Comprehensive Mutation Profile in Acute Myeloid Leukemia Patients with RUNX1-RUNX1T1 or CBFB-MYH11 Fusions

RUNX1-RUNX1T1 veya CBFB-MYH11 Füzyonları Olan Akut Myeloid Lösemili Hastalarda Detaylı Mutasyon Profili

Objective: This study was undertaken with the aim of better understanding the genomic landscape of core-binding factor (CBF) acute myeloid leukemia (AML).

Materials and Methods: We retrospectively analyzed 112 genes that were detected using next-generation sequencing in 134 patients with de novo CBF-AML, FLT3-ITD, NPM1, and CEBPA mutations were detected by DNA-PCR and Sanger sequencing.

Results: In the whole cohort, the most commonly mutated genes were c-KIT (33.6%) and NRAS (33.6%), followed by FLT3 (18.7%), KRAS (13.4%), RELN (8.2%), and NOTCH1 (8.2%). The frequencies of mutated genes associated with epigenetic modification, such as IDH1, IDH2, DNMT3A, and TET2, were low, being present in 1.5%, 0.7%, 2.2%, and 7.5% of the total number of patients, respectively. In[16](16;16) AML patients exhibited more mutations in NRAS and KRAS (p=0.001 and 0.0001, respectively) than in[8;21] AML patients. Functionally mutated genes involved in signaling pathways were observed more frequently in the in[16](16;16) AML group (p=0.016), while the mutations involved in cohesin were found more frequently in the in[8;21] AML group (p=0.011). Significantly higher white blood cell counts were found in in[16](16;16) AML patients with c-KIT (c-KITmut) or NRAS (NRASmut) mutations compared to the corresponding in[8;21] AML (c-KITmut) and in[8;21] AML/NRASmut groups (p=0.001 and 0.009, respectively).

Conclusion: The mutation profiles of in[8;21] AML patients showed evident differences from those of patients with in[16](16;16) AML. We have provided a comprehensive overview of the mutational landscape of CBF-AML.

Keywords: Core-binding factor, Acute myeloid leukemia, Mutation, Next-generation sequencing

ABSTRACT

Amaç: Bu çalışma çekirdek bağlama faktörü (CBF) akut myeloid lösemi (AML) genomik durumunu daha iyi anlamak amacıyla yapılmıştır.

Yöntemler: Thumbnail per novum ÇBF-AML hastasında yeni nesil dizileme ile tespit edilen 112 gen analiz edildi. FLT3-ITD, NPM1 ve CEBPA mutasyonları DNA-PCR ve Sanger dizileme ile tespit edildi.

Bulgular: Bütün kohortta en sık mutasyonlu genler c-KIT (33,6%) ve NRAS (33,6%) idi, ardından FLT3 (18,7%), KRAS (13,4%), RELN (8,2%), NOTCH1 (8,2%) idi. IDH1, IDH2, DNMT3A ve TET2 gibi epigenetik modifikasyonlara iliskili mutasyonlar daha fazla bulundu (p=0,001; 0,0001). İşlevsel olarak sinyal yolcularında yer alan mutasyonlar genlerin in[16](16;16) AML hastalarında NRAS ve KRAS mutasyonlarının t(8;21) AML hastalarına göre daha fazla sıklıkta idi (sarsıyla: p=0,001; 0,0001). İşlevsel olarak sinyal yolcularında yer alan mutasyonlar genlerin in[16](16;16) AML grubunda daha çok sıklıkta idi (p=0,016), kohezin içinde yer alan mutasyonlar genlerin in[8;21] AML grubunda daha çok sıklıkta idi (p=0,011). c-KIT (c-KITmut) veya NRAS mutasyonları (NRASmut) olan in[16](16;16) AML hastalarında karışımlığında t(8;21) AML/c-KITmut ve t(8;21) AML/NRASmut gruplarına göre beyaz küre sayısi daha yüksek bulundu (sarsıyla: p=0,001; 0,0009).

Sonuç: t(8;21) AML hastalarının mutasyon profillerini in[16](16;16) AML’den belirgin farklılıklar gösterdi. Bu çalışmada ÇBF-AML’in mutasyon profili kapsamlı bir biçimde incelenmiştir.

Anahtar Sözcükler: Çekirdek bağlama faktörü, Akut myeloid lösemi, Mutasyon, Yeni nesil sekanslama
Introduction
Cases of acute myeloid leukemia (AML) involving the core-binding factor (CBF) include AML with t(8;21) and inv(16)/t(16;16) chromosomal translocations, leading to the RUNX1-RUNX1T1 and CBFB-MYH11 fusions genes, respectively. Such AML patients account for approximately 25% of pediatric and 15% of adult de novo AML cases [1], and CBF-AML was recognized as a unique entity in the 2016 World Health Organization classification of myeloid neoplasms and acute leukemia [2]. Accumulating evidence has revealed that the t(8;21)(q22;q22) and inv(16)/t(16;16) CBF rearrangements are associated with favorable outcomes relative to other cytogenetic subtypes and that allogeneic hematopoietic stem cell transplantation is not generally recommended during the first complete remission (CR) [3,4]. However, relapse occurs in up to 40% of these cases, indicating clinicopathological heterogeneity within this AML subset [5,6,7,8]. Further investigation is still needed to better understand leukemogenesis and disease progression.

Previous findings have demonstrated that expressions of translocation-encoded AML1 or CBF fusion proteins are insufficient by themselves to induce a full leukemic phenotype [9]. Further evidence supporting this model comes from the fact that mutations in genes activating tyrosine kinase signaling (including KIT, N/KRAS, and FLT3) are frequent in both CBF-AML subtypes [6,10]. Nonetheless, data regarding the prognostic significance of KIT and RAS in CBF-AML are contradictory. Duployez et al. [11] reported the presence of additional aberrations in >90% of CBF-AML cases, and mutations in epigenetic modifications or cohesin genes were associated with poor prognosis in t(8;21) AML patients with TK pathway mutations using next-generation sequencing (NGS). Ishikawa et al. [12] revealed that the c-KIT exon 17 mutation and the presence of extramedullary tumors in t(8;21) AML patients were poor prognostic factors for relapse-free survival, as were the loss of chromosome X or Y and NRAS mutation in patients with inv(16)/t(16;16). These findings highlight the multilocality of CBF-AML and suggest that the prognostic impact may differ in the context of certain gene mutations between AML patients with t(8;21) and those with inv(16)/t(16;16).

Comprehensive genetic analysis using NGS may be helpful in refining our understanding of the prognosis of CBF-AML [11]. To the best of our knowledge, limited data are available regarding the impact of companion gene mutations in CBF-AML. To better characterize this subtype and to better understand the role of co-mutations in CBF-AML, we performed extensive mutational analysis by NGS for 134 CBF-AML patients. The clinical value of co-mutations in CBF-AML patients was also explored.

Materials and Methods

Patients
A total of 134 newly diagnosed de novo CBF-AML patients were selected from the Affiliated Changzhou Second Hospital of Nanjing Medical University, Wuxi Third People's Hospital, and First Affiliated Hospital of Soochow University from May 2016 to June 2021. The diagnosis of CBF-AML was based on the 2008 definition of the World Health Organization [2]. Eighty AML patients with t(8;21)/RUNX1-RUNX1T1 and 54 patients with inv(16)/t(16;16)/CBFB-MYH11 were included in the analysis. The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.

Mutational Analysis by Next-Generation Sequencing
Genomic DNA was extracted from fresh bone marrow or peripheral blood samples with the QIAamp DNA Mini Kit (QIAGEN GmbH, Germany) following the manufacturer's instructions. The NGS library was prepared using at least 200 ng of genomic DNA. Massively parallel sequencing was performed with an Illumina next-generation sequencer and a variant allele frequency of >3% was used as the threshold for calling single-nucleotide variants. A high depth of coverage (1000x) was obtained for 112 genes, including whole coding regions known to be frequently mutated in hematological malignancies, such as genes involved in epigenetic regulators, signaling pathways, transcription factors, spliceosomes, cohesin complex, tumor suppressors, and chromatin modifiers (Table 1). Searches were performed in the COSMIC database for altered DNA sequences deemed to be mutations or variants with IGV software and were confirmed in the SNP database (dbSNP). Polymerase chain reaction (PCR) followed by direct Sanger sequencing was used to detect FLT3-ITD, NPM1 (exon 12), and CEBPA to avoid possible false-negative results due to limitations of NGS, as previously described [13,14].

Other Cytogenetic and Molecular Abnormality Screening
Bone marrow (BM) cells were collected and cultured at the time of the initial diagnosis. The presence of the t(8;21) or inv(16)/t(16;16) rearrangement was determined by the conventional G/R banding method. Fluorescence in situ hybridization (FISH) of interphase nuclei and/or metaphases was performed for the chimeric genes RUNX1-RUNX1T1 and CBFB-MYH11. The fusion transcripts were identified by real-time quantitative PCR (RT-qPCR) method using bone marrow or peripheral blood samples at diagnosis, as previously described [15].

Statistical Analysis
CR was defined as <5% blast cells, no Auer rods, and no clusters of blast cells by bone marrow analysis as well as no evidence
of extramedullary leukemia. All statistical analyses of the data were carried out using IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA). The chi-square and Fisher exact tests were used for comparisons of categorical variables between different cohorts. The Student t-test was used to analyze continuous variables with normal distribution and the Mann-Whitney U test was used for data that did not comply with normal distribution. Values of p<0.05 were considered statistically significant.

**Results**

**Patient Characteristics**

The 134 CBF-AML patients enrolled in this study included 65 women and 69 men with a median age of 35.5 years (range: 16.0-73.0 years). The median white blood cell (WBC) count was 15.3x10^9/L (range: 0.9 to 156.0x10^9/L), median hemoglobin (Hb) level was 81 g/L (range: 39.0 to 124.0 g/L), and median platelet count was 28.0x10^9/L (range: 2.0 to 170.0x10^9/L). Patients with inv(16) or t(16;16) AML tended to be older than t(8;21) AML patients (41 vs. 32 years, p=0.048) and also had higher WBC counts (35.9x10^9/L vs. 7.8x10^9/L, p=0.0001). No significant differences were identified regarding gender, Hb level, or platelet count between patients with t(8;21) and inv(16)/t(16;16) AML. By conventional chromosome analysis, 41.0% (55/134) of the CBF-AML patients were found to have secondary cytogenetic abnormalities. Additional chromosomal alterations were found in 45.0% (36/80) and 35.2% (19/54) of the patients with t(8;21) and inv(16)/t(16;16), respectively. Loss of the X or Y chromosome was identified as the most common secondary alteration (33/134, 24.6%), followed by trisomy 22 (13/134, 9.7%). In patients with inv(16)/t(16;16), the most frequently identified additional cytogenetic alterations were trisomy 22 and trisomy 8, which were found in 20.1% (13/54) and 9.3% (5/54) of these patients, respectively.

**Table 1. The 112 genes analyzed in this study.**

| Number | Gene  | Number | Gene  | Number | Gene  | Number | Gene  |
|--------|-------|--------|-------|--------|-------|--------|-------|
| 1      | ABL1  | 29     | DNM2  | 57     | KMT2A | 85     | SETBP1|
| 2      | ACD   | 30     | DNMT3A| 58     | KRAS  | 86     | SETD2 |
| 3      | ANKRD26| 31     | DNMT3B| 59     | MAPK1 | 87     | SF1   |
| 4      | ARIDIA| 32     | ECT2L | 60     | KMT2D | 88     | SF3A1 |
| 5      | ASXL1 | 33     | EED   | 61     | MPL   | 89     | SF3B1 |
| 6      | ATG2B | 34     | EP300 | 62     | MYC   | 90     | SH2B3 |
| 7      | ATM   | 35     | ETNK1 | 63     | MYD88 | 91     | SMC1A |
| 8      | B2M   | 36     | EZH2  | 64     | NF1   | 92     | SMC3  |
| 9      | BCOR  | 37     | FAM46C| 65     | NOTCH1| 93     | SRP72 |
| 10     | BCOR1 | 38     | BRIP3 | 66     | NOTCH2| 94     | SRSF2 |
| 11     | BIRC3 | 39     | FAT1  | 67     | NPM1  | 95     | STAG2 |
| 12     | BRAF  | 40     | FBXW7 | 68     | NRAS  | 96     | STAT3 |
| 13     | CALR  | 41     | FGFR3 | 69     | PAX5  | 97     | SUZ12 |
| 14     | CBL   | 42     | FLT3  | 70     | PDS5B | 98     | ETV6  |
| 15     | CCND1 | 43     | GATA1 | 71     | PHF6  | 99     | TERC  |
| 16     | CCND3 | 44     | GATA2 | 72     | PIGA  | 100    | TERT  |
| 17     | CCR4  | 45     | GATA3 | 73     | PLCG1 | 101    | TET2  |
| 18     | CD79B | 46     | JAK1  | 74     | PRKCB | 102    | TNFAIP3|
| 19     | CDC25C| 47     | JAK2  | 75     | PRPF40B| 103   | TP53  |
| 20     | CDKN2A| 48     | JAK3  | 76     | PRPS1 | 104    | TPM2 |
| 21     | CEBPA | 49     | HNRNPK| 77     | PTEN  | 105    | TRAF3 |
| 22     | CREBBP| 50     | ID3   | 78     | PTPN11| 106    | U2AF1 |
| 23     | CSF3R | 51     | IDH1  | 79     | RAD21 | 107    | U2AF2 |
| 24     | CUX1  | 52     | IDH2  | 80     | RB1   | 108    | WHSC1 |
| 25     | CXC4  | 53     | IKZF1 | 81     | RBBP6 | 109    | WT1   |
| 26     | DDX3X | 54     | IL7R  | 82     | RELN  | 110    | XPO1  |
| 27     | DDX41 | 55     | KDM6A | 83     | RHOA  | 111    | ZRSR2 |
| 28     | DIS3  | 56     | KIT   | 84     | RUNX1 | 112    | ZMYM3 |
Trisomies 8 and 22 were more frequently observed in inv(16)/t(16;16) AML patients (p=0.006 and 0.0001, respectively), while loss of the X or Y chromosome was more common in patients with t(8;21) AML (p=0.0001). Del(9q) was not noted in any inv(16) patients, while trisomies 8 and 22 were not found in any t(8;21) patients. In all cases for which FISH and/or RT-qPCR testing was performed, the results were in agreement with the results of chromosome analysis (data not shown). Both CD19 and CD56 antigen expressions were observed more frequently in the t(8;21) AML group (both p=0.0001). Clinical and biological characteristics of the patients are provided in Table 2.

**Comparison of Clinical Features and Incidence of Genetic Mutations Between AML Patients with t(8;21) and inv(16)/t(16;16)**

Among the participating 134 CBF-AML patients, 68 mutated genes were detected by screening the 112-gene panel. Thirty-two of those 68 genes could be classified as transcription factor, DNA methylation, signaling, spliceosome, cohesin, or tumor suppressor genes. An average of 3.19 (range: 1-10) mutations per individual were detected among these CBF-AML cases. While 22 patients had 1 alteration, 20 had 2, 32 had 3, and 60 had 4 or more. Among all genes sequenced, the most commonly mutated genes were c-KIT (45/134, 33.6%) and NRAS (45/134, 33.6%), followed by FLT3 (25/134, 18.7%), KRAS (18/134, 13.4%), RELN (11/134, 8.2%), NOTCH1 (11/134, 8.2%), TET2 (10/134, 7.5%), and WT1 (10/134, 7.5%). The other genes had mutation prevalences of <5%. The most frequently affected functional pathway was the signaling pathway, with such mutations observed in as many as 86.6% of cases. In addition, our findings suggest that the frequencies of mutations in genes associated with epigenetic modification, such as IDH1, IDH2, DNMT3A, and TET2, are low in CBF-AML, being identified in 1.5%, 0.7%, 2.2%, and 7.5% of the total number of participating patients, respectively.

Concomitant gene abnormalities were found in 100% of the patients with t(8;21) and 100% of the patients with inv(16)/t(16;16). The patients with inv(16)/t(16;16) AML were found to have more mutations in the NRAS and KRAS genes (53.7% vs. 20.0%, p=0.001 and 27.8% vs. 3.8%, p=0.0001, respectively) compared to t(8;21) AML patients. The distributions of c-KIT, FLT3, RELN, TET2, FAT1, and NOTCH1 mutations within these two groups were similar (Table 3). Functionally mutated genes involved in signaling pathways were observed more frequently in the inv(16)/t(16;16) AML group.

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**Table 2. Clinical and biological characteristics of the CBF-AML patients at diagnosis.**

| Variable                                    | Total (n=134) | RUNX1–RUNX1T1 (n=80) | CBFB–MYH11 (n=54) | P     |
|---------------------------------------------|--------------|----------------------|------------------|-------|
| Gender                                      |              |                      |                  |       |
| Male, n (%)                                 | 69 (51.5%)   | 42 (52.5%)           | 27 (50%)         | 0.776 |
| Female, n (%)                               | 65 (48.5%)   | 38 (47.5%)           | 27 (50%)         |       |
| Age (years)                                 |              |                      |                  |       |
| Median (range)                              | 35.5 (16-73) | 32 (16-73)           | 41 (16-63)       | 0.048 |
| WBC count (x10^9/L)                         |              |                      |                  |       |
| Median (range)                              | 15.3 (0.9-156.0) | 7.8 (0.9-123)    | 35.9 (1.6-156)   | 0.0001|
| Hb (g/L)                                    |              |                      |                  |       |
| Median (range)                              | 81 (39.0-124.0) | 78 (39-120)        | 86.5 (40-124)    | 0.169 |
| PLT count (x10^9/L)                         |              |                      |                  |       |
| Median (range)                              | 28.0 (2.0-170.0) | 32 (2-170)        | 26 (3-121)       | 0.725 |
| Secondary cytogenetic abnormalities         |              |                      |                  |       |
| Loss of X or Y chromosome, n (%)            | 33 (24.6%)   | 32 (40.0%)           | 1 (1.9%)         | 0.0001|
| del(9q), n (%)                              | 4 (3.0%)     | 4 (5.0%)             | 0                | 0.095 |
| Trisomy 8, n (%)                            | 5 (3.7%)     | 0                    | 5 (9.3%)         | 0.006 |
| Trisomy 22, n (%)                           | 13 (9.7%)    | 0                    | 13 (20.1%)       | 0.0001|
| Immunophenotyping                           |              |                      |                  |       |
| CD19 expression, n (n/N, %)                 | 59 (59/116, 50.86%) | 58 (58/73, 79.5%) | 1 (1/43, 2.33%)  | 0.0001|
| NA, n                                       | 18           | 7                    | 11               |       |
| CD56 expression, n (n/N, %)                 | 51 (51/92, 55.43%) | 50 (50/58, 86.21%) | 1 (1/34, 2.94%)  | 0.0001|
| NA, n                                       | 42           | 22                   | 20               |       |

CBF: Core-binding factor; AML: acute myeloid leukemia; WBC: white blood cell; Hb: hemoglobin; PLT: platelet; NA: not available.
(p=0.016), while cohesin mutations were found more frequently in the t(8;21) AML group (11.3% vs. 0%, p=0.011). All cohesin mutations were mutually exclusive among each other (Figure 1). The mutation distribution is provided in detail in Table 3.

**Relationships Between Clinical Characteristics, CR Rate, and Mutations**

We analyzed the clinical characteristics of patients with mutations in c-KIT, NRAS, KRAS, CSF3R, TET2, and FLT3-ITD. As listed in Table 4, significantly higher WBC counts were found in inv(16)/t(16;16) AML patients with c-KIT (c-KITmut) or NRAS (NRASmut) mutations than in t(8;21) AML patients with c-KITmut and NRASmut (p=0.001 and 0.009, respectively). Patients with both inv(16)/t(16;16) AML/TET2mut and inv(16)/t(16;16) AML/FLT3-ITDmut also had higher WBC counts than those with t(8;21) AML/TET2mut and t(8;21) AML/FLT3-ITDmut, but these differences did not reach statistical significance (p=0.088 and 0.067, respectively).

No difference was found between other factors such as age, gender, Hb level, or platelet count.

This study also aimed to assess the impact of common gene mutations on the rate of CR after initial induction therapy. Among the 134 participating patients, relevant data were available for 128. The overall CR rate among these cases was 94.53% (121/128). No differences in CR rate were observed according to mutated genes (KIT, NRAS, KRAS, TET2, FLT3-ITD) between t(8;21) AML and inv(16)/t(16;16) AML patients. Clinical characteristics and CR rates of CBF-AML patients with common mutations are shown in Table 4.

**Discussion**

In this study, patients with inv(16)/t(16;16) AML had higher WBC counts than those with t(8;21) AML. Trisomies 8 and 22 were more frequently observed in inv(16) patients, while loss of the X or Y chromosome was more common in t(8;21) AML. Patients with t(8;21) AML also expressed CD19 and CD56 more frequently than those with inv(16) AML. These findings are consistent with the conclusions of previous reports [11,16].

![Figure 1. Comparisons of genetic mutations between AML patients with t(8;21) and inv(16)/t(16;16).](Image)
Table 3. Concomitant gene abnormalities of CBF-AML at diagnosis.

| Mutational genes       | Total (n=134) | RUNX1–RUNX1T1 (n=80) | CBFB–MYH11 (n=54) | p    |
|------------------------|--------------|-----------------------|-------------------|------|
| **NPM1**               |              |                       |                   |      |
| Signaling pathways, n (%) | 116 (86.6%)  | 64 (80.0%)            | 52 (96.3%)        | 0.016|
| c-KIT, n (%)           | 45 (33.6%)   | 28 (35.0%)            | 17 (31.5%)        | 0.672|
| NRAS, n (%)            | 45 (33.6%)   | 16 (20.0%)            | 29 (53.7%)        | 0.0001|
| KRAS, n (%)            | 18 (13.4%)   | 3 (3.8%)              | 15 (27.8%)        | 0.0001|
| FLT3, n (%)            | 25 (18.7%)   | 15 (18.8%)            | 10 (18.5%)        | 1    |
| CSF3R, n (%)           | 8 (6.0%)     | 7 (8.8%)              | 1 (1.9%)          | 0.143|
| RELN, n (%)            | 11 (8.2%)    | 8 (10.0%)             | 3 (5.6%)          | 0.524|
| NOTCH1, n (%)          | 11 (8.2%)    | 7 (8.8%)              | 4 (7.4%)          | 1    |
| NOTCH2, n (%)          | 8 (6.0%)     | 4 (5.0%)              | 4 (7.4%)          | 0.714|
| JAK2, n (%)            | 8 (6.0%)     | 6 (7.5%)              | 2 (3.7%)          | 0.363|
| SH2B3, n (%)           | 6 (4.5%)     | 6 (7.5%)              | 0                 | 0.081|
| PTPN11, n (%)          | 3 (2.2%)     | 1 (1.3%)              | 2 (3.7%)          | 0.565|
| Epigenetic regulators, n (%) | 15 (11.2%)  | 10 (12.5%)            | 5 (9.3%)          | 0.781|
| TET2, n (%)            | 10 (7.5%)    | 6 (7.5%)              | 4 (7.4%)          | 1    |
| IDH1, n (%)            | 2 (1.5%)     | 1 (1.3%)              | 1 (1.9%)          | 1    |
| IDH2, n (%)            | 1 (0.7%)     | 1 (1.3%)              | 0                 | 1    |
| DNMT3A, n (%)          | 3 (2.2%)     | 3 (3.8%)              | 0                 | 0.273|
| Transcription factors, n (%) | 17 (12.7%)  | 8 (10.0%)            | 9 (16.7%)         | 0.296|
| ETV6, n (%)            | 1 (0.7%)     | 1 (1.3%)              | 0                 | 1    |
| RUNX1, n (%)           | 2 (1.5%)     | 1 (1.3%)              | 1 (1.9%)          | 1    |
| GATA2, n (%)           | 1 (0.7%)     | 1 (1.3%)              | 0                 | 1    |
| SETBP1, n (%)          | 8 (6.0%)     | 3 (3.8%)              | 5 (9.3%)          | 0.267|
| CEBPA<sup>dm</sup>, n (%) | 7 (5.2%)    | 3 (3.8%)              | 4 (7.4%)          | 0.439|
| Splicecosomes, n (%)   | 4 (3.0%)     | 4 (5.0%)              | 0                 | 0.148|
| SRSF2, n (%)           | 1 (0.7%)     | 1 (1.3%)              | 0                 | 1    |
| SF3B1, n (%)           | 3 (2.2%)     | 3 (3.8%)              | 0                 | 0.273|
| Tumor suppressors, n (%) | 14 (10.4%)  | 9 (11.2%)            | 5 (9.26%)         | 0.712|
| TP53, n (%)            | 4 (3.0%)     | 4 (5.0%)              | 0                 | 0.148|
| WT1, n (%)             | 10 (7.5%)    | 5 (6.3%)              | 5 (9.3%)          | 0.516|
| Cohesin, n (%)         | 9 (6.7%)     | 9 (11.3%)             | 0                 | 0.011|
| RAD21, n (%)           | 4 (3.0%)     | 4 (5.0%)              | 0                 | 0.148|
| SMC1A, n (%)           | 3 (2.2%)     | 3 (3.8%)              | 0                 | 0.273|
| SMC3, n (%)            | 2 (1.5%)     | 2 (2.5%)              | 0                 | 0.515|
| Chromatin modifiers, n (%) | 15 (11.2%)  | 11 (13.8%)           | 4 (7.4%)          | 0.403|
| ASXL1, n (%)           | 8 (6.0%)     | 6 (7.5%)              | 3 (5.6%)          | 0.659|
| KDM6A, n (%)           | 2 (1.5%)     | 2 (2.5%)              | 0                 | 0.515|
| BCOR, n (%)            | 4 (3.0%)     | 3 (3.8%)              | 1 (1.9%)          | 0.648|
| BCORL1, n (%)          | 1 (0.7%)     | 1 (1.3%)              | 0                 | 1    |
| **Number of mutated genes** |            |                       |                   |      |
| 1                      | 22 (16.4%)   | 14 (17.5%)            | 8 (14.8%)         | 0.681|
| 2                      | 20 (14.9%)   | 10 (12.5%)            | 10 (18.5%)        | 0.338|
| 3                      | 32 (23.9%)   | 18 (22.5%)            | 14 (25.9%)        | 0.648|
| ≥4                     | 60 (44.8%)   | 38 (47.5%)            | 22 (40.7%)        | 0.440|
| **Average number (range)** | 3.19 (1-10) | 3.17 (1-8)          | 3.22 (1-10)       | 0.873|

CBF: Core–binding factor; AML: acute myeloid leukemia.
Both t(8;21) and inv(16)/t(16;16) disrupt the normal functioning of the heterodimeric transcription factor CBF complex in AML with relatively similar clinical outcomes. However, the molecular genetic abnormalities potentially explaining the differences between these two AML subtypes have not yet been explored in detail. We performed extensive mutational analysis by NGS for 134 patients with CBF-AML who ranged in age from 16 to 73 years. As expected, additional aberrations were found in 100% of these CBF-AML cases and the most commonly mutated gene was c-KIT, as seen in 35.0% of AML cases with t(8;21) and 31.5% of AML cases with inv(16)/t(16;16). This is in accordance with the previous research conducted by Duployez et al. [11]. Interestingly, a significantly different spectrum of gene mutations was demonstrated in AML between patients with

| Variables               | Age, years, median (range) | Male/female, n/n | WBC count, x10⁹/L, median (range) | Hb, g/L, median (range) | PLT count, x10⁹/L, median (range) | CR rate, n/N (%) |
|-------------------------|----------------------------|------------------|-----------------------------------|------------------------|----------------------------------|------------------|
| c-KIT mutations (n=45)  |                            |                  |                                    |                        |                                  |                  |
| RUNX1–RUNX1T1 (n=28)    | 31.5 (16–56)               | 13/15            | 10.1 (2.0–49.9)                    | 78 (38–19)             | 32.5 (11–170)                    | 50.0% (13/26) *  |
| CBFB–MYH11 (n=17)       | 32 (16–57)                 | 10/7             | 29 (5.7–137)                       | 93 (63–124)            | 40 (15–117)                      | 58.8% (10/17)   |
| p                       | 0.566                      | 0.542            | 0.001                              | 0.081                  | 0.419                            | 0.571            |
| NRAS mutations (n=45)   |                            |                  |                                    |                        |                                  |                  |
| RUNX1–RUNX1T1 (n=16)    | 30.5 (16–69)               | 9/7              | 18.6 (2.4–123)                     | 80 (57–119)            | 28 (7–105)                       | 87.5% (14/16)   |
| CBFB–MYH11 (n=29)       | 35 (18–63)                 | 14/15            | 43 (5.0–156)                       | 87 (40–122)            | 25 (3–117)                       | 92.8% (26/28) * |
| p                       | 0.618                      | 0.758            | 0.009                              | 0.506                  | 0.585                            | 0.552            |
| KRAS mutations (n=18)   |                            |                  |                                    |                        |                                  |                  |
| RUNX1–RUNX1T1 (n=3)     | 41 (16–51)                 | 3/0              | 30.9 (3.2–49.9)                    | 79 (64–110)            | 11 (7–58)                        | 100% (3/3)      |
| CBFB–MYH11 (n=15)       | 36 (19–58)                 | 9/6              | 32 (1.56–144)                      | 82 (59–110)            | 40 (15–121)                      | 80% (12/15)     |
| p                       | 0.824                      | 0.515            | 0.783                              | 0.824                  | 0.138                            | 1.000            |
| CSF3R mutations (n=8)   |                            |                  |                                    |                        |                                  |                  |
| RUNX1–RUNX1T1 (n=7)     | 21 (16–32)                 | 2/5              | 5.1 (0.9–52.4)                     | 63 (59–119)            | 28 (8–105)                       | 57.1% (4/7)     |
| CBFB–MYH11 (n=1)        | 56 (56–56)                 | 1/0              | 34.9                               | 98 (98–98)             | 14 (14–14)                       | 100% (1/1)      |
| p                       | 0.127                      | 0.375            | 0.275                              | 0.275                  | 0.275                            | 1.000            |
| TET2 mutations (n=10)   |                            |                  |                                    |                        |                                  |                  |
| RUNX1–RUNX1T1 (n=6)     | 36 (29–49)                 | 2/4              | 4.2 (0.9–74)                       | 89.5 (45–107)          | 30.5 (2–80)                      | 66.7% (4/6)     |
| CBFB–MYH11 (n=4)        | 39.5 (26–52)               | 1/3              | 28.6 (18.3–65.4)                   | 77.5 (59–98)           | 22 (22–44)                       | 100% (4/4)      |
| p                       | 1.00                       | 1                | 0.088                              | 0.593                  | 0.892                            | 0.467            |
| FLT3–ITD mutations (n=10)|                          |                  |                                    |                        |                                  |                  |
| RUNX1–RUNX1T1 (n=7)     | 43 (24–64)                 | 2/5              | 23.9 (5.6–40)                      | 75 (57–108)            | 23 (6–45)                        | 42.9% (3/7)     |
| CBFB–MYH11 (n=3)        | 40 (36–42)                 | 3/0              | 54 (33–137)                        | 82 (77–85)             | 40 (20–57)                       | 66.7% (2/3)     |
| p                       | 0.732                      | 0.167            | 0.067                              | 0.21                   | 0.138                            | 1.000            |

WBC: White blood cell; HB: hemoglobin; PLT: platelet; CR: complete remission. *: Some patients did not receive any chemotherapy or were only treated with low-dose cytosine arabinoside.
t(8;21) and inv(16)/t(16;16). We noticed that fewer signaling pathways were involved in cases of t(8;21) in comparison to inv(16)/t(16;16) (80.0% vs. 96.3%, p=0.016), while patients with inv(16)/t(16;16) AML exhibited more mutations in NRAS and NRAS compared to t(8;21) AML patients.

RAS genes encode a family of membrane-associated proteins that regulate signal transduction upon the ligand binding to a variety of membrane receptors, and they play important roles in physical processes including proliferation, differentiation, and apoptosis [17,18]. Activating point mutations of RAS genes have generally been accepted as oncogenic events in the tumorigenesis of numerous malignancies, including hematological malignancies such as AML [11,19,20]. RAS mutations seem to be particularly frequent in inv(16)/t(16;16) AML, with a reported incidence of up to 54% [11]. Duployez et al. [11] and Boissel et al. [21] reported that NRAS and KRAS mutations were more common among AML patients with inv(16)/t(16;16) than those with t(8;21). Further studies showed that RAS mutations had no effect on overall/disease-free survival, CR, or relapse rates [22,23,24]. In our cohort, both NRAS and KRAS mutations were more frequently found among inv(16)/t(16;16) AML patients than in the t(8;21) AML group, a finding previously demonstrated among other cohorts [11,21]. These data suggest that the synergic cooperation between inv(16)/t(16;16) and RAS mutations may influence the pathophysiology of CBF-AML.

Cohesin is a multimeric protein complex that is involved in the cohesion of sister chromatids, post-replicative DNA repair, and transcriptional regulation and it is composed of 4 core subunits: the SMC1A, SMC3, RAD21, and STAG proteins [25]. Cohesin mutations have been reported in about 6% of AML patients [26] and fewer than 2% of cases of CBF-AML [11]. Recent data revealed the identification of cohesin and chromatin modifier mutations in t(8;21) but not inv(16) patients [11,27]. In the present study, mutations in genes encoding members of the cohesin complex were present in 11.3% of the t(8;21) AML patients and none of the inv(16) AML patients, and all cohesin mutations were mutually exclusive among each other, which is consistent with previous studies [26,27]. It is interesting that cohesin gene mutations are more frequent in patients with RUNX1-mutated AML [28]. Indeed, Mazumdar et al. [29] demonstrated that cohesin mutations led to a state of elevated chromatid accessibility and higher levels of binding at RUNX1 binding sites. These findings suggest links between alterations in cohesin and the RUNX1-RUNX1 fusion oncprotein. In addition, our study also suggests that the frequencies of mutations in genes associated with epigenetic modification (IDH1, IDH2, DNMT3A, and TET2), are low in CBF-AML, which is in accordance with the findings of Park et al. [30] and Duployez et al. [11]. These results may support the idea that mutations involved in epigenetic modification do not contribute to leukemogenesis in CBF-AML.

Yang et al. [23] showed that AML patients with RAS mutations had significantly higher WBC counts at diagnosis than those without mutations (p=0.001). Boissel et al. [21] demonstrated that CBF-AML patients with c-KIT mutations had a significantly higher median WBC count at presentation, but this difference was mainly observed among patients with inv(16), with the mutations being less common in patients with t(8;21). Additionally, no gene mutations predicted poor response to induction in comparisons with patients without mutations for particular genes (c-KIT, RAS), except for FLT3 [21]. Jahn et al. [31] found that both DNMT3A and TET2 were associated with a significantly worse prognosis in univariate analysis. However, regarding the subgroup with c-KIT, NRAS, FLT3, CSF3R, and TET2 mutations, WBC count and CR rate were not compared between inv(16)/t(16;16) AML and t(8;21) AML patients in previous studies. Our results showed that patients with inv(16)/t(16;16) had significantly higher WBC counts than those with t(8;21) in the context of c-KIT or NRAS mutations, but no significant differences were found for CR rates.

Conclusion

This study has comprehensively analyzed the genetic mutations of 134 CBF-AML patients to characterize certain crucial genetic characteristics, compare the mutational profiles of t(8;21) AML and inv(16)/t(16;16) AML, and establish unique genetic maps. The major limitations of our study are the absence of survival data, because these patients received treatment in different medical institutions, and the fact that the prognosis of these patients was affected by diverse factors including physical status, financial situation, and the different consolidation regimens that were administered. The molecular mechanisms, exact characteristics, and clinical implications of these mutations require further study.

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Ethics

Ethics Committee Approval: The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki.
Authorship Contributions

Surgical and Medical Practices: H.H.; Concept: N.J.; Design: W.Q.;
Data Collection or Processing: X.Ch.; Analysis or Interpretation:
H.J.S., Ca.; Literature Search: Z.W.; Writing: N.J.

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