differentiation, are inhibited.[5,8] Mutations in the PML-B2 domain affect direct binding of ATO with PML-B2, and PML-RARA SUMOylation with ATO followed by multimerization and degradation is impaired.

The aim of the study was to evaluate the mutational spectrum of APL both at initial diagnosis and relapse, and identify potential genetic defects leading to treatment resistance.

We analyzed a cohort of 123 adult *de novo* APL cases including 14 cases who showed hematologic or molecular relapse. Patient characteristics are given in Table 1. All patients were proven to have t(15;17)(q24;q21)/*PML-RARA* by chromosome banding analysis (CBA) (n=113), fluorescence *in situ* hybridization (n=119), and/or RT-PCR (n=123). Sixty-nine patients were male and 54 patients were female, with a median age of 47 years (range: 16-84 years). All 123 samples were analyzed by next generation sequencing (NGS) using a 26-gene panel targeting *ASXL1, CBL, CSF3R, DNMT3A, ETNK1, ETV6, EZH2, FLT3-TKD, GATA2, IDH1, IDH2, KIT, KRAS, NPM1, NRAS, RUNX1, SETBP1, SF3B1, SRSF2, TET2, TP53, U2AF1, WT1* and *ZRSR2*. Since mutations in the genes *ARID1A* and *ARID1B* were recently described in APL,[9] NGS analysis of these genes was also included in this study. NGS was performed using the Fluidigm Access Array microfluidic chip system (Fluidigm, San Francisco, CA) in combination with MiSeq instrument (Illumina, San Diego, CA). Variants which cannot be assigned as mutation or SNP according to current knowledge were excluded from further studies (n=26)(for evaluation of variants see *Online Supplementary Methods*). *FLT3*-ITD was analyzed by gene scan. Relapse samples were additionally screened for mutations within the *PML-RARA* fusion transcript by direct Sanger sequencing as previously published.[6] The patient cohort analyzed in the present study is unique and does not overlap with study cohorts described in previous evaluations. All patients gave their written informed consent for scientific evaluations. The study was approved by the Internal Review Board and adhered to the tenets of the Declaration of Helsinki.

Table 1. Patient demographics and clinical and molecular characteristics of 123 APL patients.

Characteristic	No. of patients	% of total
Age (years)		
Median	47	
Range	16-84	
Gender		
Female	54	44
Male	69	56
Hemoglobin, [g/dL]		
Median	9	
Range	4-16	
WBC count, [x10⁹/L]		
Median	3.0	
Range	0.5-155	
Platelet count, [x10⁹/L]		
Median	32	
Range	1-228	
Cytomorphologic subtype*		
AML M3	67	54
AML M3v	55	45
PML-RARA isoform		
bcr1	63	51
bcr3	60	49
Correlating Mutations		
FLT3-ITD	50	41
FLT3-TKD	15	12
WT1	13	11
NRAS	7	6
KRAS	6	5
ARID1A	3	2
ETV6	3	2
ARID1B	2	2
ASXL1	2	2
DNMT3A	2	2
RUNX1	2	2
CBL	1	1
SF3B1	1	1
TET2	1	1

*according to FAB nomenclature; one case with missing cytomorphology.

According to risk stratification of APL (Sanz index[10]), 37/123 patients (30%) were stratified as high risk, 32/123 (26%) as intermediate risk, and 36/123 (30%) as low risk. Of the cases, 18/123 had no blood counts available for scoring, 67/123 cases (54%) had M3 subtype, and 55/123 (46%) had M3v subtype. In one case, cytomorphology was not available. Regarding treatment, 88/123 cases (72%) received ATRA in combination with chemotherapy, 12/123 cases (10%) received ATRA + ATO, and 10/123 cases (8%) received chemotherapy only. In 13 cases (10%), no detailed information about treatment was available.

Using standard CBA, additional cytogenetic aberrations were observed in 44/113 patients (27%). The most frequent secondary chromosome aberration was trisomy 8 (14/44; 32%) (figure 1A).

Using NGS, 82/123 patients (67%) had at least one mutation in addition to *PML-RARA*, and 22/82 (27%) had

≥2 additional mutations (maximum: four). As anticipated, the most common were mutations in *FLT3*, including 50/123 (41%) with *FLT3*-ITD, and 15/123 (12%) with *FLT3*-TKD. In 43/123 cases (35%), alteration of the *FLT3* gene was the sole genetic aberration. The second most common gene mutated was *WT1* (13/123, 10%). Mutations in other genes (*ARID1A, ARID1B, ASXL1, CBL, DNMT3A, ETV6, KRAS, NRAS, RUNX1, SF3B1* and *TET2*) were found in less than 10% of cases, respectively (figure 1A). Evaluating the detected mutations according to functional pathways, there is a clear predominance of mutations resulting in activated signaling (*FLT3*-ITD and *FLT3*-TKD) and mutations in genes involved in DNA methylation (*WT1*). Mutations of chromatin modifying genes (*KMT2A*-PTD, *ASXL1, EZH2*), mutations of myeloid transcription factors (*RUNX1, ETV6*) and spliceosome genes (*SF3B1, SRSF2* and *U2AF1*), mutations of tumor suppressors (*TP53*) and *NPM1* mutations were rarely or never detected. This is in line with previous studies.[11] Patients with AML M3v subtype had significantly more frequent concomitant mutations (49/55; 89%) compared to patients with AML M3 subtype (32/67; 48%) (*P*<0.001). Furthermore, patients with high risk APL had significantly more frequent concomitant mutations (34/37; 92%) compared to patients with intermediate (17/32; 53%) or low risk APL (21/36; 58%) (*P*<0.001). In detail, high risk APL was highly correlated with the presence of *FLT3*-ITD. Of the cases, 27/30

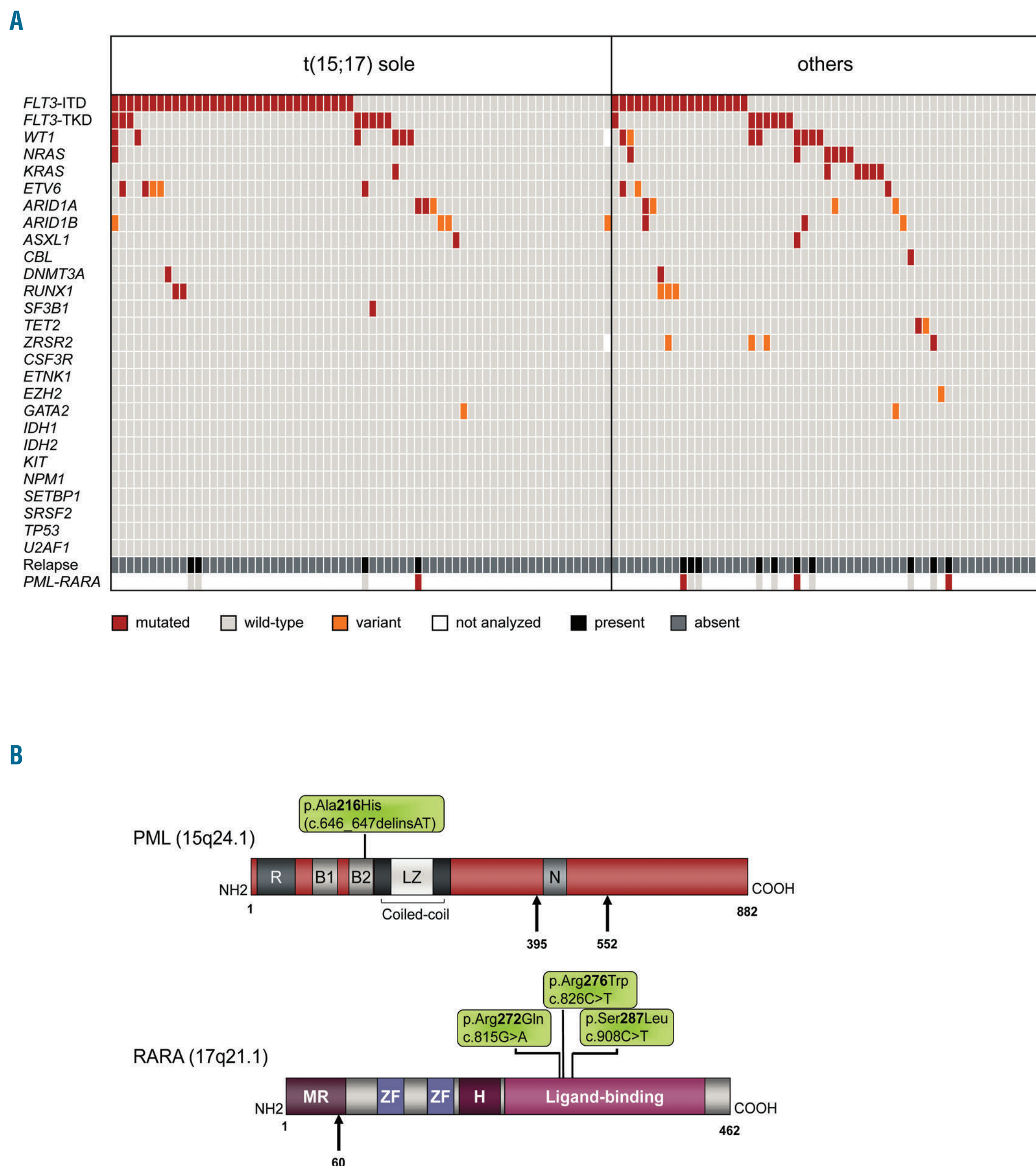

Figure 1. Pattern of molecular and cytogenetic lesions in 123 patients with APL. (A) Alignment of gene mutations for 123 patients. Each column represents one of the 123 analyzed samples. Mutations in the investigated genes are shown by colored bars. Information about relapse and cytogenetics of individual patients is included. *mutations within the PML-RARA fusion transcript of relapsed samples are indicated by red bars, *PML-RARA* fusion transcripts without mutations are indicated by grey bars (ACA: additional chromosomal abnormalities). (B) Distribution of mutations within A) the *PML* region and B) the *RARA* region of PML-RARA in 4/14 patients with relapsed APL. Breakpoints are indicated by black arrows. R: Ring finger; B1: B-box type 1; B2: B-box type2; LZ: Leucin-zipper, ZF: Zinc Finger; N: nuclear localization signal; MR: modulating region; H: Hinge.

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(71%) with high risk APL had concomitant *FLT3*-ITD compared to 16/68 cases (24%) with non-high risk APL (P<0.001). This is in line with previous studies.[12] Comparing the initial mutational pattern of patients who relapsed during the course of disease (n=14) with those staying in molecular remission (n=109) revealed no difference in concomitant molecular mutations. Solely the percentage of patients with concomitant mutations was higher in the relapse group (12/14, 86% *vs.* 70/109, 64%; n.s.). Survival analysis revealed no influence of concomitant mutations in individual genes on prognosis. Also the amount of additional mutations had no prognostic impact (*data not shown*).

In selected cases, we compared changes in the patterns of cytogenetic (n=6/123) and molecular (n=14/123) lesions between initial diagnosis and relapse. Patient characteristics are given in *Online Supplementary Table S1*. Of the 14 cases harboring relapse, 4 cases were in the high risk group, 4 cases in the intermediate risk group, and 4 cases in the low risk group. After considering other potential risk factors, such as age, presence of bcr3 isoform of *PML-RARA*, presence of concomitant *FLT3*-ITD or additional chromosomal aberrations (ACA), all four low risk cases show ACA, one case has additional *FLT3*-ITD, and one case has bcr3-isoform of *PML-RARA*. Two cases had no blood counts available for scoring. Focusing on cytogenetics, in 3/6 cases, the initial karyotype remained, but additional chromosomal aberrations were gained at relapse.

Furthermore, in 10/14 (71%) patients, the initial mutation pattern changed at relapse. Mutations gained at relapse were *DNMT3A, RUNX1* and *WT1* (each 2/14, 14%), followed by *ARID1A, ETV6, FLT3*-TKD and *TP53* (each 1/14, 7%). Loss of mutations was observed in *FLT3*-TKD, *WT1* (each 2/14, 14%) as well as in *FLT3*, *FLT3*-ITD and *NRAS* (each 1/14, 7%) (*Online Supplementary Figure S1*).

In 4/14 relapsed patients (29%), mutations within the *PML-RARA* fusion transcript were detected (figure 1B). These mutations were detectable in none of the cases at initial diagnosis by direct Sanger sequencing (sensitivity of 10%), and thus all were acquired mutations. Missense mutations in the ligand binding domain of *RARA* were detected in 3 relapsed patients (p.Arg272Gln, p.Arg276Trp and p.Ser287Leu). Patients with LBD mutations relapsed after a mean of 21 months (range: 11-40 months), whereas patients lacking LBD mutations (n= 10) relapsed after a mean of 34 months (range 12-65 months). In one relapsed APL patient, a mutation in the B2 domain of *PML* was observed (p.Ala216His) at the time point of second relapse 64 months after initial diagnosis and treatment with ATRA and ATO. No sample of first relapse was available in our laboratory, so it is unclear whether the PML B2 domain mutation was already detectable at this time point.

In conclusion, in our cohort of 123 adult *de novo* APL cases, 67% carry additional molecular mutations, with the most frequent additional molecular mutation being *FLT3*-ITD (41%), followed by *FLT3*-TKD mutations (12%), and mutations in *WT1* (10%). In 57% of relapsed APL, the molecular mutation pattern changed, but no clear driver gene/genes predicting relapse could be identified in the present study. Acquired mutations within the *PML-RARA* fusion transcript were detected in 29% of relapsed APL patients and may account for an impaired response to therapy.

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