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REPORT

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Gene rearrangements of *MLL* and *RUNX1* sporadically occur in normal CD34$^+$ cells under cytokine stimulation

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Abstract

Gene rearrangements of *MLL/KMT2A* or *RUNX1* are the major cause of therapy-related leukemia. Moreover, *MLL* rearrangements are the major cause of infant leukemia, and *RUNX1* rearrangements are frequently detected in cord blood. These genes are sensitive to topoisomerase II inhibitors, and various genes have been identified as potential fusion partners. However, fetal exposure to these inhibitors is rare. Therefore, we postulated that even a proliferation signal itself might induce gene rearrangements in hematopoietic stem cells. To test this hypothesis, we detected gene rearrangements in etoposide-treated or non–treated CD34$^+$ cells cultured with cytokines using inverse PCR. In the etoposide-treated cells, variable-sized rearrangement bands were detected in the *RUNX1* and *MLL* genes at 3 hours of culture, which decreased after 7 days. However, more rearrangement bands were detected in the non–treated cells at 7 days of culture. Such gene rearrangements were also detected in peripheral blood stem cells mobilized by cytokines for transplantation. However, none of these rearranged genes encoded the leukemogenic oncogene, and the cells with rearrangements did not expand. These findings suggest that *MLL* and *RUNX1* rearrangements, which occur with very low frequency in normal hematopoietic progenitor cells, may be induced under cytokine stimulation. Most of the cells with gene rearrangements are likely eliminated, except for leukemia-associated gene rearrangements, resulting in the low prevalence of leukemia development.

KEYWORDS

cytokine, gene rearrangements, hematopoietic stem cells, *MLL/KMT2A*, *RUNX1*

1 | INTRODUCTION

Therapy-related leukemia occurs as a result of gene abnormalities induced by chemotherapy or radiation therapy, and is associated with a poor prognosis. Gene rearrangements of *MLL/KMT2A* or *RUNX1* are considered to be the major cause of therapy-related leukemia. Etoposide, a topoisomerase II inhibitor, can induce *MLL* gene rearrangements in human CD34$^+$ cells.[1] Etoposide induces DNA double-stranded breaks that lead to errors in DNA repair and gene translocation, resulting in variable fusion partner genes.

These rearrangements are also detected in infant leukemia outside of the context of treatment; *MLL* rearrangements are detected

in up to 80% of patients with infant leukemia.[2] Moreover, in Basecke et al, the *RUNX1-RUNX1T1* fusion gene is detected in 40% of cord blood (CB) samples.[3] During fetal hematopoiesis, hematopoietic cells show rapid expansion throughout their life under cytokine stimulation. Therefore, we postulated that even a proliferation signal itself might be sufficient to induce gene rearrangements in hematopoietic stem cells.

To explore this possibility, we evaluated these gene rearrangements in CD34$^+$ cells with etoposide treatment or simply under cytokine stimulation. Rearrangements were identified with inverse PCR (IPCR), which is a highly sensitive method for identifying a small number of gene rearrangements.[4]

2 | MATERIALS AND METHODS

2.1 | Cell lines

The *RUNX1-RUNX1T1* cell lines (Kasumi-1 and SKNO-1) and *MLL*-rearranged cell lines (KOPB-26 and YACL-95, kindly provided by Dr Sugita, Yamanashi University) were used as positive controls.

2.2 | Culture of human CD34$^+$ primary cells

Human CB CD34$^+$ cells (Riken BRC and StemCell Technologies) were precultured for 3 days in expansion medium,[5] and then treated (or not treated) with etoposide (Merck) at the clinical dose (20 μmol/L) for 1 hour. After washout, the cells were cultured in Iscove modified Dulbecco's medium with 20% FBS, 100 ng/mL each of FLT-3 ligand, stem cell factor and thrombopoietin, and 20 ng/mL each of interleukin (IL)-6 and IL-3 (all from Peprotech).[5] Annexin V staining was carried out with the PE Annexin V Apoptosis Detection Kit I (BD), and cell viability and proliferation were evaluated.

2.3 | Patients

Peripheral blood stem cells (PBSC) from seven patients with diffuse large B cell lymphoma were mobilized by granulocyte colony-stimulating factor (G-CSF) administration and were harvested for autologous stem-cell transplantation. The median age at harvest was 62 years (range 54-68 years). As a positive control of *RUNX1* gene rearrangements, cells from patients with t (8;21) leukemia were examined. The study was approved by the institutional review board, and all patients provided written informed consent.

2.4 | Inverse PCR

Genomic DNA was extracted from the cells using a Gentra Puregene Cell Kit (QIAGEN). Subsequently, 2 μg of DNA was digested with

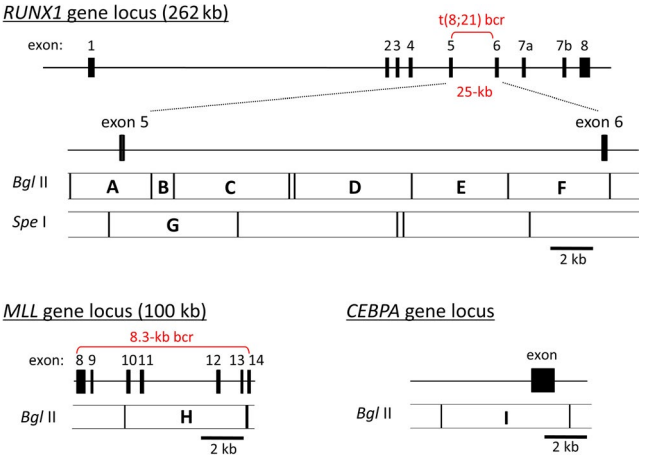

FIGURE 1 Genomic structure of *RUNX1*, *MLL* and *CEBPA*. The *RUNX1* gene spans 262 kb, and the breakpoint cluster region (bcr) of *RUNX1-RUNX1T1* is a 25-kb fragment between exons 5 and 6. The *MLL* gene spans 100-kb, and most *MLL* aberrations initiate within a particular 8.3-kb bcr between exons 8 and 14. We also analyzed the *CEBPA* gene, which is rarely involved in translocations. A to I indicate DNA fragments digested by *Bgl* II or *Spe* I

Bgl II or *Spe* I (New England Biolabs, NEB) targeting the breakpoint cluster region of the genes (Figure 1) at 37°C overnight, and purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics). The digested DNA was self-ligated with T4 DNA ligase (NEB) at 16°C overnight and purified. Gene rearrangements were assessed by IPCR using the primers listed in Table S1 and Takara LA Taq (TaKaRa). We also analyzed the *CEBPA* gene as a rarely rearranged control. The following cycles were used for IPCR: denaturing for 1 minute at 94°C, 30 cycles of 95°C (20 seconds) and 68°C (6 minutes), and final extension at 72°C for 10 minutes. Nested PCR was performed using 1 μL of the first IPCR products with 15 cycles.

2.5 | Detection of gene rearrangements

Inverse PCR products were detected using agarose gel electrophoresis. The expected sizes of germline bands are listed in Table S1. Extra bands other than those of the germline were subcloned and sequenced.[1]

3 | RESULTS AND DISCUSSION

3.1 | Effect of etoposide on the viability of CD34$^+$ cells

The positive rate of Annexin V staining peaked at 3 hours after CD34$^+$ cells were treated with etoposide. The viability and proliferation ability of the cells initially decreased, and then recovered at 7 days (Figure S1A-C). Therefore, we investigated the gene rearrangements at the time points of 3 hours and 7 days of culture with etoposide.

3.2 | Detection of gene rearrangements in cultured CB CD34$^+$ cells

Inverse PCR of the cells without translocation demonstrated exclusive germline products, whereas t (8;21) cells showed other bands besides the germline band (Figure S2A). We also detected *MLL* gene rearrangements in cell lines with 11q23 translocation (Figure S2B). However, we could not find any other bands in CB CD34$^+$ cells before culture (Figure S2C).

We ascertained gene rearrangements in CB CD34$^+$ cells cultured with cytokines with and without etoposide treatment. Representative results for eight CB samples are shown in Figure 2A. As expected, only germinal bands were detected in the *CEBPA* gene, which is rarely rearranged. However, in the *RUNX1* and *MLL* genes, etoposide-treated cells showed variable-sized bands at 3 hours of culture. These rearrangements decreased after 7 days, suggesting that most of the cells with gene rearrangements were eliminated during the culture. By contrast, only a few additional bands were detected in the non–treated cells at 3 hours. Unexpectedly, more of these additional bands were detected after 7 days of culture, with more rearrangement bands detected than in the etoposide-treated cells at this time point. This phenomenon was more apparent for *MLL* than *RUNX1* rearrangements. This result suggested that 7 days of culture without etoposide, simply under the condition of cytokine stimulation, may also induce these rearrangements.

Sequencing analysis was performed to determine the rearrangement partner genes (Table 1). We could not find any typical consensus sequences of the topoisomerase II recognition site (Tables S2 and S3). In the *MLL* rearrangements, a total of 70 fusion genes were detected, which were distributed across almost all chromosomes, with the majority identified in extragenic regions. Only one partner gene (11q24) had the same sequence in both cells with and without etoposide treatment after 7 days of culture (Figure S3). None of the partner sequences in the rearrangements matched known *MLL* partners.[4] We also analyzed a total of 52 *RUNX1* rearrangements from the etoposide-treated cells. Breakpoints associated with translocations clustered near exon 5 and exon 6. Among the 24 *RUNX1* fusion partner genes, 7 were intragenic breakpoints; however, they did not match known *RUNX1* partners. The other 17 sequences were extragenic. The cultured cells died within 1 month, which was different

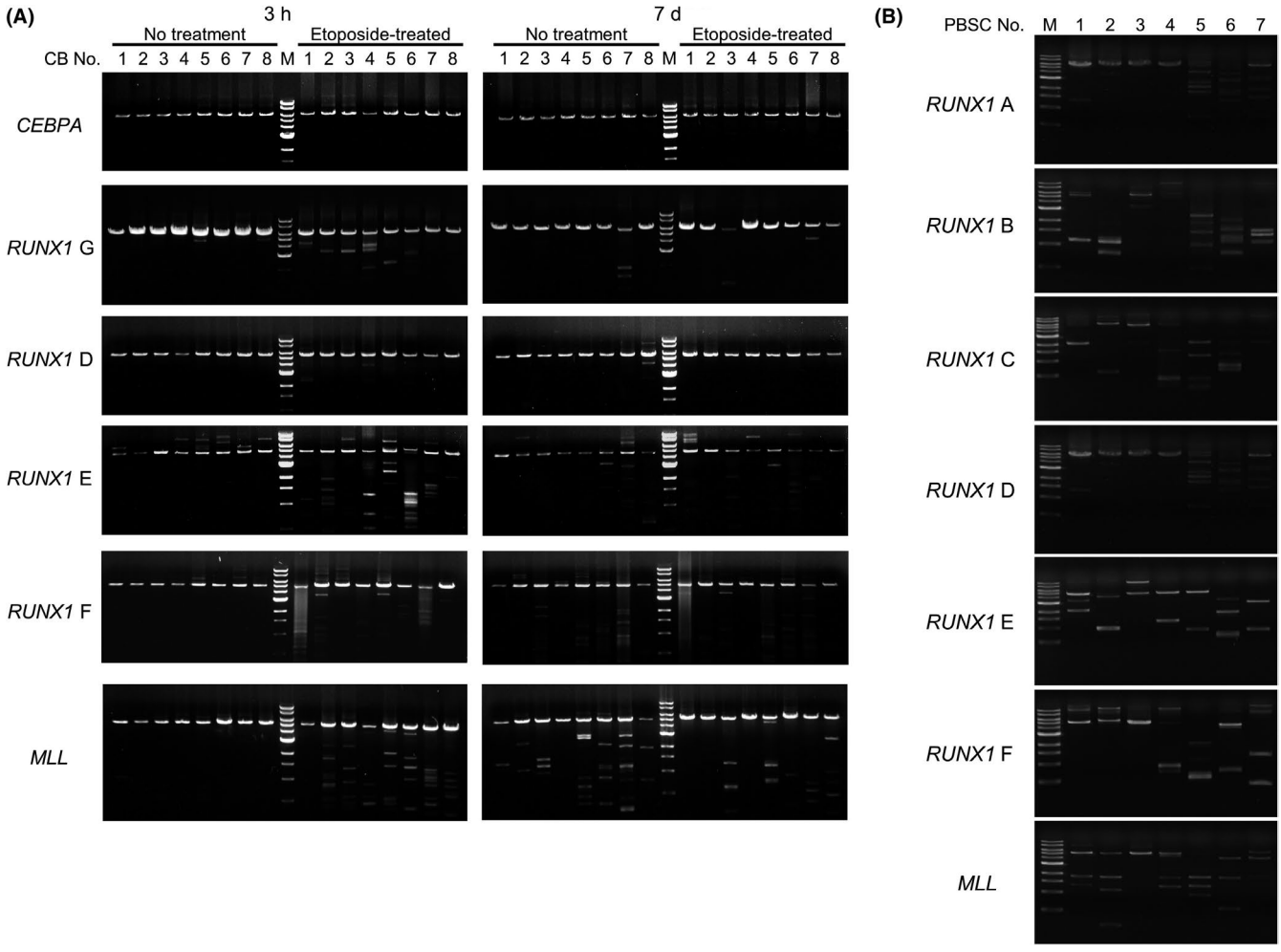

FIGURE 2 Gene rearrangements detected by inverse PCR. The size of germline bands is listed in Table S1. A, *CEBPA*, *RUNX1* and *MLL* gene rearrangements in eight representative cord blood (CB) samples. B, *RUNX1* and *MLL* gene rearrangements in peripheral blood stem cells (PBSC). M, 1-kb ladder marker

TABLE 1 The fusion partner genes of *MLL* or *RUNX1* rearrangement

	3 h		7 d	
	No treatment	**Etoposide-treated**	**No treatment**	**Etoposide-treated**
MLL				
Tandem duplication	0	0	2	1
Fusion with other genes	2	35	19	14
Location of the partner genes (intragenic)	None	1p32 (NCSTN) 2q21 (FLJ34870) 4q11-12 (SCFD2) 10q23 (KIF11) 12q24.3 (SPPL3)	1p31 (ZRANB2) 5q11.2 (LOC728103) 10p13 (C10orf97) 10p14 (CUGBP2) 14q12 (PRKD1)	3p21.1 (WDR51A) 4q35.1 (SORBS2) 7q34 (KIAA1529) 16p12 (XYLT1) 21q22 (RUNX1)
Location of the partner genes (extragenic)	17q12, Xq25	1q21, 1q23, 2p23-24, 2q36, 3p21, 4q13, 4q31.1, 6q14, 6q21, 7p11.2, 7p12, 7q13, 8q12, 8q21.3, 8q22, 8q23, 10q26, 11q23, 13q12, 13q21, 14q11.2, 15q13, 15q21-22, 18q11.2, 18q21, 19q13.2, 20p13, 22q11.2, Xq25, not mapped	2p22, 3p25, 7p15, 8q23, 8q24.2, 9p11, 9q31, 11q13, 11q24, 16q23, 18q22, Xp21, Xq24, Yp11.2	3p21, 3p21, 9p11, 9q31, 11p12, 11q24, 12p13, 17(not mapped), 20q13.2,
RUNX1				
Tandem duplication	0	26	0	2
Fusion with other genes	0	17	1	6
Location of the partner genes (intragenic)	None	8p21 (BNIP3L) 19q13.2 (HNRPUL1) Xp22.3 (CLCN4)	None	2p23.2 (BRE) 4q27 (PRDM5) 12q24.3 (KIAA1944) 14q32.12 (GOLGA5)
Location of the partner genes (extragenic)	None	1p11-12, 3q12-13.1, 3q26.2, 5q33, 8p22, 10q22, 11q13, 11q23, 12p13, 15q13, 15q25, 20q11.2, Xp11.1, Xp11.1-11.22	15q25	2q11.2, 16p11.2-12

from the case of the leukemogenic mutant-transduced cultured cells that we had analyzed previously.[5] These findings suggested that gene fusion may occur sporadically between breakpoints in easily cleaved genes, and the partner genes of rearrangements appear to be non–specific. Therefore, cells with leukemia-specific fusion genes may be selected to grow, whereas the vast majority of cells with non–specific rearrangements would be eliminated. Thus, these rearrangements are not only induced by etoposide but also sporadically occur in specific genes under cytokine stimulation.

3.3 | Detection of gene rearrangements in peripheral blood stem cells

To investigate the effect of G-CSF on the PBSC, we also performed IPCR to detect gene rearrangements. Six out of seven samples showed several abnormally rearranged bands in *MLL* (Figure 1B). Among the 13 successfully sequenced fragments, 12 were partial tandem duplications (PTD) and one fusion with an extragenic region on chromosome 11q was detected (Table S4). *Mll*-PTD alone was found to be insufficient for leukemia development in a mouse model.[6] Patients with *MLL*-PTD have other leukemogenic gene mutations and the *MLL*-PTD seem to arise after these initial mutations.[7] Therefore, if the PBSC had an initial mutation, this additional *MLL*-PTD might result in the development of leukemia.

Furthermore, numerous abnormal *RUNX1* bands were detected in all samples. Sequencing analysis of 37 rearranged fragments showed one intragenic fusion with the metabolism of cobalamin associated D gene, whereas the other 36 rearrangements were PTDs (Table S4). To date, *RUNX1*-PTD has not been reported in patients with leukemia, suggesting that cells with *RUNX1*-PTD are likely not viable.

In summary, *RUNX1* and *MLL* gene rearrangements occur in normal hematopoietic progenitor cells cultured with cytokines at the same frequency as detected in etoposide-treated cells. This may explain the mechanism of gene rearrangements in CB, infant leukemia or donor-derived leukemia. Most of these random fusion genes or partial duplications are non–functional, resulting in cell death; however, specific leukemogenic chimera genes or additional abnormalities may lead to clonal expansion followed by leukemia development. These findings provide a warning against stem cell expansion in vitro and in vivo.

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DISCLOSURE

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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