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Translocation of chromosomes 8 and 21, t(8;21), resulting in the AML1-ETO fusion gene, is associated with acute myeloid leukemia. We searched for additional genomic abnormalities in this acute myeloid leukemia subtype by performing single nucleotide polymorphism genomic arrays (SNP-chip) analysis on 48 newly diagnosed cases. Thirty-two patients (67%) had a normal genome by SNP-chip analysis (Group A), and 16 patients (33%) had one or more genomic abnormalities including copy number changes or copy number neutral loss of heterozygosity (Group B). Two samples had copy number neutral loss of heterozygosity on chromosome 6p including the PIM1 gene; and one of these cases had E135K mutation of Pim1. Interestingly, 38% of Group B and only 13% of Group A samples had a KIT-D816 mutation, suggesting that genomic alterations are often associated with a KIT-D816 mutation. Importantly, prognostic analysis revealed that overall survival and event-free survival of individuals in Group B were significantly worse than those in Group A.

Key words: t(8;21), AML1-ETO, CNN-LOH, SNP-chip, KIT, PIM1.

Severe murine models have demonstrated that AML1-ETO alone is not sufficient to induce leukemia. Murine bone marrow cells expressing tetracycline-inducible AML1-ETO transgene did not develop leukemia, but developed myeloproliferative disorders. In contrast, 30-55% of AML1-ETO-expressing mice treated with the DNA-alkylating mutagen N-ethyl-N-nitrosourea (ENU) developed AML. These findings strongly suggest that a secondary hit is necessary for the development of t(8;21) AML.

The protooncogene KIT is a receptor tyrosine kinase. Activating mutations of KIT including those in either the extracellular (exon 8) region or the protein kinase domains

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The online version of this article contains a supplementary appendix.
Genome-wide single nucleotide polymorphism genomics arrays (SNP-chip) allow the detection of copy number changes, as well as copy number neutral loss of heterozygosity (CNN-LOH) in leukemia samples. In order to screen for secondary alteration(s) that potentially could cause AML1-ETO transformed cells to develop acute myeloid leukemia, we performed SNP-chip analysis of 48 t(8;21) AML samples. The use of CGH (copy number analysis for Affymetrix GeneChips) program and an algorithm AsCNAR (allele-specific copy number analysis using anonymous references) allows identification of hidden abnormalities and novel disease-related genomic regions in the leukemia samples. Here, we found that genomic changes detected by SNP-chip analysis are associated with a poor overall and event-free survival in t(8;21) AML.

### Design and Methods

#### Patient samples, determination of mutant genes and statistical analysis

Genomic DNA of 48 anonymized samples of t(8;21) AML cells were obtained from Chang-Gung Memorial Hospital, Chang-Gung University in Taiwan after obtaining informed consent. These samples had been frozen over a span of 14 years (July 1990 to July 2004). Sample information is shown in the Online Supplementary Table S1. The study has been approved by Cedars-Sinai Medical Center (IRB number 4456).

To detect an AML1-ETO fusion transcript, RT-PCR was performed using specific primers as described previously. Mutation analysis of the KIT gene for the t(8;21) AML samples was reported previously. Statistical analysis is described in the Online Supplementary Design and Methods section.

#### SNP-chip analysis

Genomic DNA isolated from t(8;21) AML cells was subjected to GeneChip Human mapping microarray (SNP-chip, Affymetrix, Santa Clara, CA, USA) as described previously. Ten samples (cases #47, #51, #52, #54, #56, #57, #59, #60, #61 and #62) were examined with the 250 K array, and the other 38 samples were analyzed with the 500 K array, and the other 38 samples were analyzed with the 500 K array. The allele-specific copy numbers (AsCNs) were estimated using normal genomic DNA from peripheral blood of normal volunteers as controls. The array does not contain Y-chromosome probes; therefore, we summarize the SNP-chip data without sex chromosomes. Size, position and location of genes were identified with UCSC Genome Browser (http://genome.ucsc.edu/). Copy number changes previously described as copy number variants (http://projects.tcag.ca/variation/) were excluded.

### Fluorescence in situ hybridization (FISH) analysis

Interphase hybridizations were performed following the manufacturer’s instructions and standard protocols. Probes for the SNRPN gene, 15q telomere, 7p telomere and 7q telomere were obtained from Cytocell (Cambridge, United Kingdom), and probes for the MYC gene as well as the centromere of chromosome 8 were purchased from Abbott Molecular (Abbott Park, IL, USA). Fifty interphase cells were scored for each sample, with 20 cells scored in controls (bone marrow controls with normal karyotypes). Signal patterns were normal for all controls with all probe sets.

### Analysis of the PIM1 gene

Six coding exons of the PIM1 gene were amplified using specific primers from genomic DNA of cases #39 and #41. After purification of the PCR products from agarose gel, nucleotide sequences were determined. Primer sequences will be provided upon request. These 6 exons of other t(8;21) AML samples were examined by single strand conformation polymorphism (SSCP) as described in the Online Supplementary Design and Methods section.

To determine the frequency of missense mutations of the PIM1 gene within exon 4, this region of 34 t(8;21) AML samples and 40 normal blood DNA samples were amplified by PCR using specific primers (5′-TCC TGG AGG ATG-3′). The PCR product (178 bp) was treated with a restriction enzyme Hpy188III for 1h. PCR products from wild-type allele are not digested but mutated allele are digested by the restriction enzyme.

### Results and Discussion

#### SNP-chip analysis of 48 t(8;21) acute myeloid leukemia samples

SNP-chip analysis of 48 t(8;21) acute myeloid leukemia (AML) samples revealed several genomic copy number changes, as well as copy number neutral loss of heterozygosity (CNN-LOH). As shown in Table 1 and Online Supplementary Figure S1, 32 patients (67%) had a normal genome by SNP-chip analysis (Group A, mean age is 25, range 2-74). In contrast, 16 patients (33%) had one or more genomic abnormalities (Group B, mean age is 31, range 4-61). Thus, these copy number changes probably harbor dysregulated leukemia-associated genes in t(8;21) AML. Cytogenetics showed that case #33 had trisomy 4 in 2 out of 15 cells (13%), and case #34 had monosomy 18 in 6 out of 24 cells (25%) (Online Supplementary Table S1). These minor clones were not detected by SNP-chip analysis. Case #40 had tetraploidy in 23 out of 25 cells; 2-fold gene-dosage in all chromosomes was masked and detected as normal gene-dosage.

Next, we compared SNP-chip results and gene mutations. Ten out of 48 samples (18%) had a KIT-D816 mutation. Interestingly, 6 (case #7, #14, #26, #37, #40, and #52) of the 10 samples were found in Group B.
demonstrating that KIT-D816 mutation is significantly associated with Group B \((p<0.05, \chi^2\) test). This result suggests that copy number changes are often involved in cases with a KIT-D816 mutation in t(8;21) AML.

Recurrent copy number changes in t(8;21) acute myeloid leukemia samples

Two cases (#34 and #37) had a duplication on chromosome 8 from 8q22.1 to q-terminal including the MYC gene; and 2 cases (#13 and #28) had a trisomy/duplication on chromosome 15 with common duplicated region at 15q12.2-15q-terminal (53.7 Mb). Four cases (#14, #34, #43 and #60) had a deletion/monosomy on chromosome 7 with a common deleted region at 7q31.3-7q36.1 (16.2 Mb). Interestingly, a frequent large duplication was found on chromosome 4. Two cases had trisomy 4, and one case had a large region of duplication on chromosome 4 from 4p16.1 to q28.3 (131.1 Mb). All of these amplifications covered the region of the KIT gene; and 3 of these cases (#26, #37 and #39) had a KIT mutation of either D816Y, D816V or D820G. Amplification of chromosome 4 linked to KIT mutations has previously been described in systemic mastocytosis.21 Thus, the probable increased expression of the mutated form of KIT by trisomy 4 or duplication in the region of the gene should give the clone a proliferative advantage.

Validation of copy number change by fluorescence in situ hybridization (FISH)

To validate some of these copy number changes, we used an interphase FISH approach. Case #34 had a duplication of 8q22.1-8q-terminal (46.6 Mb) including the MYC gene and a deletion of 7q31.2-7q-terminal (42.0 Mb). The 8q duplication was confirmed using FISH probes for MYC and the centromere of chromosome 8 (Online Supplementary Figure S2A). In the same case, deletion of chromosome 7q (q31.2 to q-terminal) was confirmed using FISH probes for 7p telomere and
7q telomere (Online Supplementary Figure S2B). Case #38 had a large duplication of chromosome 15 (55.7 Mb). The duplication was confirmed using FISH probes for SNRPN and 15q telomere (Online Supplementary Figure S2C). These results suggest that abnormalities detected by SNP-chip analysis reflected real alterations in AML cells.

Chromosomal regions and candidate genes in genomic areas with copy number neutral loss of heterozygosity (CNN-LOH)

Four cases (8%) had CNN-LOH (Table 1 and Online Supplementary Figure S1). Case #7 has CNN-LOH at 11p-terminal-11p12 (40.5 Mb) which included the CDKN1C, HRAS, WT1 and LMO2 genes. Case #39 and #41 have CNN-LOH at 6p-terminal-6p12.3 (46.8 Mb) and at 6p-terminal-6p21.1 (44.7 Mb), respectively, and the region contained the PIM1 and CDKN1A genes (Table 1 and Online Supplementary Figure S3). Case #59 had CNN-LOH at 11q13.2-q-terminal (67.2 Mb). Raghavan et al. showed that approximately 20% AML samples had CNN-LOH, and Gondek et al. found that 20% of MDS, 23% of MDS-derived AML, and 35% of MDS/MPD patients had CNN-LOH. In additional studies, we found that 52% of normal karyotype AML samples and 15% of t(15;17) APL samples had CNN-LOH. 22,23 CNN-LOH in t(8;21) AML is less frequent than many other types of leukemia.

Acquired mutation of the PIM1 gene

The protooncogene PIM1, which encodes the serine-threonine protein kinase, is located on chromosome 6p, and 2 cases had CNN-LOH in the region. All exons of the PIM1 gene for these 2 cases were examined for mutations. As shown in Figure 1A, case #39 had a nucleic acid change of G to A at exon 4 of the PIM1 gene leading to an amino acid change of glutamic acid (E) to lysine (K) at codon 135 (E135K). The amino acid change occurred between the ATP-binding site and serine-threonine kinase domain. The wild-type amino acid is conserved between human, rat, mouse and xenopus. Importantly, the complete remission sample of the same individual showed the wild-type sequence, demonstrating that the nucleic acid change was a disease-specific acquired alteration.

The missense mutation in the PIM1 gene change produces the recognition site of a restriction enzyme, Hpy188III. A total of 34 t(8;21) AML samples and 40 normal blood DNA samples were examined for this mutation by Hpy188III digestion. The PCR product (178 bp) encompassing the mutation was only digested in case #39 (Figure 1B), but not the DNA from the other AML samples or normal blood DNA (data not shown), suggesting it is infrequent in the AML subtype. We also examined all exons of the PIM1 gene by SSCP using 34 t(8;21) AML samples, but no shifted bands were detected other than exon 4 of case #39. The PIM1 E135K mutant was also detected in B-cell diffuse large-cell lymphoma;22 and another mutant (E135Q) was discovered in primary diffuse large B-cell lymphomas.23 It remains to be clarified whether the E135K mutant is activated constitutively.

Prognostic significance of genomic change

Overall survival of t(8;21) AML patients of Group A (no genomic abnormality observed by SNP-chip) was significantly better than those in Group B (genomic abnormality observed by SNP-chip) (hazard ratio=2.992 [95% confidence interval, 1.247-7.179], p=0.0341) (Figure 2). The event-free survival of individuals of Group A was also significantly better than those in Group B (hazard ratio=2.360 [95% confidence interval, 1.037-5.372], p=0.0487). We also compared the prognosis of individuals with the KIT-D816 mutation (6 cases) to those without the alteration (10 cases) in Group B, but found no significant difference (data not shown). These results strongly suggest that genomic changes in t(8;21) AML are associated with a poor overall and event-free survival.

Figure 1. Acquired mutation of the PIM1 gene in case #39. (A) Exon 4 of the PIM1 gene in case #39 had a missense mutation in the sample at diagnosis but not at remission (top panel). The mutation leads to the amino acid change of glutamic acid (E) to lysine (K) at amino acid 135 (E135K) of PIM1 protein. This mutated amino acid is located between the ATP binding domain and serine/threonine kinase domain of the protein (middle panel). The wild-type amino acid (E) is highly conserved among human, rat, mouse and xenopus (bottom). Note, identical amino acid. (B) The mutated DNA sequence produced a Hpy188III restriction enzyme recognition sequence. The region was amplified by PCR, digested with Hpy188III, and subjected to agarose gel electrophoresis. The PCR product from only case #39 was digested.
A recent study showed that a KIT-D816V mutation is associated with a poor prognosis in t(8;21) AML patients. Also, secondary cytogenetic abnormalities including trisomy of chromosome 7 and 8, deletion/duplication of chromosome 7, as well as deletion of chromosome X and Y in t(8;21) AML have previously been reported to be associated with a poor prognosis. Taken together, these findings indicate that genomic alterations and KIT-D816 mutation confer a poor prognosis in t(8;21) AML patients. Further studies in a larger cohort of patients will begin to stratify prognostically the patients in relation to the genomic changes; and new therapeutic targets should be discovered.

Figure 2. Comparison of overall survival and event-free survival of t(8;21) acute myeloid leukemia patients either with or without genomic changes. Overall survival (left) and event-free survival (right) were compared between Groups A and B. Black and grey lines indicate Group A (no genomic abnormality by SNP-chip) and Group B (genomic abnormality by SNP-chip), respectively.

Authorship and Disclosures

TA performed research, analyzed the data and wrote the paper; LS and DL determined mutation of genes; SO, MS, and YN performed SNP-chip analysis and developed CNAG; NK, SD, and JS assisted data analysis; JG and MM performed statistical analysis; VZ and AN performed the methylation analysis; SRM and RS performed FISH analysis; and SL and HPK directed the overall study. TA, LS and SO contributed equally in this work; and SL and HPK are co-last authors.

The authors reported no potential conflicts of interest.

References