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

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

Running title: SNP-chip analysis in t(15;17) APL

Tadayuki Akagi[1,*,#,§], Lee-Yung Shih[2,3,#], Motohiro Kato[4], Norihiko Kawamata[1], Go Yamamoto[4,5], Masashi Sanada[4,5], Ryoko Okamoto[1], Carl W. Miller[1], Der-Cherng Liang[6], Seishi Ogawa[4,] and H. Phillip Koeffler[1,**]**

[1]Division of Hematology and Oncology, Cedar-Sinai Medical Center, UCLA School of Medicine; [2]Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Taipei, Taiwan; [3]School of Medicine, Chang Gung University, Taoyuan, Taiwan. [4]Department of Hematology and Oncology and [5]the 21st century COE program, Graduate School of Medicine, University of Tokyo; [6]Division of Pediatric Hematology-Oncology, Mackay Memorial Hospital, Taipei, Taiwan.

Scientific heading; Neoplasia

***Correspondence**: Tadayuki Akagi; Division of Hematology and Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Blvd, Los Angeles, CA90048, USA; Fax: +1-310-423-0225; E-mail: tadayuki@staff.kanazawa-u.ac.jp

#, These two authors have contributed equally in the study and should be considered co-first authors; **, These two authors are co-last authors in the study; §, Current address: Department of Stem Cell Biology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

Abstract

Acute promyelocytic leukemia (APL) is a hematopoietic malignant disease characterized by the chromosomal translocation t(15;17), resulting in the fusion of the *PML-RARA* gene. Here, 47 t(15;17) APL samples were analyzed with high-density single-nucleotide polymorphism microarray (50K and 250K 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 chromosome 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) only occurred 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.

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 of PML-RARα homodimerizes, binds to DNA, and works as a transcriptional repressor together with co-repressors including histone deacetylase.[3] Therefore, re-activation 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 about 30-35% APL samples;[8,9] and *NRAS* and *KRAS* mutations are found in 4-5% and 5-10% of APL samples, respectively.[9,10] Interestingly, transgenic mice co-expressing 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] and Akagi et al. submitted).

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) program[15] and a new algorithm AsCNAR (allele-specific copy-number analysis using anonymous references)[17] provide a highly sensitive technique to detect CNN-LOH, as well as, copy-number changes in APL.

Materials and 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 examined. Sample information including the form of PML-RARα (long, short or variant), gender, age, white blood cell counts (WBC), blast percentage in the bone marrow, mutational status of the *FLT3* gene, *FLT3*-ITD level and karyotype are shown in Table 1.

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, Santa Clara, CA, USA) 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 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.

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 #39 at diagnosis and complete remission, and 6 SNP sequences (rs10491032, rs363221, rs2099803, rs2104543, rs7075893 and rs7918018) in chromosome 10q of case #18 at diagnosis were determined. The genomic region of each SNP site was amplified by genomic PCR using specific primers (Supplemental Table 1), and PCR products were purified and sequenced. For determinations of *FLT3*-TKD and *FLT3*-ITD mutations, genomic PCR was performed as described previously.[28]

Cell culture, mRNA isolation and quantitative real-time PCR

APL cell lines, NB4 and PL-21 were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Atlanta Biologicals, Inc., Lawrenceville, GA, USA). Total RNA was isolated from these cells and case #48 bone marrow sample at diagnosis using RNeasy kit (QIAGEN, Valencia, CA, USA), and 1μg of 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, USA) using Sybr Green. β-actin was used as control.

Copy-number of chromosome 11p15.4 in case #39, 10q24.31 in case #18, the *MYC* gene in cases #2, #18 and #65 and the *ERG* gene in case #43 were determined by quantitative real-time PCR (Bio-Rad) using Sybr Green. 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 Supplemental Table 2.

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 250K 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 and Table 2).

One case (case #65, 2%) had 4 chromosomally altered regions; 2 cases (4%; #39 and #58) had 3 chromosomally altered regions; 8 cases (17%; #2, #50, #3, #18, #13, #37, #19, and #21) had 2 chromosomally altered regions; and 8 cases (17%; #38, #60, #66, #20, #4, #57, #43, and #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 #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 about 2-fold higher than normal genomic DNA (Figure 2B). Other copy-number changes including duplication of the *MYC* gene in cases #2 and #18, and duplication of the *ERG* gene in case #43 were also confirmed by QG RT-PCR (data not shown).

Next, we validate 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 two independent SNP sequences in case #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 were double signal at complete remission (heterozygosity) (Figure 3B). These result 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 #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 #18 was also validated by SNP sequencing and QG RT-PCR (Supplemental Figure 1). 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 #65 (4q28.1, 1.33 Mb; 7q21.11-q21.12, 1.03 Mb; and 9q12-q31.3, 47.27 Mb), case #2 (10q21.2-q21.3, 5.55 Mb), case #50 (6p25.1-p24.3, 2.51 Mb), case #37 (1q42.2, 0.02 Mb), case #19 (12p13.31 - p11.22, 22.49 Mb; and 13q14.2 - q14.3, 0.88 Mb), case #21 (7q11.21 - q-terminal, 97.03 Mb), and case #58 (17p-terminal - p11.2, 21.44 Mb). Of note, deleted region at 12p of case #19 and 17p of case #58 contained the *ETV6/TEL* and *TP53* genes, respectively.

Duplications were found in 7 cases (15%) including case #65 (8q24.13-q24.22, 9.48 Mb), case #13 (13q21.1- q-terminal, 57.27 Mb; and 15q22.2 - q-terminal, 42.94 Mb), case #57 (9q22.32, 0.27 Mb), case #37 (18p11.31 - p11.23, 0.46 Mb), case #21 (1q21 - q-terminal, 102.63 Mb), case #43 (21q22.12 - q-terminal, 10.69 Mb), and case #58 (15q24.1 – q-terminal, 27.97 Mb; and 17p11.2-q21.1, 14.05 Mb). Duplicated region at 21q of case #43 and at 17p of case #58 included the *ERG* and *ERBB2* genes, respectively. Importantly, duplicated region at 8q of case #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 #65 within the +8 group.

Of note, NB4 APL cell line had amplification of the *MYC* gene region (8q24.21), whereas PL-21 APL cell line showed duplication of the region (Supplemental Figure 2A). We compared levels of c-Myc mRNA in these cell lines, and found that NB4 cells had about 6-fold higher expression of c-Myc than did PL-21 cells (Supplemental Figure 2B). 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 #18 (10q22.2-q-terminal), case #39 (10q21.1-q-terminal) and case #66 (10q22.2-q-terminal) (Figure 1 and Supplemental Figure 3A). This region included the tyrosine kinase receptor gene *FGFR2* and the tumor suppressor gene *PTEN* (Table 2). Cases #3, #20 and #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 and

Supplemental Figure 3B) 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 (#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, germline mutations or copy-number variants, we compared chromosomal changes between diagnosis and complete remission (CR) samples in the same patients. CR samples of cases #2, #3, #18, #19, #38, #39 and #50 were available, and these CR samples were subjected to SNP-chip analysis. As shown in Figure 4, trisomy 8 of case #2 and CNN-LOH of case #18 at diagnosis were not present in the samples obtained at CR. Other alterations including trisomy 8 (cases #3, #18, #38, #39 and #50), CNN-LOH at chromosomes 10 (case #39) and 11 (cases #3 and #39), and deletions (cases #2, #19, and #50) also were not present at CR (Supplemental Figure 4). 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 samples had CNN-LOH (Akagi et al, submitted); and other investigators also demonstrated CNN-LOH in AML samples at a frequency of 15 – 20%.[22-25,27] Of interest, about 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 #39 had both 10q and 11p CNN-LOHs, suggesting that 10q and 11p might contain novel APL-related gene(s). Furthermore, 3 APL cases (#66, #20 and #4) had CNN-LOH, and these 3 cases did not contain additional chromosomal alterations. CNN-LOH is a genomic abnormality that 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 contains 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 which 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.[29-31] Interestingly, bone marrow transplantation in mice showed that *FLT3*-ITD induced an oligoclonal myeloproliferative disease;[29] whereas *FLT3*-TKD produced an oligoclonal lymphoid disorder with a long latency.[30] Furthermore, only *FLT3*-ITD caused activation of STAT5 and repression of C/EBPα and PU.1.[30,31]

Here, t(15;17) APL samples were divided into three 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, while 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 #65 had duplication localized to 8q24.13-q24.22 which included the *MYC* gene. Previous karyotype analysis showed that the PL-21 cell line, which was established from an APL patient, had a polyploidy male karyotype with 13q+

chromosome; but a translocation between chromosome 15 and 17 was not identified.[40] NB4 cells are cytogenetically very complex, with a hypotetraploid karyotype and multiple chromosomal alterations.[32,33] 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;[34,35] and AML samples with *FLT3*-ITD have increased expression of c-Myc mRNA compared with normal bone marrow.[36] 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.

Our APL cohort, "other abnormalities" group has neither *FLT3*-ITD nor trisomy 8, but have several other genomic changes including deletion of *ETV6/TEL* (case #19) and duplication of *ERG* (case #43). *ETV6/TEL* is a transcriptional repressor, and about 30% of AML patients have loss of expression of ETV6/TEL protein.[37,38] In addition, mutation of the *ETV6/TEL* gene occurs in about 2% of AML samples; and these mutants behaved in a dominant-negative fashion.[38] *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.[39] 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 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 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.

Acknowledgment

We thank members of our laboratory for helpful discussions. APL cell line PL-21 is kindly provided from Dr. Ikezoe Takayuki (Kochi University). This work was supported by NIH grant (5R01CA026038-30, H.P.K.), Inger Foundation, the Tom Collier Memorial Regatta Foundation, Parker Hughes Fund, as well as, grants NHRI-EX96-9434SI and NSC 91-2314-B-182-032 (Taiwan, L-Y.S) H.P.K. is the holder of the Mark Goodson endowed Chair in Oncology Research and is a member of the Jonsson Cancer Center and the Molecular Biology Institute, UCLA. The study is dedicated to David Golde, a mentor and friend.

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 data analysis; L-Y.S and D-C.L provided APL samples and clinicohematologic data for all APL patients, and performed *FLT3* mutation analysis; H.P.K. directed the overall study. T.A. and L-Y.S are contributed equally in the study. S.O. and H.P.K is co-last author.

Conflict-of-interest statements: The authors declare no competing financial interests.

Correspondence: Tadayuki Akagi; Division of Hematology and Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Blvd, Los Angeles, CA90048, USA; Fax: +1-310-423-0225; E-mail: tadayuki@staff.kanazawa-u.ac.jp

References

1. de Thé H, Chomienne C, Lanotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a novel transcribed locus. Nature. 1990; 347:558-561.
2. de Thé H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RAR-alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell. 1991; 66: 675-684.
3. Grignani F, De Matteis S, Nervi C, et al. Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature. 1998; 391:815-818.
4. Warrell RP Jr, de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. N Engl J Med. 1993; 329: 177-189.
5. Melnick A, Licht JD. Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood. 1999; 93: 3167-3215.
6. Grisolano JL, Wesselschmidt RL, Pelicci PG, Ley TJ. Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. Blood. 1997; 89: 376-387.
7. Brown D, Kogan S, Lagasse E, et al. A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. Proc Natl Acad Sci USA. 1997; 94: 2551-2556.
8. Reilly JT. Class III receptor tyrosine kinases: role in leukaemogenesis. Br J Haematol. 2002; 116: 744-757.
9. Callens C, Chevret S, Cayuela JM, et al. Prognostic implication of FLT3 and Ras

gene mutations in patients with acute promyelocytic leukemia (APL): a retrospective study from the European APL Group. Leukemia. 2005; 19: 1153-1160.

10. Bowen DT, Frew ME, Hills R, et al. RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years. Blood. 2005;106: 2113-2119.
11. Sohal J, Phan VT, Chan PV, et al. A model of APL with FLT3 mutation is responsive to retinoic acid and a receptor tyrosine kinase inhibitor, SU11657. Blood. 2003; 101:3188-3197.
12. Chan IT, Kutok JL, Williams IR, et al. Oncogenic K-ras cooperates with PML-RAR alpha to induce an acute promyelocytic leukemia-like disease. Blood. 2006; 108: 1708-1715.
13. Le Beau MM, Bitts S, Davis EM, Kogan SC. Recurring chromosomal abnormalities in leukemia in PML-RARA transgenic mice parallel human acute promyelocytic leukemia. Blood. 2002; 99: 2985-2991.
14. Kelly LM, Kutok JL, Williams IR, et al. PML/RARalpha and FLT3-ITD induce an APL-like disease in a mouse model. Proc Natl Acad Sci USA. 2002;99: 8283-8288.
15. Nannya Y, Sanada M, Nakazaki K et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res. 2005; 65:6071-6079.
16. Engle LJ, Simpson CL, Landers JE. Using high-throughput SNP technologies to study cancer. Oncogene. 2006; 25:1594-1601.
17. Yamamoto G, Nannya Y, Kato M, et al. Highly sensitive method for genomewide

detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. Am J Hum Genet. 2007; 81:114-126.

18. Pfeifer D, Pantic M, Skatulla I, et al. Genome-wide analysis of DNA copy number changes and LOH in CLL using high-density SNP arrays. Blood. 2007; 109:1202-1210.
19. Lehmann S, Ogawa S, Raynaud SD et al. Molecular allelokaryotyping of early-stage, untreated chronic lymphocytic leukemia. Cancer. 2008; 112:1296-305.
20. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature. 2007; 446:758-764.
21. Kawamata N, Ogawa S, Zimmermann M, et al. Molecular allelokaryotyping of pediatric acute lymphoblastic leukemias by high-resolution single nucleotide polymorphism oligonucleotide genomic microarray. Blood. 2008;111: 776-84.
22. Raghavan M, Lillington DM, Skoulakis S, et al. Genome-wide single nucleotide polymorphism analysis reveals frequent partial uniparental disomy due to somatic recombination in acute myeloid leukemias. Cancer Res. 2005; 65:375-378.
23. Fitzgibbon J, Smith LL, Raghavan M, et al. Association between acquired uniparental disomy and homozygous gene mutation in acute myeloid leukemias. Cancer Res. 2005; 65:9152-9154.
24. Serrano E, Carnicer MJ, Orantes V, Estivill C, Lasa A, Brunet S, Aventin AM, Sierra J, Nomdedeu JF. Uniparental disomy may be associated with microsatellite instability in acute myeloid leukemia (AML) with a normal karyotype. Leuk Lymphoma. 2008; 49: 1178 –1183.
25. Gupta M, Raghavan M, Gale RE, et al. Novel regions of acquired uniparental

disomy discovered in acute myeloid leukemia. Genes Chromosomes Cancer. 2008. in press.

26. Raghavan M, Smith LL, Lillington DM, et al. Segmental uniparental disomy is a commonly acquired genetic event in relapsed acute myeloid leukemia. Blood. 2008. in press.
27. Gorletta TA, Gasparini P, D'Elios MM, Trubia M, Pelicci PG, Di Fiore PP. Frequent loss of heterozygosity without loss of genetic material in acute myeloid leukemia with a normal karyotype. Genes Chromosomes Cancer. 2005;44: 334-337.
28. Shih LY, Kuo MC, Liang DC, et al. Internal tandem duplication and Asp835 mutations of the FMS-like tyrosine kinase 3 (FLT3) gene in acute promyelocytic leukemia. Cancer. 2003; 98: 1206-1216.
29. Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG.. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood. 2002; 99: 310-318.
30. Grundler R, Miething C, Thiede C, Peschel C, Duyster J. FLT3-ITD and tyrosine kinase domain mutants induce 2 distinct phenotypes in a murine bone marrow transplantation model. Blood. 2005; 105: 4792-4799.
31. Choudhary C, Schwable J, Brandts C, et al. AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. Blood. 2005; 106: 265-273.
32. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute

promyelocytic leukemia (M3). Blood. 1991;77: 1080-1086.

33. Mozziconacci MJ, Rosenauer A, Restouin A, et al. Molecular cytogenetics of the acute promyelocytic leukemia-derived cell line NB4 and of four all-trans retinoic acid-resistant subclones. Genes Chromosomes Cancer. 2002; 35:261-270.
34. Tickenbrock L, Schwäble J, Wiedehage M, et al. Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction. Blood. 2005;105: 3699-3706.
35. Li L, Piloto O, Kim KT, et al. FLT3/ITD expression increases expansion, survival and entry into cell cycle of human haematopoietic stem/progenitor cells. Br J Haematol. 2007;137: 64-75.
36. Kim KT, Baird K, Davis S, et al. Constitutive Fms-like tyrosine kinase 3 activation results in specific changes in gene expression in myeloid leukaemic cells. Br J Haematol. 2007;138: 603-615.
37. Hernandez JM, Gonzalez MB, Garcia JL, et al. Two cases of myeloid disorders and a t(8;12)(q12;p13). Haematologica. 2000; 85:31-34.
38. Barjesteh van Waalwijk van Doorn-Khosrovani S, Spensberger D, de Knegt Y, Tang M, Löwenberg B, Delwel R. Somatic heterozygous mutations in ETV6 (TEL) and frequent absence of ETV6 protein in acute myeloid leukemia. Oncogene. 2005; 24:4129-4137.
39. Marcucci G, Baldus CD, Ruppert AS, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. J Clin Oncol. 2005; 23: 9234-9242.
40. Kubonishi I, Machida K, Niiya K, et al. Establishment of a new peroxidase-positive

human myeloid cell line, PL-21. Blood. 1984; 63:254-259.

Figure legends

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 denotes 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 one sample in +8 group and 5 samples in other abnormalities group had *FLT3* point mutations which are shown by their amino acid change at codon 835 from D (aspartic acid) to either Y (tyrosine), E (glutamic acid) or H (histidine).

Figure 2. Validation of copy-number change in case #65. (A) SNP-chip data of chromosome 8 in case #65. Red dots are SNP sites as probes and indicate total copy-number (CN). Blue line is an average of the copy-number and shows gene dosage. Green bars are heterozygous (hetero) SNP calls. Red and green lines show allele-specific copy-number (AsCN). (B) Duplication of the *MYC* gene region in case #65. Copy-number of the *MYC* gene in case #65 was compared to 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 ± SD.

Figure 3. Validation of CNN-LOH in case #39. (A) SNP-chip data of chromosome 11 in case #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 #39. Two 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 #39 at diagnosis was compared to 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 ± SD.

Figure 4. Comparison of chromosomal changes between diagnosis and complete remission samples. (A) Trisomy 8 in case #2. Case #2 had trisomy 8 at diagnosis; whereas chromosome 8 was 2n at complete remission (CR). (B) CNN-LOH in case #18. Case #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.

Legends for Supplemental Figures

Supplemental Figure 1. Validation of CNN-LOH in case #18. (A) Determination of SNP sequence in 10q CNN-LOH region. Six independent SNP sites (rs10491032, rs363221, rs2099803, rs2104543, rs7075893 and rs7918018) were sequenced. All 6 SNP sites showed only a single nucleotide; no SNP sites showed heterozygosity consistent with CNN-LOH. (B) Determination of copy-number in the 10q region. Copy-number of 10q24.31 (CNN-LOH region) in case #18 was compared to DNA from normal white blood cells using quantitative genomic real-time PCR. Levels of the copy-number were determined as a ratio between 10q24.31 and the reference genomic DNA, 2p21. Results represent the mean of 3 experiments ± SD.

Supplemental Figure 2. Expression of c-Myc mRNA in NB4 and PL-21 APL cell lines. (A) SNP-chip results of NB4 and PL-21 APL cell lines, as well as, bone marrow sample of case #48. NB4 cells had amplification of 8q24.21 region, and this region included the *MYC* gene. On the other hand, the region had a duplication in the PL-21 cells and had a normal copy-number in case #48. (B) Expression of c-Myc mRNA. Levels of c-Myc mRNA in cells from NB4, PL-21, and case #48 were determined by quantitative real-time PCR. Results represent the mean of 3 experiments ± SD.

Supplemental Figure 3. Chromosomal region of CNN-LOH in t(15;17) APL samples. When one allele copy-number (red-line) increases and the other allele copy-number (green-line) decreases, the region has loss of heterozygosity; and if the

region has normal-copy-number, the sample has copy-number-neutral loss of heterozygosity (CNN-LOH). (A) Chromosome 10q CNN-LOH was found in three cases including #18 (10q22.2 - q-terminal, 58.2 Mb), #39 (10q21.1 - q-terminal, 75.7 Mb) and #66 (10q22.2 - q-terminal, 59.0 Mb). (B) Chromosome 11p CNN-LOH was noted in three cases including #3 (11p-terminal - p11.12, 47.9 Mb), #20 (11p-terminal - p11.12, 47.4 Mb), and #39 (11p-terminal - p14.1, 28.7 Mb). Chromosomal and physical localization, size, as well as, genes in the regions are described in Table 2.

Supplemental Figure 4. Comparison of chromosomal changes between diagnosis and complete remission samples. Chromosomal status of DNA of matched complete remission samples of cases #2, #3, #18, #19, #38, #39 and #50 were compared to samples obtained at diagnosis. Chromosomal alterations including copy-number changes and CNN-LOH were not present in the complete remission samples. Pink, green, and red boxes indicate CNN-LOH (copy-number-neutral loss of heterozygosity), deletion, and duplication including trisomy, respectively. Abbreviations: D, diagnosis; C, complete remission.

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

Group	Case #	PML-RARα isoform	Sex	Age	WBC (x 10^6/L)	Blast (%)	*FLT3* D835	*FLT3* ITD	*FLT3*-ITD Level (%)	Chromosome
NC	5	S	F	43	1900	88	-	-		46,XX,t(15;17)(q22;q21)
	48	S	M	45	1300	76	-	-		46,XY,t(15;17)(q22;q21)
	23	L	F	38	400	84	-	-		ND, RT-PCR(+)
	28	L	F	36	9900	87	-	-		ND, RT-PCR(+)
	35	L	F	60	1000	94	-	-		46,XX,t(15;17)(q22;q21)
	40	L	M	32	900	75	-	-		46,XY,t(15;17)(q22;q21)
	12	L	M	54	1300	97	-	-		46,XY,t(15;17)(q22;q21)
	55	L	M	36	2000	76	-	-		46,XY,t(15;17)(q22;q21)
	56	L	M	17	1900	85	-	-		ND, RT-PCR(+)
	24	V	M	32	900	77	-	-		46,XY,t(15;17)(q22;q12)
	46	V	M	33	2800	62	-	-		ND, RT-PCR(+)
	33	S	M	42	31400	88	+H	-		46,XY,t(15;17)(q22;q21)
	1	L	F	68	1300	96	+Y	-		46,XX,t(15;17)(q22;q21)
	9	L	F	30	1400	87	+Y	-		46,XX,t(15;17)(q22;q21)
	11	L	M	30	4300	78	+D	-		ND, RT-PCR(+)
	53	L	M	36	36200	96	+E	-		46,XY,t(15;17)(q22;q21)
	61	L	M	32	300	93	+E	-		ND, RT-PCR(+)
	7	S	F	31	120000	95	-	+	42	46,XX,t(15;17)(q22;q12)
	8	S	M	49	4490	96	-	+	37	46,XY,t(15;17)(q22;q21)

	14	S	M	9	27980	80	-	+	40	46,XY,t(15;17)(q22;q21)
	17	S	F	47	51100	98	-	+	24	46,XX,t(15;17)(q22;q21)
	29	S	M	75	1500	88	-	+	35	ND, RT-PCR(+)
	63	S	F	57	9700	96	-	+	42	46,XX,t(15;17)(q22;q21)
	64	S	M	28	24800	90	-	+	57	46,XY,t(15;17)(q22;q21) [25/26]
										46,XY[1/26]
	6	L	F	37	29500	93	-	+	32	ND, RT-PCR(+)
	42	L	F	24	1200	87	-	+	4	46,XX,t(15;17)(q22;q21)
	51	L	M	51	45400	78	-	+	47	46,XY,t(15;17)(q21;q12)
	62	L	F	46	45000	89	-	+	42	46,XX,t(15;17) (q22;q21)
+8	65	S	F	58	1500	87	-	-		46,XX,RT-PCR(+)
	38	L	F	22	7000	78	-	-		47,XX,+8,t(15;17)(q22;q12)
	2	S	M	42	2400	79	-	-		47,XY,+8,t(15;17)(q22;q21)
	50	S	F	58	19800	84	-	-		47,XX,+8,t(15;17)(q22;q21)
	60	L	M	22	9900	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	3200	67	-	-		47,XY,+8,t(15;17)(p22;q12)
	39	L	M	38	1000	51	-	-		47,XY,+8,t(15;17)(q22;q21)
	18	L	M	23	18100	94	-	-		ND, RT-PCR(+)
Other	66	S	F	41	15600	89	+Y	-		46,XX,t(15;17)(q22;q11.2)
	20	L	F	51	77900	88	-	-		46,XX,t(15;17)(q22;q21)
	13	L	F	7	1600	99	+Y	-		ND, RT-PCR(+)

4	S	M	68	700	91	+E	-	46,XY,t(15;17)(q22;q21)
37	S	F	26	2000	75	+Y	-	46,XX,t(15;17)(q22;q12)
57	L	F	30	1400	87	+Y	-	46,XX,t(15;17)(q22;q21)
19	L	M	51	1100	73	-	-	46,XY,t(15;17)(q22;q21)
21	L	M	45	4690	87	-	-	ND, RT-PCR(+)
43	L	M	7	8640	75	-	-	46,XY,t(15;17)(q22;q21)
52	L	M	54	8600	82	-	-	46,XY,t(15;17)(q21;q12) [19/20]
58	L	F	27	6900	90	-	-	47,XY, +21, t(15;17)(q21;q12) [1/20] 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 (WBC) and bone marrow blast percents 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 three groups based on SNP-chip analysis: normal-copy-number (CN), trisomy 8 including duplication of the *MYC* gene region (+8), and other abnormalities (other).

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

Group	Case #	Status	Location	Physical localization		Size	Gene(s) in the region
				Proximal	Distal	(Mb)	
+8	65	Del	4q28.1	125190507	126521903	1.33	*KIAA1223*
		Del	7q21.11-q21.12	85414972	86445002	1.03	*GRM3*, *KIAA1324L* , and *DMTF1*
		Dup	8q24.13-q24.22	122607785	132092760	9.48	> 10 genes including *MYC*
		Del	9q12-q31.3	64207745	111479523	47.27	> 10 genes
	38	Tri	Trisomy 8	-	-	-	-
	2	Tri	Trisomy 8	-	-	-	-
		Del	10q21.2-q21.3	62496958	68046104	5.55	>10 genes
	50	Del	6p25.1-p24.3	5545437	8054930	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.	56784440	114051465	57.27	> 10 genes

	Dup	15q22.2 - q-ter.	57244668	100182183	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	227843862	227867765	0.02	*EGLN1*
	Dup	18p11.31 - p11.23	7,192,739	7,657,575	0.46	*PTPRM*
57	Dup	9q22.32	94435025	94710006	0.27	*FBP2, FBP1*, and *C9orf3*
19	Del	12p13.31 - p11.22	6755671	29248257	22.49	> 10 genes including *ETV6* and *CDKN1B*
	Del	13q14.2 - q14.3	49630676	50510777	0.88	*FAM10A4, DLEU7, FLJ11712*, and *GUCY1B2*
21	Dup	1q21 - q-ter.	142487224	245120412	102.63	> 10 genes
	Del	7q11.21 - q-ter.	61522282	158554645	97.03	> 10 genes
43	Dup	21q22.12 - q-ter.	36234195	46924583	10.69	>10 genes including *ERG*
52	Tri	Trisomy 21	-	-	-	-
58	Dup	15q24.1 – q-ter.	72224840	100192115	27.97	> 10 genes
	Del	17p-ter. - p11.2	18901	21459693	21.44	> 10 genes including *TP53*
	Dup	17p11.2 - q21.1	21491135	35542587	14.05	> 10 genes including *NF1* and *ERBB2*

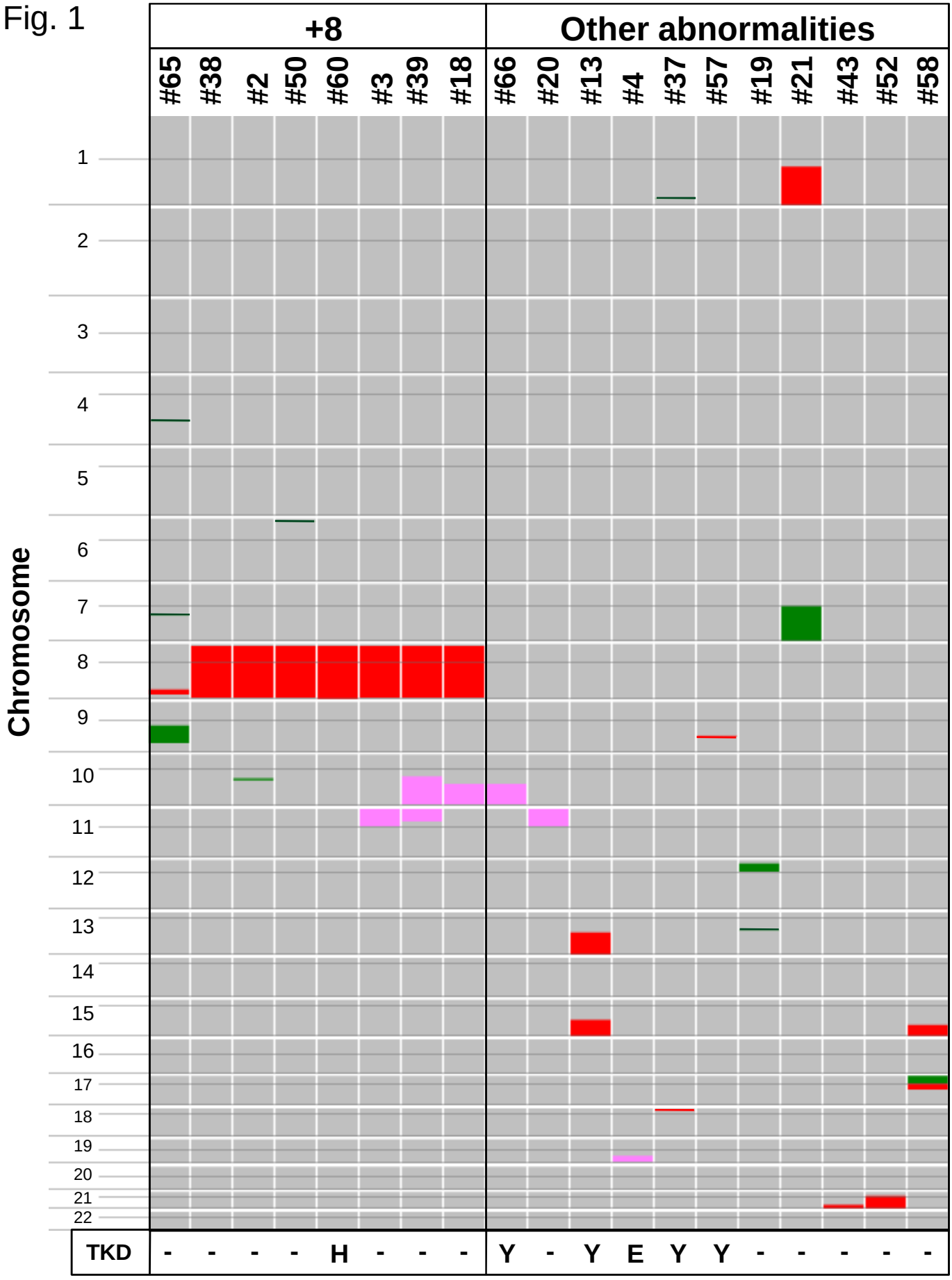
Physical localization, size (Mb), and gene(s) at the chromosomal regions were obtained from UCSC Genome Browser. If known gene(s) in the chromosomal regions are less than 10, all gene names are displayed. Abbreviations: Del, deletion; Dup, duplication; Tri, trisomy; ter, terminal; CNN-LOH, copy-number-neutral loss of heterozygosity. Copy number changes as previously described as copy number variant at Database of Genomic Variants <http://projects.tcag.ca/variation/> and UCSC Genome Browser <http://genome.ucsc.edu/> were excluded.

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

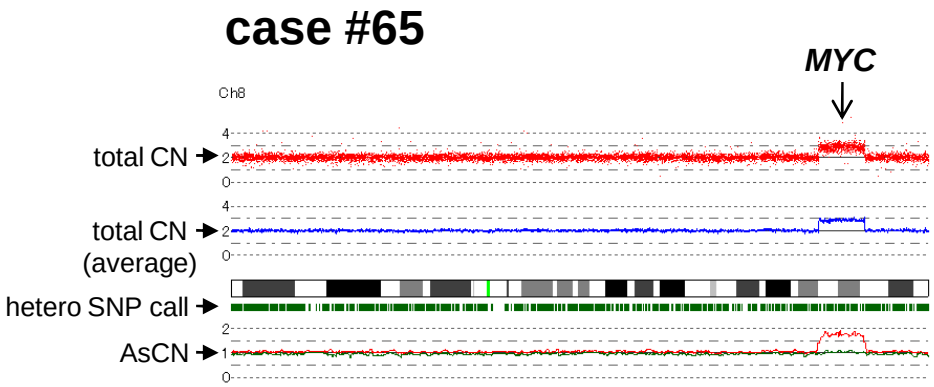
	Group			
	NC	+8	other	Total
	28 (60%)	8 (17%)	11 (23%)	47 (100%)
FLT3 WT	11	7	6	24 (51%)
FLT3 TKD	6	1	5	12 (26%)
FLT3 ITD	11	0	0	11 (23%)

Mutational status of the *FLT3* gene is shown. Abbreviations: NC, normal-copy-number; +8, trisomy 8 or duplication of the *MYC* gene region; other, other abnormalities; WT, wild-type.

Fig. 1


+8
Other abnormalities
#65
#38
#2
#50
#60
#3
#39
#18
#66
#20
#13
#4
#37
#57
#19
#21
#43
#52
#58
Chromosome
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
TKD
- - - - H - - -
Y - Y E Y Y - - - - -

A


case #65
MYC
Ch8
total CN
total CN (average)
hetero SNP call
AsCN

B

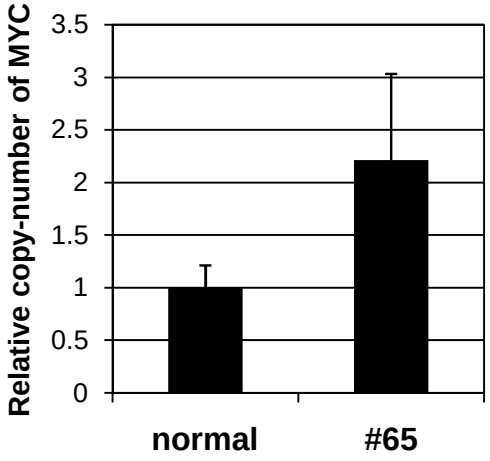

Relative copy-number of MYC
3.5
3
2.5
2
1.5
1
0.5
0
normal
#65

Figure 2

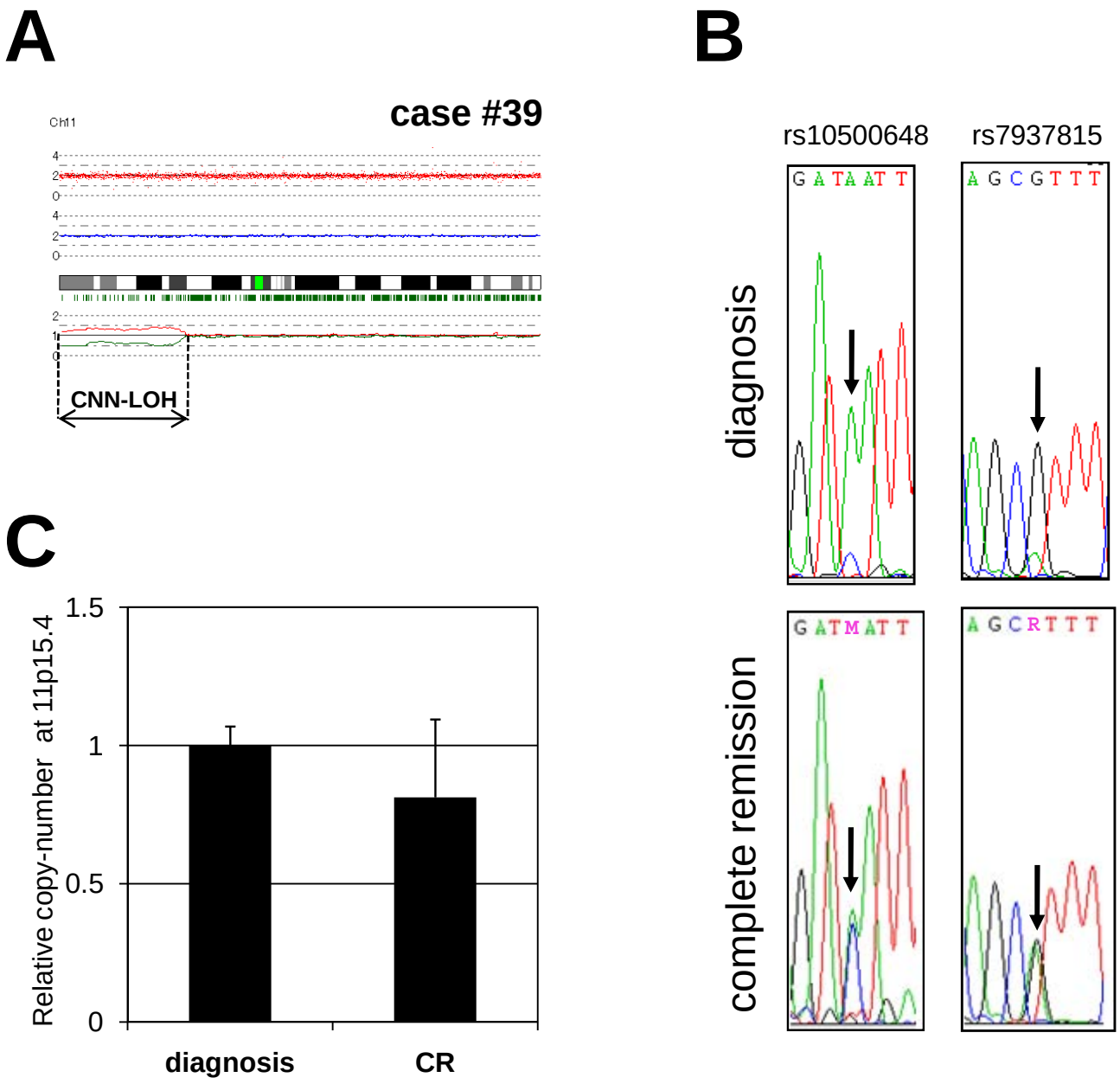

A
case #39
Ch11
CNN-LOH
B
rs10500648
rs7937815
diagnosis
complete remission
C
Relative copy-number at 11p15.4
1.5
1
0.5
0
diagnosis
CR

Figure 3

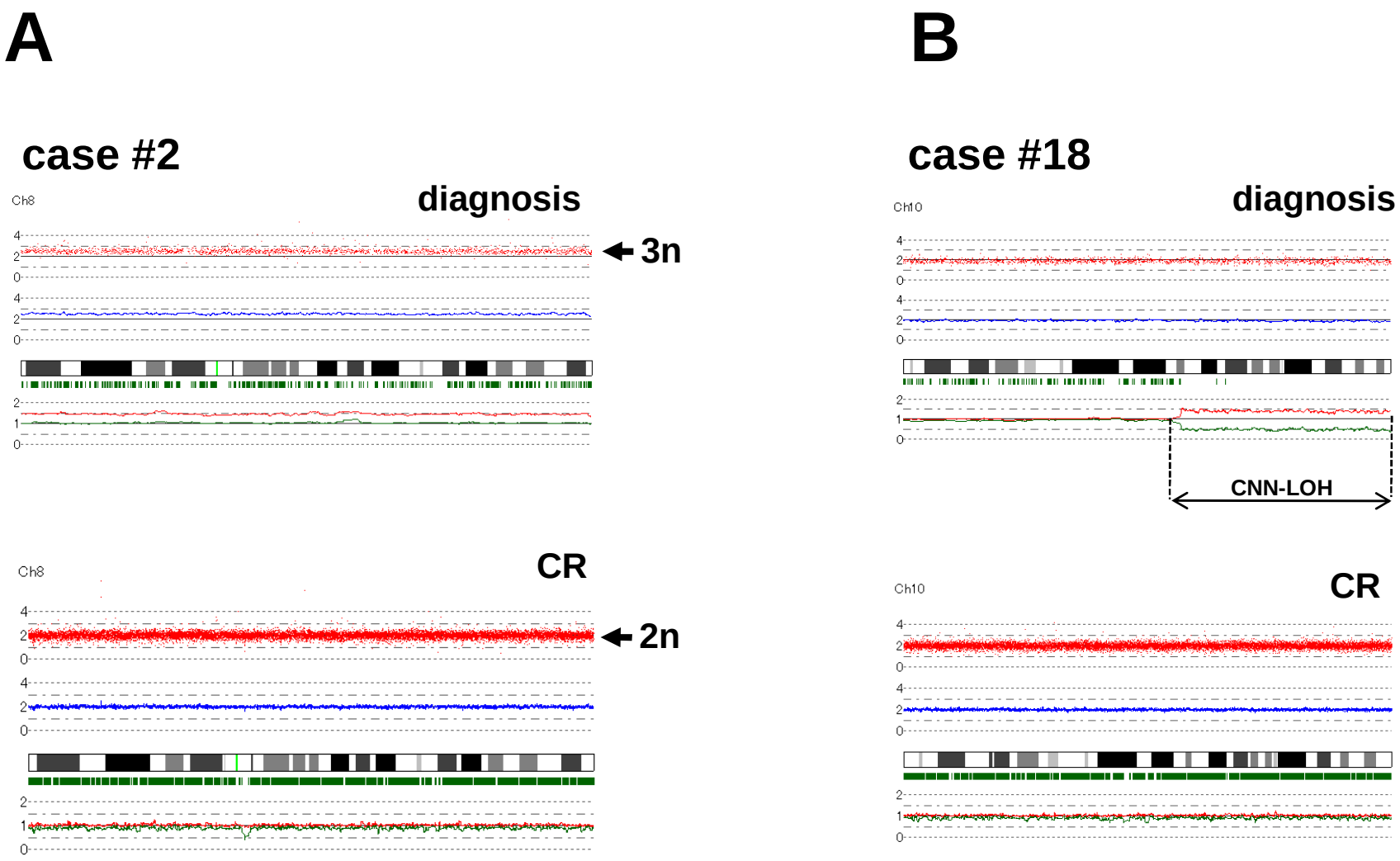

A
case #2
diagnosis
Ch8
3n
CR
Ch8
2n
B
case #18
diagnosis
Ch10
CNN-LOH
CR
Ch10

Figure 4