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Original contribution

An integrative approach reveals genetic complexity and epigenetic perturbation in acute promyelocytic leukemia: a single institution experience☆

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Summary Acute promyelocytic leukemia (APL) is a distinct type of acute myeloid leukemia that is defined by the presence of the translocations that mostly involve the *RARA* gene. The most frequent translocation is the t(15;17), which fuses the *RARA* gene with the *PML* gene. Previous studies have shown that other cooperative mutations are required for the development of APL after the initiating event of the t(15;17). In this study, we combined cytogenetics with next-generation sequencing and single-nucleotide polymorphism array to study the genetic complexity in 20 APL cases diagnosed in our institution. All but 3 cases had additional genetic aberrations. Our study demonstrated that somatic mutations are frequent events in APL. In addition to the previously reported recurrent cooperative mutations in the *FLT3*, *WT1*, and *RAS* genes, we identified mutations in several epigenetic modifiers, including *TET2*, *EZH2*, and *DNMT3A*, co-occurring with either *FLT3* or *WT1* mutations. Mutations of the *WT1* gene and chromosome 11p copy neutral loss of heterozygosity affecting *WT1* are present in a third of the cases in our series. Two-thirds of APL cases in our study demonstrated a global reduction but focal accumulation of H3K27 methylase (H3K27me) expression, indicating a disorganized chromatin methylation pattern with generally more accessible chromatin status. Our study confirmed genetic complexity of APL and revealed that epigenetic aberrations are more common than previously expected. Although epigenetic modulation is not a common treatment strategy in APL, targeting this pathway may have some clinical utility in refractory or relapsed APL cases.

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1. Introduction

Acute promyelocytic leukemia (APL) is a unique subtype of acute myeloid leukemia that is characterized by a balanced reciprocal translocation between chromosomes 15 and 17, resulting in the fusion transcript *PML-RARA* fusion in most APL cases. Although this translocation is the genetic hallmark of APL, studies from animal models with *PML-RARA* fusion suggested that rearrangement of the *RARA* gene alone is not sufficient for leukemogenesis, and the cooperative mutations may contribute to the development of APL [1]. Recent advent in sequencing technologies allowed for landscape analysis of somatic mutations in APL. Several studies using whole exome or targeted panels identified *FLT3*, *WT1*, and *KRAS* mutations as common recurrent abnormalities in APL [2,3]. To expand our knowledge of genetic alterations that might cooperate along with the *PML-RARA* fusion in the leukemogenic process of APL, we performed comprehensive genetic studies including next-generation sequencing (NGS), fluorescence in situ hybridization (FISH), and/or conventional cytogenetic analyses, as well as CytoScan HD (Thermo Fisher Scientific, Santa Clara, CA) single-nucleotide polymorphism (SNP) microarray analysis on a series of 20 consecutive APL cases in our institution.

2. Materials and methods

2.1. Cases

After approval by the Northwestern University Institutional Review Board, cases of APL were identified by searching the database of the Department of Pathology, Northwestern Memorial Hospital. The diagnoses were confirmed by examining the peripheral blood (PB) smear, bone marrow (BM) aspirate, and core biopsies, and reviewing all ancillary studies including immunohistochemistry, flow cytometric analysis, FISH, and/or cytogenetic studies. Only the cases with available DNA material were included in this study, and the corresponding data sets were assigned study numbers and deidentified.

2.2. Flow cytometric analysis

Flow cytometric analysis was performed on fresh BM samples. Samples were processed using the whole blood lysis. The white blood cell suspension was mixed with the following fluorochrome-conjugated antihuman monoclonal antibodies from Becton Dickinson Biosciences (Franklin Lakes, NJ): CD34-BV421, CD13-PE, CD3-APC-H7, CD5-APC, CD11b-APC, and HLADR-APC-H7. The following antibodies were from Beckman Coulter (Brea, CA): CD45-krome orange, CD117-PC7, MPO-PE, CD33-PC5.5, CD64-FITC, CD2-PC7, CD7-PE, and TDT-FITC. The cells were incubated at room temperature for 20 minutes, washed in phosphate-buffered saline with 0.2% NaAzide, and resuspended in 400 μL of 4% paraformaldehyde. Data acquisition was performed on a standard Canto II 8-color flow cytometer (Becton Dickinson Biosciences) using 405-, 488-, and 635-nm excitation lasers using a standard optical detection configuration. Data analysis was performed using Kaluza software (Beckman Coulter) and FACSDiva software (Becton Dickinson Biosciences).

2.3. Cytogenetic and FISH studies

Cytogenetic analysis for karyotyping was performed on fresh BM aspirate samples after 24- or 48-hour unstimulated BM cultures using standard procedures. The slides were stained using G-bands via trypsin using Giemsa banding technique. The International System for Human Cytogenetic Nomenclature 2016 was followed. In each case, 20 metaphases were analyzed to make karyogram using Cytovision System (Leica Biosystem, Buffalo Grove, IL).

Interphase FISH analysis for the *PML-RARA* fusion was performed using commercially available *PML* (15q22, SpectrumOrange) and *RARA* (17q21, SpectrumGreen) dual-color dual-fusion probes (Vysis/Abbott Molecular, Abbott Park, IL) following standard laboratory procedures. A total of 200 interphase cells were evaluated independently by 2 technologists. An abnormal result is determined if the percentage of the cells with abnormal FISH signal patterns is above the relevant cutoff values at 95% confidence (0.0%).

2.4. Microarray analysis

SNP array analysis was performed using the Affymetrix CytoScan HD arrays (Thermo Fisher Scientific), following the manufacturer's recommendations. The same DNA samples extracted from BM aspirate were used. The CytoScan HD arrays contain 2.67 million probes for comprehensive whole genomic coverage, including 750 000 biallelic SNP probes and 1.9 million nonpolymorphic probes. Briefly, 200 ng of genomic DNA extracted from fresh BM or PB samples was digested with Dsp1, followed by ligation of Dsp1 adaptors and polymerase chain reaction (PCR) amplification. Purified amplicons were fragmented to the range of 25 to 125 bp, labeled, and hybridized to the CytoScan chips for 16 to 18 hours. The chips were scanned, and the data were analyzed with the Chromosome Analysis Suite software from Affymetrix and Nexus software from BioDiscovery (El Segundo, CA). Copy number alterations (CNAs) including gain or loss, and copy neutral loss of heterozygosity (CN-LOH), particularly for those loci encoding genes associated with leukemia and other cancer, were manually analyzed.

2.5. Next-generation sequencing

NGS was performed using the Ion Torrent platform (Life Technologies, Carlsbad, CA) according to the manufacturer's

specifications. Genomic DNA was extracted from BM aspirate and was sequenced with a custom design panel, which is a hybridization- and amplification-based NGS test (Haloplex custom kit; Agilent Technologies, Santa Clara, CA), designed to detect mutations within hotspot or full exon regions of known genes associated with myeloid neoplasms. The targeted regions of these genes included the following: *ASXL1* (exon 12), *BRAF* (exon 15), *CALR* (exon 9), *CBL* (exons 8, and 9), *CSF3R* (exons 14 and 17), *DNMT3A* (exons 4, 8, 13, 15, 16, 18, 19, 20, 22, and 23), *FLT3* (exons 14, 15, and 20), *GATA1* (exon 2), *GATA2* (exons 2-5), *HRAS* (exons 2 and 3), *IDH1* (exon 4), *IDH2* (exon 4), *JAK2* (exons 12 and 14), *JAK3* (exons 11, 13, and 15), *KIT* (exons 9 and 17), *KRAS* (exons 2 and 3), *MPL* (exon 10), *NPM1* (exon 12), *NRAS* (exons 2 and 3), *PTPN11* (exons 3 and 13), *SETBP1* (codons 799-965), *SF3B1* (exons 13-16), *SRSF2* (exon 1), *TET2* (exons 3-11), *TP53* (exons 2-11), *U2AF1* (exons 2 and 6), *WT1* (exons 7 and 9), *CEBPA* (full exons), *ETV6* (all exons), *EZH2* (all exons), *IKZF1* (all exons), *PHF6* (all exons), and *RUNX1* (all exons). The sequencing was performed on an Ion Torrent Personal Genome Machine (PGM) with the Ion PGM Hi-Q sequencing kit according to the manufacturer's instructions (Life Technologies).

The raw data were analyzed using the torrent suite software v5.2.1 (Life Technologies). Human genome build 19 was used as the reference for alignment of the sequence reads. Identification of sequence variants was facilitated via IT Variant Caller Plugin software v5.2.1. (Life Technologies), and coverage of each amplicon was obtained by the Coverage Analysis Plugin software v5.2.1 (Life Technologies). The Integrative Genomics Viewer from the Broad Institute (http://www.broadinstitute.org/igv/) was used to visualize the reads alignment and the presence of variants against the reference genome and the presence of large indels in *FLT3* and *CALR* as well as to confirm variant calls by checking for strand biases and sequencing errors. Variants reported in the COSMIC (Sanger Institute Catalogue of Somatic Mutations in Cancer, http://www.sanger.ac.uk/cosmic) database as confirmed somatic mutations were taken into account; silent or intronic changes were not reported. The sensitivity of this assay (Limit of Detection) is 5% allele frequency. An average sequencing depth of 500× was consistently achieved in >95% of the targets.

Complementary fragment length analysis for FLT3-ITD was also preformed according to previously described method [4], using ITD F-primer FAM-GCAATTTAGG TATGAAAGCCAGC and ITD R-primer HEX-CTTTCA GCATTTTGACGGCAACC. PCR products were visualized by capillary gel electrophoresis on an Applied Biosystems (Waltham, MA) 3130xl Genetic Analyzer.

2.6. Immunohistochemistry

Immunohistochemical stains for *EZH2* and H3K27me were performed on the formalin-fixed, paraffin-embedded BM particle clots. Antigen retrieval and immunohistochemical stains were performed on an automated Leica Bond Max (Leica Microsystems, Buffalo Grove, IL) using the Bond polymer refine detection horseradish peroxidase (Leica Biosystems; DS9800) method. The primary antibodies against *EZH2* and H3K27me3 were obtained from BD Biosciences (clone: 11/*EZH2*, catalog no. 612666, mouse) and Active Motif (clone: MABI 0323, catalog no. 61017, mouse), respectively. Dilution was performed at 1:500 for *EZH2* and 1:800 for H3K27me. Normal BM particle clot tissue was used as control.

3. Results

3.1. Case characteristics

We identified 20 newly diagnosed APL cases, including 18 de novo APL and 2 therapy-related APL. One therapy-related APL (case 5) developed in a patient with a history of diffuse large B-cell lymphoma status after 5 cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone). The other therapy-related APL (case 20) developed in a patient with stage IV ovarian cancer and breast-infiltrating ductal carcinoma who received carboplatin/taxane 8 years before and platinum/taxane a year before APL onset. The 20 patients included 15 women and 5 men, ranging from 26 to 69 years old with a median age of 48 years. The diagnoses were established by reviewing the PB and BM aspirate smears, BM core biopsies, along with the flow cytometric analysis and molecular and cytogenetic confirmation, that is by the presence of t(15;17), PML-RARA fusion by either conventional cytogenetic analysis, and/or FISH or reverse transcriptase PCR. Two cases (case 1 and case 4) showed features of hypogranular variants. At the time of diagnosis, all 20 patients had thrombocytopenia, most (17/20) had variable degree of anemia, and approximately half (11/20) of the patients had neutropenia. The most common presentations were pancytopenia and bleeding. Other symptoms such as fatigue, headache, and weight loss were also reported.

The patient's cohort fell into 3 prognostic groups: 5 with high-risk and 15 with low/intermediate-risk disease based on white blood cell count and platelet count [5]. The induction, consolidation, and maintenance regimens were summarized in Table 1. All patients except one achieved complete remission after initial induction. One patient achieved partial remission after induction and complete remission after consolidation. All other patients, except one who was lost for follow-up, were in complete remission with no relapse at the time of last follow-up. Interestingly, one patient (case 1) developed therapy-related myelodysplastic syndrome with multilineage dysplasia and 5% to 8% blasts. Cytogenetic analysis reported loss of chromosome 7 but no evidence of PML/RARA fusion by cytogenetics, FISH, and reverse transcriptase PCR. The patient received allogeneic stem cell transplant and had no evidence of acute leukemia or therapy-related myelodysplastic syndrome 4 years after the transplant.

Table 1 Clinical features of APL

Case	Sex/ age (y)	Presentation	WBC/ HGB/PLT (k/μL)	Risk stratification	Induction	Consolidation	Maintenance	Response (CR/PR/no)	Disease status relapse (yes/no)
1	F/44	Fatigue, easy bruising, and intermittent headache, (hypogranular variant)	17/7/9	High	ATRA/idarubicin	AIDA 2000	ATRA, 6-MP, and MTX	CR	Developed t-MDS, status after stem cell transplant
2	M/ 42	Epistaxis and ecchymosis	2.4/9.1/20	Low/intermediate	MSKCC phase II ATRA + ATO + idarubicin	ATO + ATRA	None	CR	No
3	F/48	Bruise and thrombocytopenia	9.6/9.4/45	High	ECOG S0535 gemtuzumab/ ATRA/ATO	ATO + IT MTX + cytarabine	ATRA, 6-MP, and MTX	CR	No
4	F/52	Thrombocytopenia (hypogranular variant)	10.5/8.1/ 25	High	ATRA + ATO + idarubicin	Delayed due to very poor mental/physical performance	Refused	CR	Loss to follow-up
5[a]	M/ 62	Bleeding	19/13.6/15	High	ATRA + ATO + idarubicin	ATRO/ATO + IT MTX interchanged with cytarabine	ATRA, 6-MP, and MTX	CR	No
6	M/ 35	Tender mandible mass, fever/chills, cytopenia	0.9/13/1/ 65	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
7	M/ 42	Pancytopenia, weight loss	1.6/13.7/ 125	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
8	F/46	Recurrent nose bleeding	2.2/6.9/13	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
9	F/69	Fatigue, bruising, pancytopenia	1.5/NA/28	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
10	F/31	Easy bruising, menorrhagia, fatigue	7.2/8.9/17	Low/intermediate	ATRA + ATO + idarubicin	ATRA + ATO	ATRA, 6-MP, and MTX	CR	No
11	F/57	Pancytopenia	2.1/8.6/54	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
12	F/54	Pancytopenia	1.2/9.9/ 122	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	Loss to follow-up
13	F/26	Pancytopenia	2.2/9.7/27	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
14	F/60	Epistaxis, pancytopenia	0.6/8.3/13	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
15	F/54	Pancytopenia	1.1/7.1/8	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
16	F/65	Pancytopenia	0.8/7.1/5	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
17	F/56	DIC	15.9/8.1/ 56	High	ATRA + ATO + idarubicin	ATRA + ATO + IT cytarabine	ATRA, 6-MP, and MTX	CR	No
18	F/48	Vaginal bleeding	0.9/8/29	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR	No
19	M/ 48	Gum bleeding, bruising, fatigue, pancytopenia	2/8/13	Low/intermediate	ATRA + ATO + idarubicin	ATRA + ATO	IT methotrexate	CR	No
20[a]	F/62	Pancytopenia	1.4/9.7/47	Low/intermediate	ATRA + ATO	ATRA + ATO	None	CR (minimal disease)	No

Abbreviations: CR, complete response; F, female; HGB, hemoglobin; M, male; PLT, platelet; PR, partial response; t-MDS, therapy-related myelodysplastic syndrome; WBC, white blood cell.
[a]Therapy-related APL DIC, Disseminated intravascular coagulation; ATRA, All-trans retinoic acid; ATO, Arsenic trioxide; MTX, Methotrexate ; 6-MP, 6-Mercaptopurine; IT, Intrathecal; AIDA, All-trans retinoic and idarubicin; MSKCC, Memorial Sloan Kettering Cancer Center; ECOG, Eastern Cooperative Oncology Group..

3.2. Cytogenetic and SNP array studies

Chromosome analysis was performed in 17 of 20 cases in this study. Of these, 11 cases showed the classic t(15;17), 1 (case 2) had a 3-way translocation of t(15;16;17)(q24;p11.2;q21) involving chromosomes 15, 16, and 17, and 4 cases showed (cases 3, 5, 17, and 18) additional cytogenetic aberrations including 2 cases with trisomy 8 as a single-cell abnormality, likely representing clonal evolution. Case 5 had complex karyotype, which may reflect the patient's history of therapy-related APL. Interestingly, case 15 showed an apparently normal karyotype; however, a cryptic insertion of ins(15;17) was identified by the concurrent metaphase FISH analysis. All 3 cases without available karyotypic analysis were all confirmed by concurrent FISH analysis showing the typical PML-RARA fusion (Table 2).

Cytoscan SNP microarray analysis showed copy number aberrations and/or CN-LOH in 11 (55%) of 20 cases tested (Table 2). Cases 3 and 5 showed complex copy number aberrations. Recurrent deletions of chromosome Xp and 6p were found in 4 cases (4/20; 20%). Six cases (30%) had CN-LOH, including 3 with CN-LOH in the short arm of chromosome 11 (11p), which is known to be common in AML, encoding the *WT1* gene. No cases showed copy number aberrations at the breakpoint regions of the t(15;17), indicating that the t(15;17) is balanced at the molecular level. Interestingly, 1 case (case 17) had both *WT1* c.1384C > T (p.R462W) on chromosomal band 11p13 and a stretch of CN-LOH at 11p (Fig. 1), 2 potentially consecutive events, resulting in *WT1* mutations in homozygous state. The *WT1* p.R462W is a well-recognized pathogenic variant, and functional assays have demonstrated that this variant abolishes DNA binding [6], likely resulting in a loss of *WT1*-mediated cellular function. In addition to the therapy-related APL (case 5), case 2 also showed relatively complex changes with multiple deletions and duplications. The remaining cases had a few submicroscopic deletions or duplications; however, no consistent CNAs or stretches of CN-LOH, other than at 11p, were identified. SNP array did not identify additional CNAs in the 2 cases with trisomy 8 as a single-cell abnormality, likely due to the sensitivity limitation (10%) of the assay.

3.3. Next-generation sequencing

Using a hybridization-based targeted NGS panel, we identified somatic mutations in genes most frequently mutated in myeloid neoplasms in 11 (55%) of 20 cases, including 4 cases (20%) with 1 or 2 somatic mutations, and 7 cases (35%) with 3 or more mutations. The most common mutations were identified in the *FLT3* (7/20; 35%) and *WT1* (5/20; 25%) genes. *FLT3* mutations included 6 internal tandem duplication and 2 tyrosine kinase domain mutations (codons 835 and 839). *WT1* mutations were mostly missense mutations affecting nucleotides coding for residues important for interaction with target DNA in exon 9 (4/5; NM_024426.4, GRCh37 assembly, Swiss-Prot: P19544). The remaining change in *WT1* was a missense mutation in exon 7. *ETV6* mutations were present in 3 (15%) of 20 cases, including 2 frameshift and 1 substitution. Three (15%) of 20 cases showed *RAS* mutations including 2 variants in *NRAS* and 1 variant in *KRAS*. Mutations involving epigenetic modifiers (*TET2*, *EZH2*, *DNMT3A*, *SETBP1*) were identified in 4 cases, all co-occurring with either *WT1* or *FLT3* mutations and mostly presented with lower variant allele frequencies, raising the possibility for their presence in subclones (summary of cohort characteristics found in Table 2). The *TP53* mutations were uncommon and present in only 1 case (5%).

3.4. *EZH2* and H3K27me expression

Immunostains for *EZH2* and H3K27me were performed on the BM particle clot sections, as the core biopsies were suboptimal staining due to the decalcification process. Immunostains were also performed as a control using normal BM obtained for lymphoma staging but with no evidence of lymphoma involvement. The hematopoietic cells in normal BMs showed strong expression of *EZH2*. K3K27me, on the other hand, was expressed mostly in the myeloid precursors with intense nuclear staining with decreasing intensity during maturation. The BM particle clots were available in 11 APL cases (cases 1, 2, 3, 7, 8, 10, 11, 13, 15, 16, and 19). In most of the cases, the BM cellularity consisted of predominantly APL blasts with rare erythroid precursors. All cases except case 15 showed high *EZH2* nuclear expression in the blasts. The APL cases demonstrated 2 staining pattern for H3K27me. Approximately a third of the cases (4/11) showed intense nuclear staining of H3K27me, similar to the early myeloid precursors in the normal BM. The remaining 7 cases (cases 1, 3, 8, 10, 11, 13, and 15) demonstrated an abnormal H3K27me staining pattern in the APL blasts with weaker nuclear staining and abnormal accentuation within the nucleus (Fig. 2). Among the 7 cases, 5 had either *WT1* mutations (cases 3 and 10), aberrations involving 11p (cases 11 and 13), or *TET2* mutation (case 1), and had a mixture of high- (3 cases) and intermediate/low-risk diseases (3 cases). Although one could speculate that prior ATRA therapy may impact nuclear H3K27me3 distribution, this does not seem to explain the abnormal H3K27me3 staining in these cases. In the 4 cases with normal H3K27me3 staining pattern, all patients received ATRA therapy for 1 day before BM biopsy. In the 7 cases with abnormal H3K27me3 staining pattern, 3 patients had not received ATRA therapy yet. Two patients had ATRA for 1 day, and the other 2 had ATRA for 2 or 3 days at the time of BM biopsy.

4. Discussion

APL is a unique type of AML with clinical, morphologic, and genetic features that are distinctive from other AMLs. APL is characterized by the t(15;17)/*PML-RARA* fusion or

Table 2 Cytogenetic and molecular features of APL

Case no.	NGS or PCR (*FLT3* ITD)-Allele frequency (%)	Cytogenetics/FISH	Cytoscan microarray
1	*FLT3* ITD *TET2* c.884C>G (p.A295G)—6.5% *RUNX1* c.494_495insGG (p.R166fs*11)—56%	46,XX,t(15;17)(q24;q21)[20]	Normal
2	*NRAS* c.176C>A (p.A59D)—29% *WT1* c.1381_1383delTCC (p.S461del)—44% *WT1* c.1375A>G (p.K459E)—44%	46,XY,t(15;16;17)(q24;p11.2;q21)[20].ish t(15;16;17)(PML+,RARA+;PML+;RARA+)[3]	del(X)(p22.31) (567 kb)
3	*FLT3* ITD *WT1* c.1394A>G (p.H465R)—46% *FLT3* c.2516A>G (p.D839G)—30%	46,XX,dup(2)(q21q33),t(15;17)(q24;q21)[20]	del(2)(q34-qter) (34 Mb); gain of 8q13.3-qter, (75 Mb, minor clone); gain 17q(q24.3-q25.3) (2 Mb); gain Xp22.31-pter, (10.7 Mb, minor clone).
4	*FLT3* ITD *ETV6* c.962_963insT (p.V322fs*5)—41% *SETBP1* c.1168G>A (p.A390T)—50%	46,XX,t(15;17)(q24;q21)[20]	Normal
5	*FLT3* ITD *EZH2* c.2187_2188insT (p.D730*)—1.5% *WT1* c.1393C>T (p.H465Y)—13.6%	46,XY,del(9)(q22q34),t(15;17)(q24;q21)[2]/46,idem,der(1)inv(1)(p36.3q21)t(1;14)(q21;q24),del(8)(p21p23),del(10)(q22q26),der(14)t(1;14)(q21;q24),add(21)(q22),del(22)(q11.2q13)[19]	CN-LOH 3p24.3-p21.3 (27.7 Mb); del(9q12-q22.31); CN-LOH 21q11.21-q22.1 (8.9 Mb)
6	*TP53* c.1125_1126insA (p.S376fs*6)—3.4%	Karyotype ND; nuc ish(PML,RARA)x3(PML con RARAx2)[190/200]	Normal
7	Negative	46,XY,t(15;17)(q24;q21)[18]	Normal
8	Negative	46,XX,t(15;17)(q24;q21)[18]	CN-LOH 12p11.22-pter (27.6 Mb)
9	Negative	Karyotype: ND; nuc ish(PML,RARA)x3(PML con RARAx2)[172/200]	Normal
10	*FLT3* ITD *WT1* c.1109G>T (p.R370L)—51% *ETV6* c.446_447insTC (p.Q150fs*60)—24%	46,XX,t(15;17)(q24;q21)[20]	del(6p)
11	Negative	46,XX,t(15;17)(q24;q21)[20]	CN-LOH 11p
12	*FLT3* ITD *NRAS* c.35G>A (p.G12D)—4.5% *ETV6* c.1171 T>A (p.Y391N)—20%	Karyotype: ND; FISH is t(15;17) positive (per outside report)	Normal
13	Negative	46,XX,t(15;17)(q24;q21)[20]	del(6p12.3) (4.9 Mb); CN-LOH 11p11.12-pter
14	Negative	46,XX,t(15;17)(q24;q21)[20]	Gain Xq25
15	*FLT3* c.2503G>T (p.D835Y)—7.9%	46,XX[20] Ish ins(15;17)(q24;q21q21)(PML+,RARA+;RARA+)[4]	Normal
16	Negative	46,XY,t(15;17)(q24;q21)[20]	del(17q21.2) (RARA) (355 Kb)
17	*DNMT3A* c.941G>A (p.W314*)—44% *WT1* c.1384C>T (p.R462W)—86%	46,XX,t(15;17)(q24;q21)[18]/47,idem,+8[1]/46,idem,t(3;4)(q27;q21)[1]	CN-LOH 11p (43.7 Mb)
18	*KRAS* c.108A>G (p.I36M)—42%	46,XX,t(15;17)(q24;q21)[17]/47,idem,+8[1]/46,XX[2]	Normal
19	Negative	46,XY,t(15;17)(q24;q21)[20]	CN-LOH 8q (12.5 Mb)
20	Negative	Clone 1: 46,XX,t(15;17)(q24.1;q21.2)[2] Normal: 46,XX[4] nuc ish(PML,RARA)x3(PML con RARAx2)[47/200]	Normal

Abbreviation: ND, not done

rarely by other variant translocations involving the *RARA* gene on chromosome 17. Accumulating data suggest that the t(15;17)/*PML-RARA* fusion by itself may not be sufficient for development of APL, and other cooperating genetic events contribute to leukemogenesis. In this study, we used an integrated approach to evaluate the mutational profile, cytogenetic abnormalities, and genome-wide copy number changes or CN-LOH in a series of newly diagnosed APL in our institution. Similar to previous reports, our study has confirmed that somatic mutations are common in APL. Fifty-five percent

of our APL cases had at least one somatic mutation, with approximately a third of cases harbored 3 or more mutations. This is in line with the larger series published by Fasan et al [7], who reported that 67% of APL to have at least one mutation. Similar to several published studies, the most common somatic mutations in our series were in *FLT3* and *WT1*. Interestingly, in addition to the previously reported recurrent cooperative mutations in APL such as *FLT3*, *WT1*, and *RAS*, we also identified mutations in several epigenetic modifiers, including *TET2*, *EZH2*, *DNMT3A*, and *SETBP1*, co-occurring with either *FLT3* or *WT1* mutations. The *TET2* and *EZH2* mutations are present with low allele frequencies, raising the possibility of being a secondary event present in a subclone of the leukemia cells, although the possibility of clonal hematopoiesis cannot be completely rule out. In addition, mutations involving transcription factors, such as *RUNX1* or *ETV6*, were also present. *TP53* mutations were uncommon in our series, and mutations involving pre-mRNA splicing machinery were not detected.

Our study identified recurrent aberrations affecting the *WT1* gene, including 5 cases with mutations and 3 cases with stretches of CN-LOH involving 11p13, a chromosomal location of the *WT1* gene. One case (case 2) had 2 mutations in cis in the *WT1* gene. Another case (case 17) had both *WT1* mutation and a stretch of CN-LOH at 11p13, resulting in a *WT1* mutation in homozygous state, consistent with a high variant allele frequency of that particular mutation.

WT1 mutations are found in approximately 6% to 15% of de novo AMLs [8-10]. The *WT1* gene encodes a zinc finger DNA-binding protein that can act either as a transcriptional activator or tumor repressor. The role of *WT1* in AML is still elusive and somewhat paradoxical. *WT1* is often overexpressed in AML and myelodysplastic syndrome [11,12]; however, most of the *WT1* mutations reported in AML are thought to be loss of function mutations. *WT1* mutations in AML include substitution, insertion, deletion, or frameshift mutations resulted in a truncation form without the functional zinc finger domain [10], suggestive of a loss of function of the *Wt1* protein in those cases. Interestingly, recent studies of global methylation profiles reported that *WT1*-mutant AMLs share similar epigenetic alterations to those in *TET2* and *IDH1/2* mutant AMLs [13]. Additional studies also demonstrated that *WT1* mutations and *TET2* or *IDH1/2* mutations are mutually exclusive in AMLs, and *TET2* binds to transcription start sites and CpG islands of *WT1* target genes [14]. These findings raise the possibility of *WT1* as an epigenetic modifier. Overexpression or mutations of *WT1* may disrupt the *WT1*/*TET2* interaction. More recently, Sinha et al [15] have shown that mutant *WT1* is associated with DNA hypermethylation particularly polycomb repressor complex 2 (PRC2) target genes. In cell line studies, treatment of *WT1* mutated AML cells with PRC2/EZH2 inhibitors promoted myeloid differentiation.

APL seems to have higher frequency of *WT1* mutations than non-APL de novo AML. Madan et al [16] reported that primary APL has distinct mutational profiles with recurrent mutations in *FLT3*, *WT1*, and *RAS*, whereas other common AML mutations were either rare or present at similar frequency to that in other AML entities. *WT1* mutations were reported in 14% to 26% of APL patients [16,17]. Our case series had similar high frequency of *WT1* mutations (5/20; 25%). In addition to the 5 cases with *WT1* mutations clustered in the commonly mutated exon 7 or 9 [18], there are 2 cases with stretches of CN-LOH of 11p, including the segment where the *WT1* gene maps, and one case with *WT1* mutation and a stretch of CN-LOH of 11p, resulting in homozygous mutation in the *WT1* gene. The role of *WT1* in APL is unknown, although prior studies suggesting that *WT1* abnormalities may be

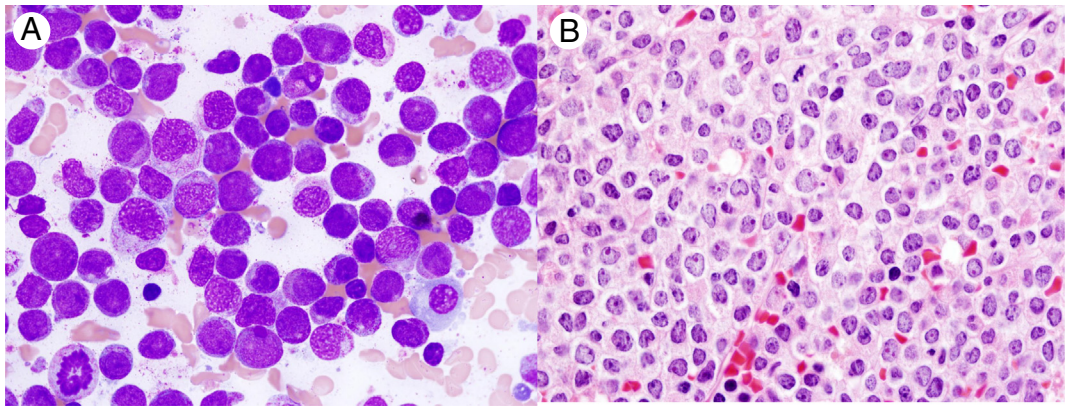
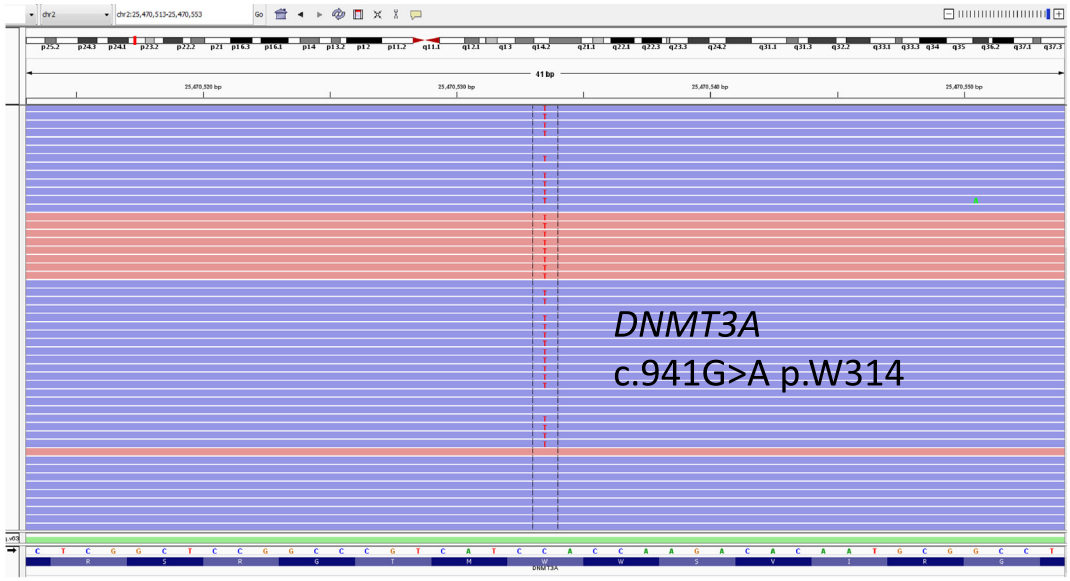


Figure 1 A representative case of APL (case 17) demonstrating *WT1*, *DNMT3A* mutations, and CN-LOH in 11p. A, The BM aspirate smear contains hypercellular particles with sheets of abnormal promyelocytes that are medium to large in size, with indented nuclei, abundant granular cytoplasm, some with bundles of Auer rods (Wright-Giemsa, original magnification ×1000). B, The BM core biopsy is markedly hypercellular and is replaced by sheets of abnormal promyelocytes with indented nuclei, prominent nucleoli, and abundant granular cytoplasm (hematoxylin-eosin, ×600). Cytogenetic analysis reported 46,XX,t(15;17)(q24;q21) and a single metaphase with an additional +8 (not shown). C and D, Molecular studies reported a stopgain mutation involving *DNMT3A* c.941G>A; p.W314* (variant allele frequency, 44%) and a missense mutation in *WT1* c.1384C>T, p.R462W (variant allele frequency, 86%). E, SNP microarray identified a 43.7-Mb CN-LOH in the short arm of chromosome 11 containing the *WT1* locus.

C



D

E

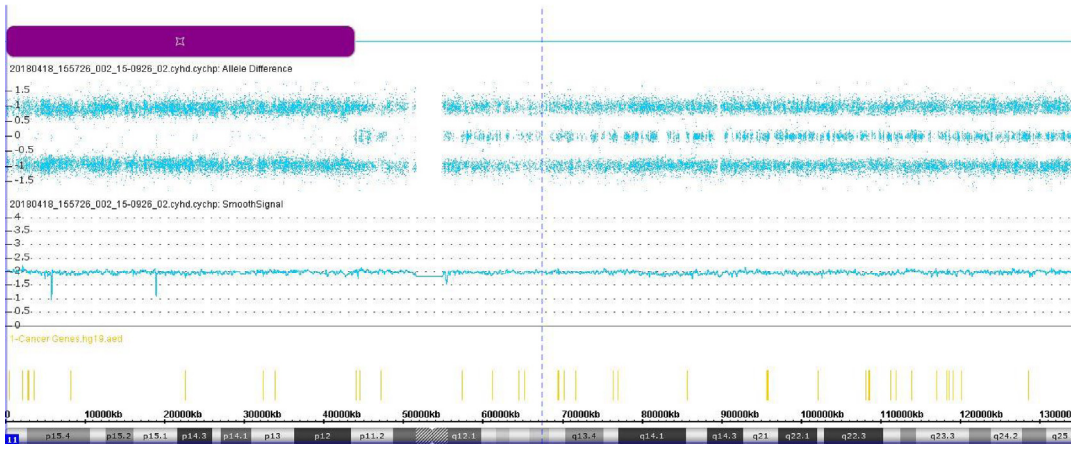


Figure 1. (*continued*).

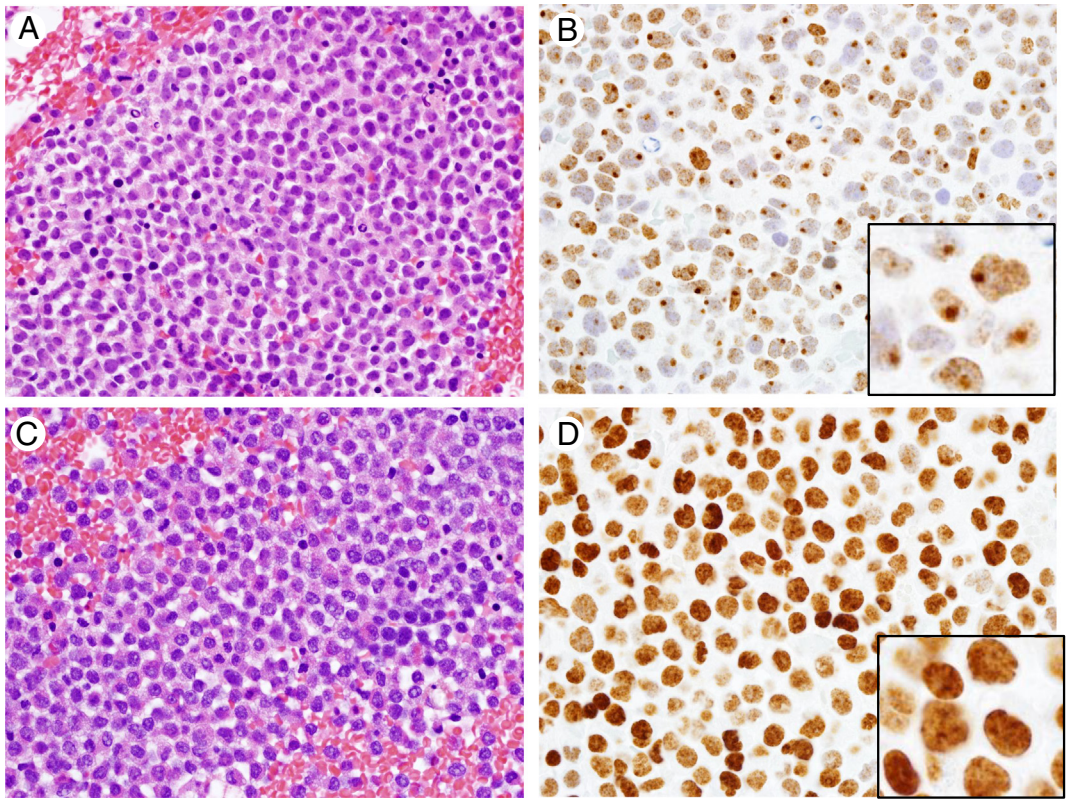


Figure 2 H3K27me expression in APL. A and B, A representative case of APL (case 1) with a global reduction and focal accumulation of H3K27me expression within the nucleus (A: hematoxylin-eosin, original magnification ×600; B: peroxidase, ×600). C and D, A representative case of APL (case 2) with intact strong nuclear expression of H3K27me (A: hematoxylin-eosin, ×600; B: peroxidase, ×600).

important for the development of APL in addition to the t (15;17). Christopher et al [17] used an inducible knockout mouse model to demonstrate that *WT1* loss-of-function mutations can cooperate with *PML-RARA* fusion to drive APL, and interestingly, *PML-RARA* fusion can induce the wild-type *WT1* expression, possibly as a physiological, inhibitory response to oncogenic events. To further explore the role of WT1 mutations or aberrations as epigenetic modifiers in APL, we analyzed the *PRC2/EZH2*-mediated hypermethylation by immunohistochemistry. *EZH2* is the catalytic component of PRC2 complex, a highly conserved H3K27 methyltransferase. Most of our APL cases (except case 15) demonstrated strong *EZH2* expression in the APL blasts. Case 5, which harbors an *EZH2* stop gain mutation, also showed strong *EZH2* staining, probably due to the very low allele frequency (variant allele frequency, 1.5%). In approximately two-thirds of the APL cases, the blasts demonstrated globally reduced methylation of lysine27 on histoneH3 (H3K27me) expression, but with abnormal accumulation of H3K27me within the nucleus. The role of H3K27me has been well studied and implicated as a global epigenetic modulator. Methylation of lysine27 on histoneH3 (H3K27me), particularly at the gene promotor regions, results in gene repression and impairs the balance between cell differentiation and proliferation. Our results demonstrated that a significant number of APL cases had global reduction in H3K27me expression, indicating a generally more accessible chromatin status. Interestingly, despite the global reduction, there was focal accumulation of H3K27me, suggesting perturbation of constitutive heterochromatin formation, which was marked by abnormal distribution of H3K27me. Five of the 8 cases with reduced H3K27me expression had *WT1* mutations, 11p aberrations, or *TET2* mutation. These results suggest an association between reduced/altered H3K27me3 distributions and epigenetic alteration. However, the number of cases in our series is too small to make this association statistically significant.

In summary, by taking an integrated genetic approach, we identified frequent genetic alterations in APL particularly those involving *FLT3* and *WT1* genes. Other than the balanced translocation between chromosomes 15 and 17, APL cases demonstrated less complex chromosomal copy number changes compared with other myeloid neoplasms, except in rare therapy-related APL cases. However, aberrations involving chromosomal region 11p, which also includes the *WT1* locus, seem to be a recurrent abnormality in our series, although studies in a larger cohort of APL cases are needed to confirm this result. A proper investigation of methylation status of 11p region will help to better understand the role of the *WT1* in the development of APL. The finding of a disturbed chromatin organization characterized by reduced or abnormal H3K27me distribution is of particular interesting and maybe of clinical implication. Although epigenetic modulation is not a common

treatment strategy in APL, ours and other recent studies raise the possibility of targeting the epigenetic pathway in the treatment of APL.

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