Association of CDKN2A/2B deletion with relapse after hematopoietic stem cell transplantation for acute lymphoblastic leukemia

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Abstract

The most important prognostic factor for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) is minimal residual disease (MRD). Previous studies have reported copy number variants of genes such as IKZF1, CDKN2A/2B, and PAX5. These gene mutations can be analyzed using multiplex ligation-dependent probe amplification (MLPA), which is less costly and easier to perform than large-scale gene mutation analyses. In this study, we performed copy number variant analysis of leukemia cells at the first onset of Ph+ALL in a case series of allogeneic hematopoietic stem cell transplantation (allo-HSCT) using the MLPA method. We analyzed how it influenced allo-HSCT prognosis together with MRD information.

CDKN2A/2B copy number variations significantly increased the rate of post-transplant recurrence (P=0.025) and significantly reduced disease-free survival (P=0.015). Additionally, patients with IKZF1 deletions had a significantly higher post-transplant recurrence rate (P=0.042). Although they were positive for pre-transplant MRD, no relapse was observed in patients with wild-type copy number variations in IKZF1 or CDKN2A/2B. CDKN2A/2B copy number variation is a crucial factor that can be confirmed at initial onset as a post-transplant prognostic factor of Ph+ALL.

Key words Ph+ALL, CDKN2A/2B, IKZF1, copy number alteration

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Introduction

Treatment outcomes for Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) have improved owing to the emergence of tyrosine kinase inhibitors (TKI)1,2. Gene abnormalities affecting Ph+ALL prognosis have been reported in genes other than BCR-ABL, such as IKZF1, PAX5, and CDKN2A/2B. It has also been clarified that each is a poor prognostic factor1–4. Recently, excellent treatment outcomes have been reported for Ph+ALL treated with blinatumomab and TKI without chemotherapy. However, these gene defects were also poor prognostic factors in the protocol1. Multiplex ligation-dependent probe amplification (MLPA) is a convenient method for detecting genetic abnormalities such as copy number variations. In this study, we analyzed the treatment outcomes of Ph+ALL after allogeneic hematopoietic stem cell transplantation (allo-HSCT) using the MLPA method to measure defects in eight genes, including IKZF1, PAX5, and CDKN2A/2B.

Minimal residual disease (MRD) is the most powerful and important prognostic factor of ALL5. However, it has been evaluated during treatment. Negative measurable MRD at the end of the initial induction chemotherapy is a reliable and good prognostic factor6,7. However, there are cases where ALL relapses, although MRD disappears early, and cases where ALL does not relapse, even if MRD persists before allo-HSCT. If genetic mutations in leukemia cells at the initial stage of the disease are identified as risk factors, in addition to MRD, the selection of optimal treatment may be possible. In addition, allo-HSCT during the first phase of Ph+ALL remission is currently recommended as the
standard treatment by the Japanese Society of Transplantation and Cellular Therapy Ph+ALL guidelines. However, considering the high rate of transplant-related mortality, selecting cases where allo-HSCT can be avoided should be considered.

Furthermore, if Ph+ALL with poor allo-HSCT results could be extracted at the time of disease onset, it would be helpful to optimize chemotherapy until allo-HSCT is performed or transplantation methods are considered. In this regard, risk assessment at disease onset is considered more important than MRD because MRD is assessed during treatment. In this study, we believe gene mutation analysis at the beginning of the disease might be an effective prognostic factor other than MRD; hence, we conducted a retrospective analysis at a single center.

Materials and Methods

Patients
Among the Ph+ALL cases in which allo-HSCT was performed at our institution between December 2006 and October 2021, 21 cases where leukemia cells were preserved at the time of onset or recurrence were analyzed in this study. The observation period was from March 2022. Chemotherapy before transplantation included hyper-CVAD (fractionated cyclophosphamide, vincristine, adriamycin, and dexamethasone) therapy in three patients and Japan Adult Leukemia Study Group (JALSG) multidrug chemotherapy in 18 patients.

Of the 21 patients, 11 received imatinib, and 10 received dasatinib. Pre-transplant treatment included three cases of reduced-intensity conditioning (RIC) and 18 cases of myeloablative conditioning (MAC). For RIC, we used fludarabine+melphalan±total body irradiation (TBI) of 4 Gy in 3 cases. For MAC, we used cyclophosphamide (CY)+TBI of 12 Gy in 10 cases, etoposide (VP)+CY+TBI of 12 Gy in 7 cases, and cytarabine (CA)+CY+TBI of 10 Gy in 1 case. For graft-versus-host disease prophylaxis, cyclosporine (CsA) and short-term methotrexate (sMTX) were administered to HLA-matched siblings, and tacrolimus (Tac)+sMTX was used as an unrelated donor source. In one case, with the mother as the donor, Tac+sMTX was used because the HLA disparity was 4/6. The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Institutional Review Board of Tokai University Hospital (101-61/121-09).

Sample collection and methods
Mononuclear cells were isolated from the nucleated bone marrow cells at the time of initial onset and stored in liquid nitrogen until analysis. DNA was extracted from stored samples (Qiagen DNA Blood Kit; Qiagen Inc., Redwood City, CA, USA) and adjusted to a Tris-EDTA buffer concentration of 50 ng/L. According to the MLPA kit protocol (MRC Holland, Amsterdam, Netherlands), denaturation, hybridization, ligation, and polymerase chain reaction (PCR) were performed in a thermal cycler and analyzed using a capillary sequencer. As in the previous report, a probe ratio between 0.75 and 1.3 was within the normal range. A probe ratio <0.75 or >1.3 indicated a deletion or gain, respectively. A probe ratio <0.25 or >1.8 indicated biallelic deletion or amplification, respectively. SALSA MLPA Probesmix P335 ALL-IKZF1 (MRC Holland, Amsterdam, Netherlands) was used to detect alterations in EBF1, IKZF1, CDKN2A, CDKN2B, JAK, PAX5, ETV6, RB1, and BTG1 expression. IKZF1+ was defined as IKZF1 associated with PAX5, CDKN2A, or CDKN2B deletions, or both.

MRD measurements were evaluated as the first MRD immediately before consolidation therapy after remission induction therapy and were performed 2-4 weeks before allo-HSCT. The first MRD measurement was quantitative using real-time PCR or qualitative using nested PCR. The second measurement before transplantation was performed using nested PCR. The second PCR product was confirmed using electrophoresis and defined as MRD-negative when no minor or major BCR-ABL bands were identified. Regarding MRD status, the first and second negatives were designated as early responders, the first positive and second negative were designated as late responders, and the second positive or non-remission was designated as poor responders.

Statistics analyses
All categorical variables were compared using Fisher’s exact test, including patient characteristics, disease status, transplantation characteristics, and MRD status. Quantitative variables, such as age, white blood cell (WBC) count, and blast ratio, were compared using t-tests. Time-to-event analyses were performed using the Cox regression model to determine the association between gene deletion and overall survival (OS) and disease-free survival (DFS). Other clinical features such as age, WBC count, blast count at diagnosis, and MRD status were also analyzed using the Cox regression model to determine the relationship between OS and DFS.

Gene deletions, other clinical features, and relapse were analyzed using a competing risk regression model to determine death without recurrence as a competing event. MLPA and post-transplantation results were evaluated for IKZF1, CDKN2A/2B, PAX5, and IKZF1+ abnormalities. The number of gene deletions (0-6) was...
classified and evaluated as 0, 1, 2, 3, and more. Relapse was considered a hematological recurrence, whereas molecular genetic recurrence was not a recurrence event. Statistical significance was defined as $p<0.05$. All statistical analyses were performed using Stata software version 16 (College Station, TX, USA).

**Results**

**Outcomes of transplantation**

The transplantation information for each patient is presented in Table 1. The median follow-up period for all patients was 1,022 days (2.8 years), ranging from 30 months to 14.7 years. On the last day of the observation period, 11 of the 21 patients survived, 2 had primary disease deaths, and 8 had transplant-related deaths. Pre-transplant disease status was observed in 18 patients in remission and 3 in non-remission. Additionally, 4 patients were negative in the initial MRD measurement, and 17 were positive. The second MRD measurement before allotransplantation showed 8 negative and 10 positive cases. Therefore, the MRD status was as follows: 4 cases of early responders, 4 of late responders, 3 not in complete remission (non-CR), and 10 poor responders. OS, DFS, and recurrence rates were assessed according to age, initial WBC count, initial blast count, and MRD status, none of which had a significant effect (age: $P=0.61$, $P=0.97$, and $P=0.87$; WBC count: $P=0.68$, $P=0.96$, and $P=0.67$; blast count: $P=0.70$, $P=0.99$, and $P=0.069$; MRD: $P=0.82$, $P=0.96$, and $P=0.75$). Furthermore, 3 patients had significantly worse OS and DFS [hazard ratio (HR), 11.3; 95% confidence interval (CI), 1.8-72.5, $P=0.010$ and HR, 14.9; 95% CI, 2.1-107.3, $P=0.007$]; all these patients died from transplant-related complication without relapse of disease.

**MLPA analysis**

MLPA tests showed that 71.4% of patients (15/21) had at least one abnormality involving *IKZF1, CDKN2A/2B, JAK, PAX5, EBF1, ETV6, BTG1*, or *RB1*, whereas the remaining 28.6% (6/21) did not have any of these abnormalities. Copy number analysis showed that *IKZF1* deletion was the most frequent variation (11 patients [52.4%]), followed by *CDKN2A* or *CDKN2B* (10 patients [47.6%]), *PAX5* (7 patients [33.3%]), *RB1* (4 patients [19%]), *BTG1* (6 patients [28.6%]), *ETV6* (2 patients [9.5%]), and *JAK* and *EBF1* (1 patient [4.8%]) (Figure 1). Of the 21 patients, 47.6% were classified as *IKZF1+*. The features of Ph+ALL, *IKZF1, CDKN2A/2B, PAX5*, and *IKZF1+* cells are shown in Table 2.
Table 2. Characteristics of patients and leukemia by IKZF, CDKN2A/2B, and PAX5

<table>
<thead>
<tr>
<th></th>
<th>IKZF deletion</th>
<th>CDKN2A/2B deletion</th>
<th>PAX5 deletion</th>
<th>IKZF+ deletion</th>
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<tr>
<td></td>
<td>Deletion</td>
<td>Wild type</td>
<td>P-values</td>
<td>Deletion</td>
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<tr>
<td>Age (years)*</td>
<td>35 (18-52)</td>
<td>42 (20-59)</td>
<td>0.32</td>
<td>37.5 (18-53)</td>
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<tr>
<td>Sex (n)</td>
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<td></td>
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<td></td>
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<tr>
<td>Male</td>
<td>9</td>
<td>5</td>
<td>0.18</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>5</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>WBC at diagnosis (×10⁶/µL)*</td>
<td>6.4 (2.9-9.9)</td>
<td>6.0 (1.7-10.2)</td>
<td>0.86</td>
<td>6.4 (2.6-10.2)</td>
</tr>
<tr>
<td>Blast at diagnosis (%)*</td>
<td>88.0 (79.5-96.6)</td>
<td>87.9 (79.4-96.4)</td>
<td>0.98</td>
<td>87.3 (77.9-96.7)</td>
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<tr>
<td>Disease status (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
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<td>9</td>
<td>0.09</td>
<td>6</td>
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<tr>
<td>non CR</td>
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<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MRD status (n) **</td>
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</tr>
<tr>
<td>Early responder</td>
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<td>3</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Late responder</td>
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<td>1</td>
<td></td>
<td>2</td>
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<tr>
<td>Poor responder</td>
<td>5</td>
<td>5</td>
<td>0.42</td>
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</table>

*Values are median (range)
**This analysis excluded non CR patients hematologically.
CR, complete remission; MRD, minimal residual disease.
Transplantation outcomes and MLPA results

The effects of IKZF1, CDKN2A/2B, and PAX5 deletions on the OS, DFS, and recurrence rates were analyzed. Similar analyses were performed for IKZF1+ and all seven gene copy number variations. Gene defects that significantly affected OS were not observed in any of the analyses (IKZF1; P=0.75; CDKN2A/2B; P=0.25; PAX5; P=0.82; JAK; P=0.056; ETV6; P=0.12; RB1; P=0.086; BTG1; P=0.32; and EBF1; P=1.0). The DFS rate was significantly lower in patients with CDKN2A/2B deletion only than wild-type CDKN2A/2B (HR, 4.1; 95% CI, 1.20-13.8; P=0.024) (Figure 2). Relapse rates were significantly higher for than each wild-types IKZF1, CDKN2A/2B, and IKZF+ defects (P=0.042, 0.025, and 0.025, respectively). However, PAX5 deletion was not associated with the relapse rate (Figure 3). Similarly, the other gene defects were not associated with the relapse rate (ETV6; P=0.25, BTG1; P=0.058, and JAK, RB1, and EBF1 were not calculable by zero events). A comparison of the total number of genetic mutations measured using MLPA did not affect the recurrence rate.

Next, the relative CDKN2A/2B deletion, DFS, and relapse rates were analyzed (excluding the four non-CR cases). In these 18 cases, CDKN2A/2B deletion was not significantly associated with DFS (P=0.098). However, the deletion was significantly associated with the relapse rate (P=0.0032).

Despite the late or poor MRD response, no patient had relapsed leukemia when genetic aberrations in IKZF1 or CDKN2A/2B were wild-type. The MRD status at allo-HSCT was not significantly associated with relapse after transplantation (P=0.75).

Discussion

Although the number of cases was small, the treatment outcomes of allo-HSCT for Ph+ALL at a single institution were analyzed based on gene defect analysis using MLPA. DFS and recurrence rates were significantly higher in patients with CDKN2A/2B deficiency, and recurrence rates were significantly higher in cases with IKZF1 and IKZF1+ gene defects. In this cohort, the presence or absence of MRD immediately before transplantation did not affect the transplant outcomes, suggesting that CDKN2A/2B deficiency may be a...
stronger prognostic factor than MRD deficiency.

The results of allo-HSCT for Ph+ALL have shown that the presence or absence of CDKN2A/2B deficiency affects DFS more strongly than IKZF1 deficiency. Furthermore, IKZF1 deficiency did not affect prognosis in adults with Ph+ALL and Ph-ALL disease. Moreover, IKZF1+ with CDKN2A/2B or PAX5 deficiency is a poor prognostic factor compared to IKZF1 alone, suggesting that CDKN2A/2B deficiency is an important prognostic factor. Although this study was conducted at a single institution, the same trend as that previously reported was confirmed for allo-HSCT for Ph+ALL in Japan.

CDKN2A encodes p16 (INK4a) and p14 (ARF), and INK4a controls cell cycle progression by blocking the activities of CDK4 and CDK6. ARF is a physiological inhibitor of MDM2, an E3 ubiquitin ligase that controls the activity and stability of P53. CDKN2B encodes a cyclin-dependent kinase inhibitor that forms a complex with CDK4 or CDK6 and prevents the activity of CDK, controlling cell cycle progression through the G1 phase. In ALL, CDKN2A/2B deletion is associated with a high WBC count at the first onset, advanced age at diagnosis, and Ph-like ALL. Thus, CDKN2A/2B deletion may be an important prognostic factor of ALL.

This study aimed to determine whether genetic defect analysis at the onset of Ph+ALL could be used to determine transplantation indications. Most post-transplant recurrences of Ph+ALL occur within 2 years of transplantation. In this regard, the observation period in our study was 2.5-14.7 years, and we believe that we have obtained a sufficient analysis period to confirm the relapse. Moreover, there was no single recurrence in this analysis but rather transplant-related death that led to reduced survival rates among approximately 30% of the cases in whom the MLPA method did not detect a gene defect. These patients may be candidates for cases where allotransplantation can be avoided. However, we could not observe recurrence due to NRM. Thus, our results should be interpreted with caution owing to the small number of cases analyzed.

The limitation of this analysis was the small number of cases; therefore, we did not evaluate the relation OS, DFS and MRD, which has the most significant impact on treatment outcomes after allo-HSCT of ALL. Previous study findings, and our results, have shown that...
early MRD negativity is a positive prognostic factor\textsuperscript{9, 15}. The importance of MRD was not demonstrated in our study cohort, which included only cases in which samples for MLPA analysis were obtained, owing to the small number of cases.

In contrast, there were no cases of recurrence in MRD-positive poor responders before allo-HSCT in patients without IKZF1 or CDKN2A/2B defects. Owing to the small cohort size, other gene defects might not significantly affect the transplant outcome. Conversely, the fact that CDKN2A/2B is a factor affecting prognosis, despite the small number of cases, indicates that this gene defect is a poor prognostic factor, as shown in prior reports\textsuperscript{3, 9}. Because Ph+ALL treatment has changed markedly owing to the advent of molecular-targeted drugs, we believe that a meaningful analysis is possible in this cohort analysis, which has a relatively uniform treatment history.

In this study, we confirmed that gene defect analysis using MLPA effectively predicted the prognosis at the onset of Ph+ALL in a single-center Japanese cohort. As the number of cases increases in the future, gene defect analysis using the MLPA method can help effectively select cases in which allogeneic transplantation can be avoided, together with MRD information.

**Author Contributions**

MO, EK, and KA conceived and designed the study; MO, SM, MT, RS, DO, YA, JA, KH, RH and SS collected samples and clinical data; MO and EK performed laboratory assessments; MO and KA performed statistical analyses; MO, YO, HK, and KA interpreted the data; MO, EK, and KH wrote the manuscript and created the figures and tables.

**Conflicts of Interest**

The authors declare no conflict of interest. Disclosure forms provided by the authors are available on the website.

**References**