Hidden abnormalities and novel classification of t(15;17) acute promyelocytic leukemia (APL) based on genomic alterations

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Acute promyelocytic leukemia (APL) is a hematopoietic malignant disease characterized by the chromosomal translocation t(15;17), resulting in the formation of the *PML-RARA* gene. Here, 47 t(15;17) APL samples were analyzed with high-density single-nucleotide polymorphism microarray (50-K and 250-K SNP-chips) using the new algorithm AsCNAR (allele-specific copy-number analysis using anonymous references). Copy-number-neutral loss of heterozygosity (CNN-LOH) was identified at chromosomes 10q (3 cases), 11p (3 cases), and 19q (1 case). Twenty-eight samples (60%) did not have an obvious alteration (normal-copy-number [NC] group). Nineteen samples (40%) showed either one or more genomic abnormalities: 8 samples (17%) had trisomy 8 either with or without an additional duplication, deletion, or CNN-LOH (+8 group); and 11 samples (23%) had genomic abnormalities without trisomy 8 (other abnormalities group). These chromosomal abnormalities were acquired somatic mutations. Interestingly, *FLT3*-ITD mutations (11/47 cases) occurred only in the group with no genomic alteration (NC group). Taken together, these results suggest that the pathway of development of APL differs in each group: *FLT3*-ITD, trisomy 8, and other genomic changes. Here, we showed for the first time hidden abnormalities and novel disease-related genomic changes in t(15;17) APL. (Blood. 2009;113:1741-1748)

Introduction

Acute promyelocytic leukemia (APL) is a hematopoietic malignant disease characterized by the chromosomal translocation t(15;17), resulting in the fusion of the promyelocytic leukemia (*PML*) gene and retinoic acid receptor α (*RARA*) gene (*PML-RARA*).[1,2] The fusion product PML-RARα homodimerizes, binds to DNA, and works as a transcriptional repressor together with corepressors including histone deacetylase.[3] Therefore, reactivation of RARα-dependent transcription is one of the major strategies to treat APL patients. In fact, all-*trans* retinoic acid (ATRA), which binds to RARα and leads to the activation of the transcription factor, is a highly effective compound for the induction of remission of APL patients.[4,5]

Transgenic mice revealed that PML-RARα is necessary but not sufficient for the development of APL.[6,7] APL occurred in these mice only after a long latency (8.5 to 12 months) and penetrance was 15% to 30%.[6,7] These findings suggest that additional genetic mutations are also required for the development of APL. Candidate genes include the tyrosine kinase receptor gene, *FLT3,* and the oncogene, *RAS*. Activating *FLT3* mutations occur in approximately 30% to 35% APL samples[8,9]; and *NRAS* and *KRAS* mutations are found in 4% to 5% and 5% to 10% of APL samples, respectively.[9,10] Interestingly, transgenic mice coexpressing PML-RARα and either FLT3^{W51} (constitutively activated form of murine FLT3), FLT3-ITD, or K-Ras (K12D) develop APL with a short latency and a high penetrance.[11-14]

Comparative genomic hybridization (CGH) is one of the genome-wide screening methods to identify chromosomal abnormalities. However, CGH analysis cannot detect copy-number-neutral loss of heterozygosity (CNN-LOH). Single-nucleotide polymorphism microarray (SNP-chip) is a powerful method to examine genomic alterations including small copy-number changes and/or CNN-LOH in several cancers.[15-17] SNP-chip analysis has been used for chronic lymphocytic leukemia (CLL),[18,19] childhood acute lymphoblastic leukemia (ALL),[20,21] acute myeloid leukemia (AML),[22-26] and AML with normal karyotype (Gorletta et al[27]; Akagi et al[28]).

In the present study, we focused on t(15;17) APL and examined whether additional genomic alterations could be found to subcategorize this disease on the basis of genomic status. The use of CNAG (copy-number analysis for Affymetrix GeneChips; Santa Clara, CA) program[15] and a new algorithm AsCNAR (allele-specific copy-number analysis using anonymous references)[17] provides a highly sensitive technique to detect CNN-LOH as well as copy-number changes in APL.

Methods

Patient samples

DNA from the bone marrow of 47 anonymized cases of t(15;17) APL at diagnosis as well as 7 complete remission bone marrow samples were

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examined. Sample information including the form of PML-RARα (long, short, or variant), sex, age, white blood cell counts (WBCs), blast percentage in the bone marrow, mutational status of the *FLT3* gene, *FLT3*-ITD level, and karyotype are shown in Table 1. This study received IRB approval from the Cedars-Sinai Medical Center and informed consent was obtained in accordance with the Declaration of Helsinki.

High-density SNP-chip analysis

Genomic DNA was isolated from bone marrow samples from t(15;17) APL patients at diagnosis and complete remission, as well as APL cell lines NB4 and PL-21. The DNA was subjected to GeneChip Human mapping 50-K or 250-K microarray (SNP-chip; Affymetrix) as described previously.[15,17] Hybridization, washing, and signal detection were performed on GeneChip Fluidics Station 400 and GeneChip scanner 3000 according to the manufacturer's protocols (Affymetrix). Microarray data were analyzed for determination of both total and allelic-specific copy number (AsCN) using the CNAG program as previously described[15,17] with minor modifications, where the status of copy numbers as well as CNN-LOH at each SNP was inferred using the algorithms based on hidden Markov models.[15,17] For clustering of AML samples with regard to the status of copy-number changes, as well as CNN-LOH, GNAGraph software (Tokyo University, Tokyo, Japan) was used.[21] Size, position, and location of genes were identified with UCSC Genome Browser (http://genome.ucsc.edu/). Germline copy-number changes previously described as copy-number variant at Database of Genomic Variants (http://projects.tcag.ca/variation/) and UCSC Genome Browser were excluded. This microarray data are available for public viewing in the Gene Expression Omnibus (GEO) database[29] under accession number GSE14016.

Determination of SNP sequences in cases of CNN-LOH and *FLT3* mutations

To validate CNN-LOH, 2 SNP sequences (rs10500648 and rs7937815) in chromosome 11p of case no. 39 at diagnosis and complete remission, and 6 SNP sequences (rs10491032, rs363221, rs2099803, rs2104543, rs7075893, and rs7918018) in chromosome 10q of case no. 18 at diagnosis were determined. The genomic region of each SNP site was amplified by genomic polymerase chain reaction (PCR) using specific primers (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), and PCR products were purified and sequenced. For determinations of *FLT3*-TKD and *FLT3*-ITD mutations, genomic PCR was performed as described previously.[30]

Cell culture, mRNA isolation, and quantitative real-time PCR

APL cell lines, NB4 and PL-21, were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA). Total RNA was isolated from these cells and case no. 48 bone marrow sample at diagnosis using RNeasy kit (QIAGEN, Valencia, CA), and 1 μg total RNA was converted into cDNA by reverse transcription with Superscript III (Invitrogen). Gene expression of c-Myc mRNA was quantified with real-time quantitative PCR (iCycler; Bio-Rad, Hercules, CA) using Sybr Green. β-Actin was used as control.

Copy number of chromosome 11p15.4 in case no. 39, 10q24.31 in case no. 18, the *MYC* gene in cases no. 2, no. 18, and no. 65, and the *ERG* gene in case no. 43 were determined by quantitative real-time PCR (Bio-Rad) using Sybr Green. The region on chromosome 2p21 was used as control.[21] Copy number of the 2p21 region was normal as determined by SNP-chip analysis in these samples. The delta threshold cycle value (ΔCt) was calculated from the given Ct value by the formula ΔCt = (Ct sample − Ct control). The fold change was calculated as $2^{-\Delta Ct}$. Primer sequences are shown in Table S2.

Results

SNP-chip analysis of t(15;17) APL samples

We examined the genomic changes in 47 samples of t(15;17) APL using 50-K and 250-K SNP-chip analyses. A total of 28 patients (60%) showed no detectable genomic abnormalities (normal-copy-number [NC] group). In contrast, 19 patients (40%) had one or more genomic abnormalities: 8 patients (17%) had trisomy 8 or duplication on chromosome 8 in the region of the *MYC* gene either with or without other genomic abnormalities (+8 group), and 11 patients (23%) had genomic abnormalities without trisomy 8 (other abnormalities group; Figure 1; Table 2).

One case (case no. 65, 2%) had 4 chromosomally altered regions; 2 cases (4%; no. 39 and no. 58) had 3 chromosomally altered regions; 8 cases (17%; no. 2, no. 50, no. 3, no. 18, no. 13, no. 37, no. 19, and no. 21) had 2 chromosomally altered regions; and 8 cases (17%; no. 38, no. 60, no. 66, no. 20, no. 4, no. 57, no. 43, and no. 52) had 1 chromosomally altered region. Importantly, 6 patients (13%) had CNN-LOH.

Validation of SNP-chip analysis

As proof of principal, we validated SNP-chip results using quantitative genomic real-time PCR (QG RT-PCR) and nucleotide sequencing of SNP sites. Case no. 65 had a duplicated region at chromosome 8, and this region contained the *MYC* gene (Figure 2A). QG RT-PCR showed that levels of the *MYC* copy number were approximately 2-fold higher than normal genomic DNA (Figure 2B). Other copy-number changes including duplication of the *MYC* gene in cases no. 2 and no. 18, and duplication of the *ERG* gene in case no. 43 were also confirmed by QG RT-PCR (data not shown).

Next, we validated CNN-LOH detected by SNP-chip analysis (Figure 3). If a chromosome has LOH, SNP sequences in this region should have homozygosity at diagnosis but heterozygosity at complete remission. Therefore, we examined 2 independent SNP sequences in case no. 39 on chromosome 11p in the CNN-LOH region using diagnosis and complete remission samples. Two SNP sites (rs10500648 and rs7937815) clearly showed a single signal at diagnosis (homozygosity), whereas, the sites showed a double signal at complete remission (heterozygosity; Figure 3B). These results demonstrated that this region had LOH. Next, we determined copy number of the region to exclude the possibility of a hemizygous deletion. As shown in Figure 3C, level of DNA at the 11p15.4 region of case no. 39 at diagnosis was almost the same as level of the complete remission sample, indicating that this region had a normal copy number and the region represented CNN-LOH. CNN-LOH region of case no. 18 was also validated by SNP sequencing and QG RT-PCR (Figure S1). Taken together, these results indicated that SNP-chip analysis clearly reflected real chromosomal abnormalities.

Copy-number changes in t(15;17) APL samples

As shown in Table 2, several copy-number changes were detected by SNP-chip analysis. Deletions were found in 7 cases (15%) including case no. 65 (4q28.1, 1.33 Mb; 7q21.11-q21.12, 1.03 Mb; and 9q12-q31.3, 47.27 Mb), case no. 2 (10q21.2-q21.3, 5.55 Mb), case no. 50 (6p25.1-p24.3, 2.51 Mb), case no. 37 (1q42.2, 0.02 Mb), case no. 19 (12p13.31-p11.22, 22.49 Mb; and 13q14.2-q14.3,

Table 1. Baseline clinical characteristic of 47 t(15;17) APL patients

	PML-RARα					*FLT3*		*FLT3*-ITD	
Group/case no.	Isoform	Sex	Age, y	WBC, $\times 10^9$/L	Blast, %	D835	ITD	Level, %	Chromosome
NC									
5	S	F	43	1.9	88	-	-		46,XX,t(15;17)(q22;q21)
48	S	M	45	1.3	76	-	-		46,XY,t(15;17)(q22;q21)
23	L	F	38	0.4	84	-	-		ND, RT-PCR(+)
28	L	F	36	9.9	87	-	-		ND, RT-PCR(+)
35	L	F	60	1.0	94	-	-		46,XX,t(15;17)(q22;q21)
40	L	M	32	0.9	75	-	-		46,XY,t(15;17)(q22;q21)
12	L	M	54	1.3	97	-	-		46,XY,t(15;17)(q22;q21)
55	L	M	36	2.0	76	-	-		46,XY,t(15;17)(q22;q21)
56	L	M	17	1.9	85	-	-		ND, RT-PCR(+)
24	V	M	32	0.9	77	-	-		46,XY,t(15;17)(q22;q12)
46	V	M	33	2.8	62	-	-		ND, RT-PCR(+)
33	S	M	42	31.4	88	+H	-		46,XY,t(15;17)(q22;q21)
1	L	F	68	1.3	96	+Y	-		46,XX,t(15;17)(q22;q21)
9	L	F	30	1.4	87	+Y	-		46,XX,t(15;17)(q22;q21)
11	L	M	30	4.3	78	+D	-		ND, RT-PCR(+)
53	L	M	36	36.2	96	+E	-		46,XY,t(15;17)(q22;q21)
61	L	M	32	0.3	93	+E	-		ND, RT-PCR(+)
7	S	F	31	120.0	95	-	+	42	46,XX,t(15;17)(q22;q12)
8	S	M	49	4.5	96	-	+	37	46,XY,t(15;17)(q22;q21)
14	S	M	9	28.0	80	-	+	40	46,XY,t(15;17)(q22;q21)
17	S	F	47	51.1	98	-	+	24	46,XX,t(15;17)(q22;q21)
29	S	M	75	1.5	88	-	+	35	ND, RT-PCR(+)
63	S	F	57	9.7	96	-	+	42	46,XX,t(15;17)(q22;q21)
64	S	M	28	24.8	90	-	+	57	46,XY,t(15;17)(q22;q21) [25/26]
									46,XY[1/26]
6	L	F	37	29.5	93	-	+	32	ND, RT-PCR(+)
42	L	F	24	1.2	87	-	+	4	46,XX,t(15;17)(q22;q21)
51	L	M	51	45.4	78	-	+	47	46,XY,t(15;17)(q21;q12)
62	L	F	46	45.0	89	-	+	42	46,XX,t(15;17) (q22;q21)
+8									
65	S	F	58	1.5	87	-	-		46,XX,RT-PCR(+)
38	L	F	22	7.0	78	-	-		47,XX,+8,t(15;17)(q22;q12)
2	S	M	42	2.4	79	-	-		47,XY,+8,t(15;17)(q22;q21)
50	S	F	58	19.8	84	-	-		47,XX,+8,t(15;17)(q22;q21)
60	L	M	22	9.9	90	+H	-		46,XY,t(15;17)(q22;q21) [16/21]
									47XY,+8,t(15;17) (q22;q21) [4/21]
									46,XY [1/21]
3	V	M	33	3.2	67	-	-		47,XY,+8,t(15;17)(p22;q12)
39	L	M	38	1.0	51	-	-		47,XY,+8,t(15;17)(q22;q21)
18	L	M	23	18.1	94	-	-		ND, RT-PCR(+)
Other									
66	S	F	41	15.6	89	+Y	-		46,XX,t(15;17)(q22;q11.2)
20	L	F	51	77.9	88	-	-		46,XX,t(15;17)(q22;q21)
13	L	F	7	1.6	99	+Y	-		ND, RT-PCR(+)
4	S	M	68	0.7	91	+E	-		46,XY,t(15;17)(q22;q21)
37	S	F	26	2.0	75	+Y	-		46,XX,t(15;17)(q22;q12)
57	L	F	30	1.4	87	+Y	-		46,XX,t(15;17)(q22;q21)
19	L	M	51	1.1	73	-	-		46,XY,t(15;17)(q22;q21)
21	L	M	45	4.7	87	-	-		ND, RT-PCR(+)
43	L	M	7	8.6	75	-	-		46,XY,t(15;17)(q22;q21)
52	L	M	54	8.6	82	-	-		46,XY,t(15;17)(q21;q12) [19/20]
									47,XY, +21, t(15;17)(q21;q12) [1/20]
58	L	F	27	6.9	90	-	-		46,XX,t(15;17)(q22;q21)ider(17) (q10)t(15;17) [23/26]
									46,XX [3/26]

Chromosomal translocation of t(15;17) was determined by karyotype studies and/or RT-PCR analysis specific for PML-RARα fusion products. Three types of PML-RARα (long, short, and variant) are shown as L, S, and V, respectively. The recorded number of white blood cells (WBCs) and bone marrow blast percentages were obtained at diagnosis. Mutations of *FLT3* were either tyrosine kinase domain (TKD) at codon 835 or internal tandem repeat (ITD). The APL samples are divided into 3 groups based on SNP-chip analysis: normal-copy-number (NC), trisomy 8 including duplication of the *MYC* gene region (+8), and other abnormalities (other).

0.88 Mb), case no. 21 (7q11.21–q-terminal, 97.03 Mb), and case no. 58 (17p-terminal–p11.2, 21.44 Mb). Of note, deleted region at 12p of case no. 19 and 17p of case no. 58 contained the *ETV6/TEL* and *TP53* genes, respectively.

Duplications were found in 7 cases (15%) including case no. 65 (8q24.13-q24.22, 9.48 Mb), case no. 13 (13q21.1–q-terminal, 57.27 Mb; and 15q22.2–q-terminal, 42.94 Mb), case no. 57 (9q22.32, 0.27 Mb), case no. 37 (18p11.31–p11.23, 0.46 Mb), case

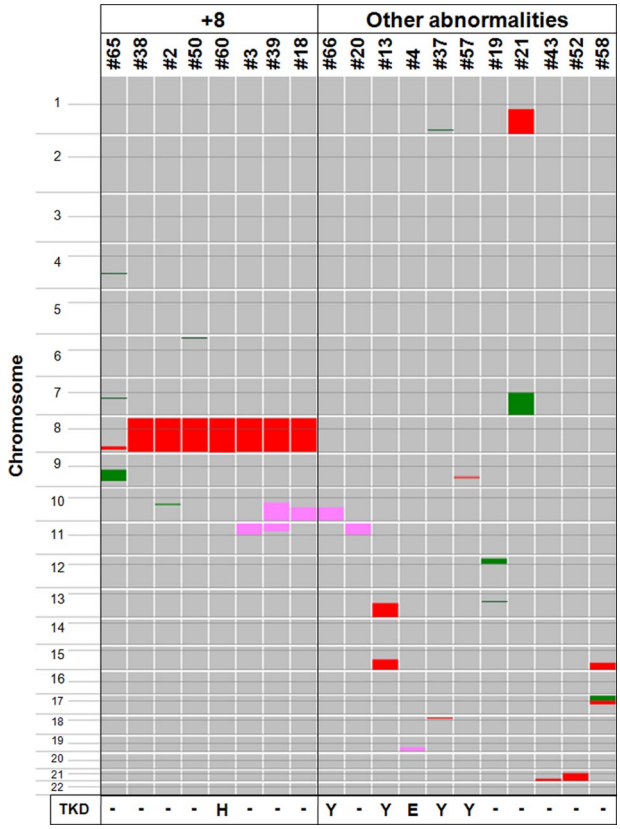

Figure 1. Summary of genomic abnormalities in t(15;17) APL samples. Genomic DNA of 47 t(15;17) APL samples were subjected to SNP-chip analysis, and genomic abnormalities are summarized. Color boxes are used to denote the type and size of abnormalities: pink (copy-number-neutral loss of heterozygosity; CNN-LOH); green (deletion); and red (duplication including trisomy). A total of 28 patients (60%) showed no detectable genomic abnormalities (data not shown). In contrast, 19 patients (40%) had one or more genomic abnormalities: trisomy 8 or duplication of the *MYC* gene region either with or without genomic abnormalities was found in 8 patients (17%, referred as "+8") and 11 patients (23%, referred as "other abnormalities") had genomic abnormalities without trisomy 8. Six patients (13%) had CNN-LOH; and 1 sample in +8 group and 5 samples in other abnormalities group had *FLT3* point mutations that are shown by their amino acid change at codon 835 from D (aspartic acid) to either Y (tyrosine), E (glutamic acid), or H (histidine).

no. 21 (1q21–q-terminal, 102.63 Mb), case no. 43 (21q22.12–q-terminal, 10.69 Mb), and case no. 58 (15q24.1–q-terminal, 27.97 Mb; and 17p11.2–q21.1, 14.05 Mb). The duplicated region at 21q of case no. 43 and at 17p of case no. 58 included the *ERG* and *ERBB2* genes, respectively. Importantly, the duplicated region at 8q of case no. 65 contained the *MYC* gene (Figure 2A). Seven cases had trisomy 8, and one of the candidate genes on chromosome 8 is the *MYC* gene; therefore, we classified case no. 65 within the +8 group.

Of note, the NB4 APL cell line had amplification of the *MYC* gene region (8q24.21), whereas the PL-21 APL cell line showed duplication of the region (Figure S2A). We compared levels of c-Myc mRNA in these cell lines, and found that NB4 cells had approximately 6-fold higher expression of c-Myc than did PL-21 cells (Figure S2B). This result indicated that copy-number change was associated with mRNA levels of the target gene.

CNN-LOH in t(15;17) samples

Several cases had the same chromosomal region involved in CNN-LOH. Chromosome 10q CNN-LOH was found in 3 cases (6%) including case no. 18 (10q22.2–q-terminal), case no. 39 (10q21.1–q-terminal), and case no. 66 (10q22.2–q-terminal; Figure 1; Figure S3A). This region included the tyrosine kinase receptor gene *FGFR2* and the tumor suppressor gene *PTEN* (Table 2). Cases no. 3, no. 20, and no. 39 had CNN-LOH on 11p-terminal–p11.12, 11p-terminal–p11.12, and 11p-terminal–p14.1, respectively; and the common region was 28.7 Mb (Figure 1; Figure S3B) containing the tumor suppressor genes *WT1* and *CDKN1C,* and the oncogene *HRAS* (Table 2). CNN-LOH of 19q13.2–q-terminal (17.3 Mb) occurred in one case (no. 4, Table 2).

Comparison of chromosomal changes between diagnosis and complete remission samples

To assess whether chromosomal alterations detected by SNP-chip analysis were acquired abnormalities, germ-line mutations, or copy-number variants, we compared chromosomal changes between diagnosis and complete remission (CR) samples in the same patients. CR samples of cases no. 2, no. 3, no. 18, no. 19, no. 38, no. 39, and no. 50 were available, and these CR samples were subjected to SNP-chip analysis. As shown in Figure 4, trisomy 8 of case no. 2 and CNN-LOH of case no. 18 at diagnosis were not present in the samples obtained at CR. Other alterations including trisomy 8 (cases no. 3, no. 18, no. 38, no. 39, and no. 50), CNN-LOH at chromosomes 10 (case no. 39) and 11 (cases no. 3 and no. 39), and deletions (cases no. 2, no. 19, and no. 50) also were not present at CR (Figure S4). Taken together, these results showed that chromosomal alterations detected by SNP-chip analysis were acquired somatic changes.

Relationship between genomic abnormalities and FLT3 mutations

Finally, we compared genomic abnormalities and *FLT3* status. Twenty-four samples (51%) had wild-type *FLT3;* whereas 12 samples (26%) had *FLT3*-TKD mutation (aspartic acid at codon 835, D835) and another 11 samples (23%) had *FLT3*-ITD form. Interestingly, all 11 samples with *FLT3*-ITD were found only in the normal-copy-number (NC) group (Tables 1 and 3). One sample in the trisomy 8 group had a *FLT3*-TKD mutation. Samples in the "other abnormalities" group did not have *FLT3*-ITD; and 6 samples in NC group and 5 samples in the "other abnormalities" group had a *FLT3*-TKD mutation. These results suggested that the pathway of development of APL differs in each group; in a mutually exclusive fashion, *FLT3*-ITD, trisomy 8, and unknown factor(s) were involved in each group.

Discussion

Our genome-wide SNP-chip analysis showed that 40% of t(15;17) APL samples had one or more genomic abnormalities including deletions, duplications, and/or CNN-LOH. Since the PML-RARα fusion protein is probably not sufficient to cause APL in murine model systems, the additional genetic changes that we found may be necessary to cause the leukemia.

Our analysis revealed that 6 samples (13%) of t(15;17) APL samples had CNN-LOH. Previously, we analyzed AML with normal karyotype samples and found that 32% of cases had CNN-LOH (Akagi et al[28]); and other investigators also demonstrated CNN-LOH in AML samples at a frequency of 15% to 20%.[22-25,27] Of interest, approximately 40% of relapse AML had CNN-LOH.[26] CNN-LOH in t(15;17) APL is about half as frequent as the other AMLs. Two CNN-LOH regions occurred in multiple samples: chromosomes 10q (58.2 Mb, 3 cases) and 11p (28.7 Mb, 3 cases). Of note, case no. 39 had both 10q and 11p CNN-LOHs, suggesting that 10q and 11p might contain novel APL-related gene(s). CNN-LOH is a genomic abnormality that

Table 2. Chromosomal alterations in t(15;17) APL samples

Group/case no.	Status	Location	Physical localization		Size, Mb	Gene(s) in the region
			Proximal	Distal		
+8						
65	Del	4q28.1	125 190 507	126 521 903	1.33	*KIAA1223*
	Del	7q21.11-q21.12	85 414 972	86 445 002	1.03	*GRM3, KIAA1324L,* and *DMTF1*
	Dup	8q24.13-q24.22	122 607 785	132 092 760	9.48	> 10 genes including *MYC*
	Del	9q12-q31.3	64 207 745	111 479 523	47.27	> 10 genes
38	Tri	Trisomy 8				
2	Tri	Trisomy 8				
	Del	10q21.2-q21.3	62 496 958	68 046 104	5.55	>10 genes
50	Del	6p25.1-p24.3	5 545 437	8 054 930	2.51	> 10 genes
	Tri	Trisomy 8				
60	Tri	Trisomy 8				
3	Tri	Trisomy 8				
	CNN-LOH	11p.ter–p11.12	1 938 894	49 879 899	47.9	> 10 genes including *WT1*, *CDKN1C* and *HRAS*
39	Tri	Trisomy 8				
	CNN-LOH	10q21.1–q-ter	59 576 047	135 228 726	75.7	> 10 genes including *PTEN* and *FGFR2*
	CNN-LOH	11p.ter–p14.1	1 938 894	30 627 880	28.7	> 10 genes including *CDKN1C* and *HRAS*
18	Tri	Trisomy 8				
	CNN-LOH	10q22.2–q-ter	76 995 152	135 228 726	58.2	> 10 genes including *PTEN* and *FGFR2*
Other						
66	CNN-LOH	10q22.2–q-ter	76 289 513	135 295 604	59.0	> 10 genes including *PTEN* and *FGFR2*
20	CNN-LOH	11p.ter–p11.12	1 938 894	49 330 228	47.4	> 10 genes including *WT1*, *CDKN1C* and *HRAS*
13	Dup	13q21.1–q-ter	56 784 440	114 051 465	57.27	> 10 genes
	Dup	15q22.2–q-ter	57 244 668	100 182 183	42.94	> 10 genes including *PML*
4	CNN-LOH	19q13.2–q-ter	46 160 099	63 437 743	17.3	> 10 genes
37	Del	1q42.2	227 843 862	227 867 765	0.02	*EGLN1*
	Dup	18p11.31-p11.23	7 192 739	7 657 575	0.46	*PTPRM*
57	Dup	9q22.32	94 435 025	94 710 006	0.27	*FBP2, FBP1,* and *C9orf3*
19	Del	12p13.31-p11.22	6 755 671	29 248 257	22.49	> 10 genes including *ETV6* and *CDKN1B*
	Del	13q14.2-q14.3	49 630 676	50 510 777	0.88	*FAM10A4, DLEU7, FLJ11712,* and *GUCY1B2*
21	Dup	1q21–q-ter	142 487 224	245 120 412	102.63	> 10 genes
	Del	7q11.21–q-ter	61 522 282	158 554 645	97.03	> 10 genes
43	Dup	21q22.12–q-ter	36 234 195	46 924 583	10.69	>10 genes including *ERG*
52	Tri	Trisomy 21				
58	Dup	15q24.1–q-ter	72 224 840	100 192 115	27.97	> 10 genes
	Del	17p-ter–p11.2	18 901	21 459 693	21.44	> 10 genes including *TP53*
	Dup	17p11.2-q21.1	21 491 135	35 542 587	14.05	> 10 genes including *NF1* and *ERBB2*

Physical localization, size (Mb), and gene(s) at the chromosomal regions were obtained from UCSC Genome Browser. If known gene(s) in the chromosomal regions are less than 10, all gene names are displayed.

Copy number changes as previously described as copy number variant at Database of Genomic Variants (http://projects.tcag.ca/variation/)[29] and UCSC Genome Browser (http://genome.ucsc.edu/)[32] were excluded.

Del indicates deletion; Dup, duplication; Tri, trisomy; ter, terminal; and CNN-LOH, copy-number-neutral loss of heterozygosity.

normally cannot be detected by conventional cytogenetic analysis. These regions usually contain a mutation of a key gene. For example, a constitutively active form of either *JAK2* V617F mutant, *FLT3*-ITD, *AML1/RUNX1* frameshift, and/or mutations of *WT1* and *NPM1* were found in CNN-LOH regions in AML.[22-25] CNN-LOH regions identified in this study contain genes coding for several tyrosine kinase and/or tumor suppressors. Further studies are required to identify the key dysregulated gene(s) in these regions. In addition to CNN-LOH, we also found several copy-number changes that may be sites containing novel disease-related genomic regions in t(15;17) APL. Although we cannot rule-out copy-number variants (CNVs) at several sites, we think it is unlikely. We had 7 genomic DNA at complete remission samples and confirmed for each that the chromosomal changes were only in the leukemia cells. Furthermore, for each of these sites, we interrogated a collated library of CNVs (Database of Genomic Variants and UCSC Genome Browser) to assure that these regions were not known CNVs.

FLT3 is a tyrosine kinase receptor involved in normal hematopoiesis, and mutations of the gene often occur in AML. Incidence of *FLT3*-ITD and *FLT3*-TKD was 23% and 26% in our samples, respectively. Experiments have shown that *FLT3*-ITD and *FLT3*-TKD have differences in their downstream signaling.[33-35] Interestingly, bone marrow transplantation in mice showed that *FLT3*-ITD induced an oligoclonal myeloproliferative disease,[33] whereas *FLT3*-TKD produced an oligoclonal lymphoid disorder with a long latency.[34] Furthermore, only *FLT3*-ITD caused activation of STAT5 and repression of C/EBPα and PU.1.[34,35]

Here, t(15;17) APL samples were divided into 3 groups based on genomic status detected by SNP-chip analysis: normal-copy-number group (NC group, 28 samples); trisomy 8 group (+8 group, 8 samples); and other abnormalities group (11 samples). Notably, our subclassifications did reveal an interesting relationship between genomic status and *FLT3* mutation. Eleven samples of NC group (39% of the NC group samples) had *FLT3*-ITD, whereas no *FLT3*-ITD occurred in samples from the other 2 groups. In contrast, one good candidate gene in the +8 cohort is the oncogene *MYC*. In fact, case no. 65 had duplication localized to 8q24.13-q24.22 that included the

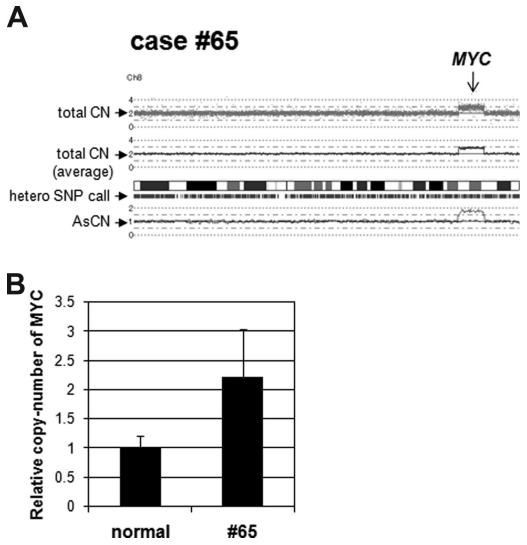

Figure 2. Validation of copy-number change in case no. 65. (A) SNP-chip data of chromosome 8 in case no. 65. Top dots are SNP sites as probes and indicate total copy number (CN). Middle line is an average of the copy number and shows gene dosage. Bars are heterozygous (hetero) SNP calls. Bottom 2 lines show allele-specific copy number (AsCN). (B) Duplication of the *MYC* gene region in case no. 65. Copy number of the *MYC* gene in case no. 65 was compared with normal genomic DNA with quantitative genomic real-time PCR. Level of the copy number was determined as a ratio between the *MYC* gene and the reference genomic region 2p21. Results represent mean of 3 experiments plus or minus SD.

MYC gene. Previous karyotype analysis showed that the PL-21 cell line, which was established from an APL patient, had a polyploid male karyotype with 13q+ chromosome, but a translocation between chromosome 15 and 17 was not identified.[36] NB4 cells are cytogenetically very complex, with a hypotetraploid karyotype and multiple chromosomal alterations.[37,38] Our SNP-chip analysis of NB4 cells also showed ploidy = 3.67, indicating that the karyotype is hypotetraploid. Of interest, NB4 cells have amplification of the *MYC* gene. Importantly, expression of c-Myc mRNA is stimulated by *FLT3*-ITD[39,40]; and AML samples with *FLT3*-ITD have increased expression of c-Myc mRNA compared with normal bone marrow.[41] These data indicate that the *MYC* gene may be dysregulated by either copy-number change or *FLT3*-ITD as a secondary abnormality to enhance the development of APL. Of course, our sample population is small, therefore, additional studies are needed to confirm these findings.

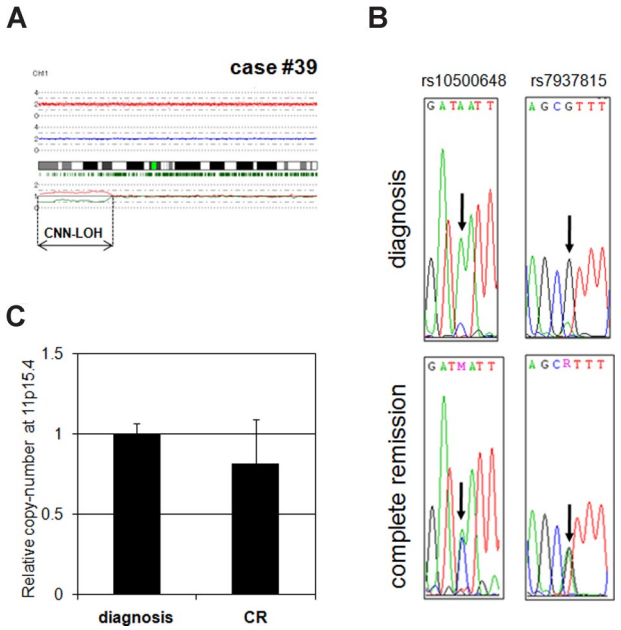

Figure 3. Validation of CNN-LOH in case no. 39. (A) SNP-chip data of chromosome 11 in case no. 39. The samples had CNN-LOH on chromosome 11 (11p-terminal–p14.1, 28.7 Mb). (B) Determination of SNP sequence in 11p CNN-LOH region in case no. 39. 2 SNP sites (rs10500648 and rs7937815) were sequenced. Both SNP sites showed heterozygosity in the complete remission sample, whereas they showed homozygosity in the diagnosis sample. (C) Determination of copy number in the 11p15.4 region. Copy number of 11p15.4 (CNN-LOH region) in case no. 39 at diagnosis was compared with complete remission (CR) sample with quantitative genomic real-time PCR. Levels of the copy number were determined as a ratio between 11p15.4 and the reference genomic DNA, 2p21. Results represent the mean of 3 experiments plus or minus SD.

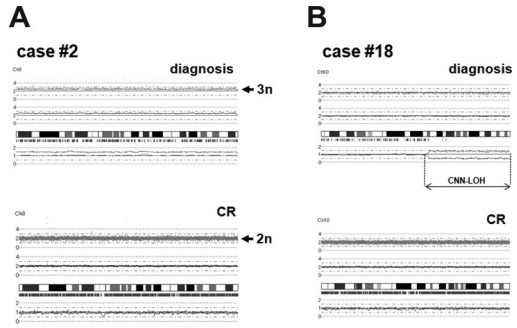

Figure 4. Comparison of chromosomal changes between diagnosis and complete remission samples. (A) Trisomy 8 in case no. 2. Case no. 2 had trisomy 8 at diagnosis, whereas chromosome 8 was 2n at complete remission (CR). (B) CNN-LOH in case no. 18. Case no. 18 had CNN-LOH in chromosome 10 (10q22.2–q-terminal, 58.2 Mb), and the alteration was not present in the matched CR sample.

Our APL cohort "other abnormalities" group has neither *FLT3*-ITD nor trisomy 8, but has several other genomic changes including deletion of *ETV6/TEL* (case no. 19) and duplication of *ERG* (case no. 43). ETV6/TEL is a transcriptional repressor, and approximately 30% of AML patients have loss of expression of ETV6/TEL protein.[42,43] In addition, mutation of the *ETV6/TEL* gene occurs in approximately 2% of AML samples, and these mutants behaved in a dominant-negative fashion.[43] ERG is a member of the ETS family of transcription factors and is a proto-oncogene. Overexpression of ERG predicts a worse outcome in AML with normal karyotype.[44] Taken together, our observed copy-number changes in these regions may be involved in development of APL.

Our findings extend those of Le Beau et al[13] who recently reported elegant models of APL using transgenic mice coexpressing PML-RARα and either BCL2, IL3, activated IL3R, or activated murine FLT3 (FLT3^{W51}). *PML-RARα/BCL2* mice developed leukemia, and these cells had a complex karyotype including trisomy 15 (100% of these mice), where the oncogene *MYC* is located. In contrast, *PML-RARα/FLT3*^{W51} mice develop

Table 3. Relationship between chromosomal abnormality and *FLT3* mutations

	Group			Total (%), 47 (100%)
	NC, 28 (60%)	+8, 8 (17%)	Other, 11 (23%)	
FLT3 WT	11	7	6	24 (51)
FLT3 TKD	6	1	5	12 (26)
FLT3 ITD	11	0	0	11 (23)

Mutational status of the *FLT3* gene is shown.

NC indicates normal-copy-number; +8, trisomy 8 or duplication of the *MYC* gene region; other, other abnormalities; and WT, wild-type.

leukemia, and these cells had normal karyotype except for trisomy of either chromosomes 8 (29%), 10 (43%), or 15 (43%), and monosomy X (86%). These models suggest that different cooperating events are involved in the development of murine APL. Taken together, these findings strongly suggest that the pathway of development of APL differs in each of our cohorts; *FLT3*-ITD, *MYC*, and unknown factor(s) are involved in the development of APL; and these finding should facilitate the screening for novel therapeutic targets in each case.

Further studies in a larger cohort of patients will begin to stratify prognostically the APL patients in relation to the genomic changes of their leukemic cells; and new therapeutic targets, which are involved in the development of APL, should be discovered.

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Authorship

Contribution: T.A. performed research, analyzed the data, and wrote the paper; M.K., S.O., G.Y., and M.S. performed SNP-chip analysis and developed CNAG; N.K., R.O., and C.W.M. assisted in data analysis; L.-Y.S. and D.-C.L. provided APL samples and clinicohematologic data for all APL patients, and performed *FLT3* mutation analysis; and H.P.K. directed the overall study.

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