Purification of leukemic blast cells from blood smears using laser microdissection

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Abstract

In treatment of acute myeloid leukemia (AML), prognostic factors, including gene mutation and abnormal gene expression, enable risk stratification of patients. However, in the case of a small proportion of leukemic blast cells, such as AML associated with Down syndrome (AML-DS), it is not possible to examine prognostic factors precisely due to the large proportion of normal cells. Here, we present a novel method for examining prognostic factors by making a smear on a membrane slide glass from a small amount of diagnostic specimen and collecting highly pure leukemic blast cells by laser microdissection (LMD). We verified the effectiveness of this method using 10% KPAM1 cell line suspension and peripheral blood containing 20% blast cells obtained from a patient with transient abnormal myelopoiesis (TAM). After making blood smears, approximately 100 cells were collected and analyzed by direct sequencing. Frameshift mutations (2 bp deletion and 17 bp duplication, respectively) in GATA-1 were detected in each sample, suggesting KPAM1 and TAM blast cells were accurately purified. This novel method enables us to precisely examine prognostic factors in many cases, even in cases with a small proportion of leukemic blast cells or small specimens to preserve.

Background

Currently, the treatment of leukemia is risk stratified according to the abnormality of genes and chromosomes of cells [1]. Our group has reported several prognostic factors in pediatric acute myeloid leukemia (AML) [2–5]. Most of the prognostic factors have been investigated by extracting nucleic acid from diagnostic specimens, followed by examining gene mutations or expression levels. However, in case of a small proportion of leukemic blast cells, it is difficult to examine prognostic factors precisely because of a large proportion of normal cells. In our recent study, we identified CXCR4 overexpression as an adverse prognostic factor in low-risk AML [5]. However, in the groups with a smaller number of patients (intermediate and high-risk AML), no statistically significant difference was found. There are variations in the proportions of leukemic blast cells among patients; therefore, we believe the purification of leukemic blast cells leads us to find prognostic significance of gene abnormalities in groups with smaller number of patients. Moreover, sometimes it is difficult to collect abundant bone marrow specimens to preserve for examination due to myelofibrosis. Especially prognostic factors in AML associated with Down syndrome (AML-DS) are not well understood because of the above reasons. Laser microdissection (LMD) is a method to procure subpopulations of tissue cells under direct microscopic visualization [6]. After preparing pathological tissue specimens on the membrane slide glass, we can dissect and analyze a target region. LMD had been used for pathological studies, for example, investigation of gene expression levels in particular tissues [7, 8]. Single cell isolation from a blood smears according to the morphological characteristics is theoretically possible, however, there has been no examination of applying this method for purifying leukemic blast cells. Here, we present a novel method to examine prognostic factors by making a blood smear on a membrane slide glass from a small amount of diagnostic specimen and collecting highly pure leukemic blast cells by LMD. This workflow enables us to examine prognostic factors in many cases precisely even in cases with a small proportion of leukemic blast cells or small specimens to preserve.

Methods

Patient sample and cell culture

Peripheral blood of transient abnormal myelopoiesis (TAM) patient was obtained with written informed consent. The study was approved by the Ethics

Committees of Kyoto University (G-516) and was conducted in accordance with the principles set down in the Declaration of Helsinki. KPAM1 cells were cultured in RPMI1640 (NISSUI, Tokyo, Japan) supplemented with heat-inactivated 10% FBS (Gibco/Invitrogen, Tokyo, Japan), penicillin/streptomycin and 50 ng/ml SCF at 37 °C/5% CO2. KO52 cells were cultured in RPMI1640 supplemented with heat-inactivated 10% FBS, penicillin/streptomycin at 37 °C/5% CO2.

Blood smear preparation and microdissection

10% KPAM1 cell suspension and peripheral blood obtained from TAM patient were mounted on Membrane Slides (Leica, Tokyo, Japan). The air-dried slides were stained with May–Grünwald's stain solution (NACALAI, Kyoto, Japan) for 5 min, then washed with diethyl pyrocarbonate (DEPC)-treated water and stained with Giemsa's stain solution (NACALAI, Kyoto, Japan) for 20 min. Slides were washed again and dried at room temperature. Approximately 100 targeted cells were dissected under direct microscopic visualization using AS LMD (Leica, Tokyo, Japan). Slides were preserved at room temperature in a dark space.

Detection of GATA-1 mutations

Dissected cells were suspended in Buffer ATL (Qiagen, Hilden, Germany), and then DNA was extracted using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First, we performed polymerase chain reaction (PCR) with a pair of primers targeting GATA-1 gene, G1_geno_5′ ; GCCAACAGCCACGGTCGCCTACATCTGA and G1_geno_3′ ; GCCCGTTTACTGACAATCTAGGGGACGA. The reaction was performed in a 50 μL of volume containing 25 μL 2× PCR Buffer for KOD FX Neo, 10 μL dNTP mix (2 mM each), 1 U KOD FX Neo polymerase (Toyobo, Osaka, Japan), and 1 μL (10 μM) of each primer. After an initial denaturation step at 94 °C for 2 min, target DNA was amplified in 30 cycles. Each cycle consisted of denaturation at 98 °C for 10 s, annealing at 66 °C for 30 s, and extension at 68 °C for 3 min. A final extension was performed at 68 °C for 5 min. After the first PCR, we performed nested PCR using a pair of primers targeting the second exon of the gene, GATA-1_S2; TGAGGTGATGGAGTGGGAGGAGGG and GATA-1_AS2; GGTCGGCACATCCATTTGAGAAGC with same condition of the first PCR. PCR products were analyzed by agarose gel electrophoresis and purified using

a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The sequences of the purified PCR-amplified fragments were determined by direct sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on a 310 genetic analyzer (Applied Biosystems).

Results and discussion

The whole workflow is presented in Fig. 1. First, we verified the capability of this method for examining prognostic factors using KPAM1 cell line. KPAM1 is a growth factor dependent cell line established by our group from a female patient with AML-DS [9]. GATA-1 is one of the transcription factors which recognize a GATA sequence and bind DNA. It plays an essential role in differentiation and maturation of erythrocytes and megakaryocytes [10]. GATA-1 exists on the X chromosome and its mutation is associated with a pathogenic mechanism of TAM and its progression to AML-DS in patients with Down syndrome [11–13]. GATA-1 mutations are basically detected in TAM and AML-DS blast cells. Thus, this mutation can be a marker for purity in dissecting TAM and AML-DS blast cells. We dissected KPAM1 from large proportion of other cells according to the morphological characteristics by LMD, followed by GATA-1 mutation analysis. The 10% KPAM1 cell suspension was obtained by mixing KPAM1 with KO52, one of the acute myeloblastic leukemia cell lines, at a rate of 1:9. Several blood smear samples were made on the membrane slide glasses using 10% KPAM1 cell suspension. The air-dried smear samples were stained with May–Grünwald–Giemsa working solution. KPAM1 can be distinguished from KO52, because of its larger size, basophilic cytoplasm, and bleb formation on the edge of cells (Fig. 2a). Using LMD, approximately 100 KPAM1 cells were collected according to the morphological characteristics. DNA was isolated and GATA-1 mutation was analyzed by PCR and direct sequencing. The frameshift mutation due to 2 bp deletion was detected (Fig. 2b). The reason that the wild-type signal is overlapping is thought to be that GATA-1 exists on the X chromosome and KPAM1 was established from a female patient [9]. We also compared the result of direct sequencing between KPAM1 cells dissected from 10% KPAM1 smear and 100% KPAM1 cells without dissection. We found identical results (data not shown) which implies that the KPAM1 cells were accurately purified. The results suggest that we can analyze gene mutation to investigate new prognostic factors, even if the proportion of leukemic blast cells is approximately 10%.

Next, we examined whether this method can be applied to specimens derived from patients. After informed consent was obtained, we collected peripheral blood from one patient with TAM. At diagnosis, TAM patient's WBC count was 54.1 × 109/L (blast 77%) and at collection of specimen, WBC count was decreased to 24.5 × 10^9/L (blast 20%) by treatment. Blood smear samples were made as described above using peripheral blood obtained from the TAM patient. TAM blast cells can be distinguished from bulk normal cells, because their nuclear-cytoplasmic ratio is considerably higher, nucleus is large and round or oval, and the contained chromatin is dispersed in fine strands or tiny granules (Fig. 2c). Two medical technologists who specialize in blood cell morphology concurred when determining the type of cells. Approximately 100 TAM blast cells were dissected and GATA-1 mutation analysis was performed as mentioned above. The 17 bp duplication results in a frameshift mutation were detected (Fig. 2d). It is a noteworthy that the wild-type signal was hardly detected, which implies the TAM blast cells were accurately purified. The result suggests that we can enhance the sensitivity of gene mutation analysis using this workflow. We also confirmed the capability of examining mRNA expression using this method (data not shown). Using this method, it is possible to analyze cases which previously could not be examined due to the small proportion of leukemic blast cells and the small amount of specimen. Some groups investigate prognostic factors after sorting blast cells using fluorescence-activated cell sorting (FACS) [14, 15]. FACS may be relatively useful in cases in which leukemic blast cells are extremely rare; however, our method is advantageous in two points. First, antibodies are not required in our method and we can purify cells at low cost. Second, the required amount of specimen is smaller in our method. Approximately 20 μL of blood is sufficient to make one blood smear. We have confirmed at least 30 cells is the acceptable number for mutation analysis. Moreover, we can use the blood smears repeatedly and apply them for purifying other blood cells as well as leukemic blast cells. We have ascertained the acceptable period to preserve the blood smears for examining gene mutations is at least one year. The Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) has already started a project to investigate new prognostic factors in pediatric AML using our method. By applying our method not only to AML but also other blood diseases, we may elucidate the new pathological mechanisms.

Conclusions

To summarize, the results suggest that LMD enables us to purify leukemic blast cells and analyze gene mutations even in cases with a small proportion of leukemic blast cells or little specimen to preserve. Using this method, we can examine prognostic factors in more cases and more precisely than more conventional methods could.

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Author's contributions

Conceived and designed the experiments: HM, TI, YK, TT, KT, EI, and SA. Performed the experiments and analyzed the data: HM, SS, TT, and KT. Wrote the paper: HM.

Conflict of interest

The authors declare that they have no competing interest.

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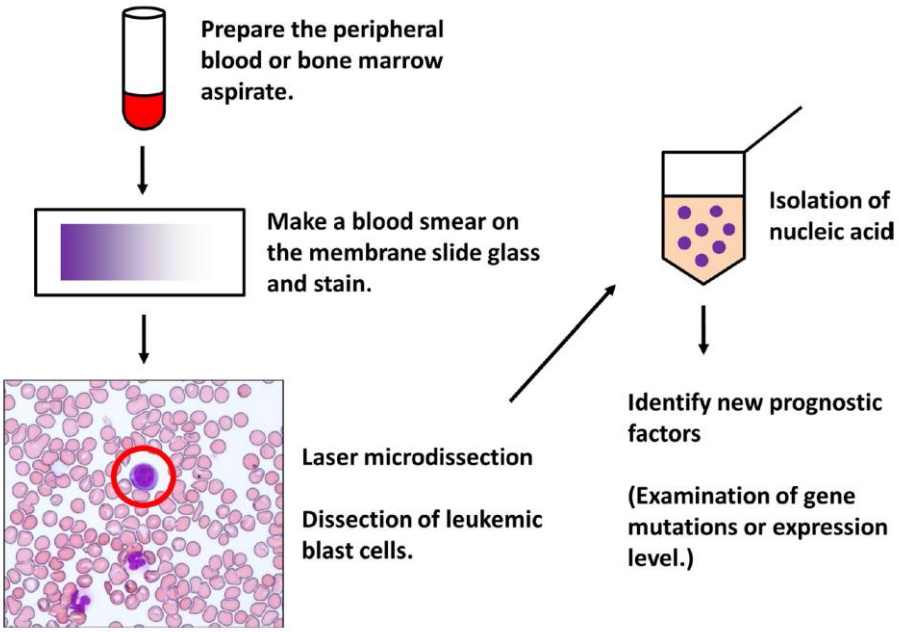

Fig. 1 Outline of procedure to identify new prognostic factors. Patient sample is mounted on membrane slides for LMD. The air dried slides were stained with May–Grünwald–Giemsa's stain solution. Cytogenetic analysis is followed by dissection of the leukemic blast cells, then isolation of nucleic acid and examination of gene mutation or expression level using the dissected material.

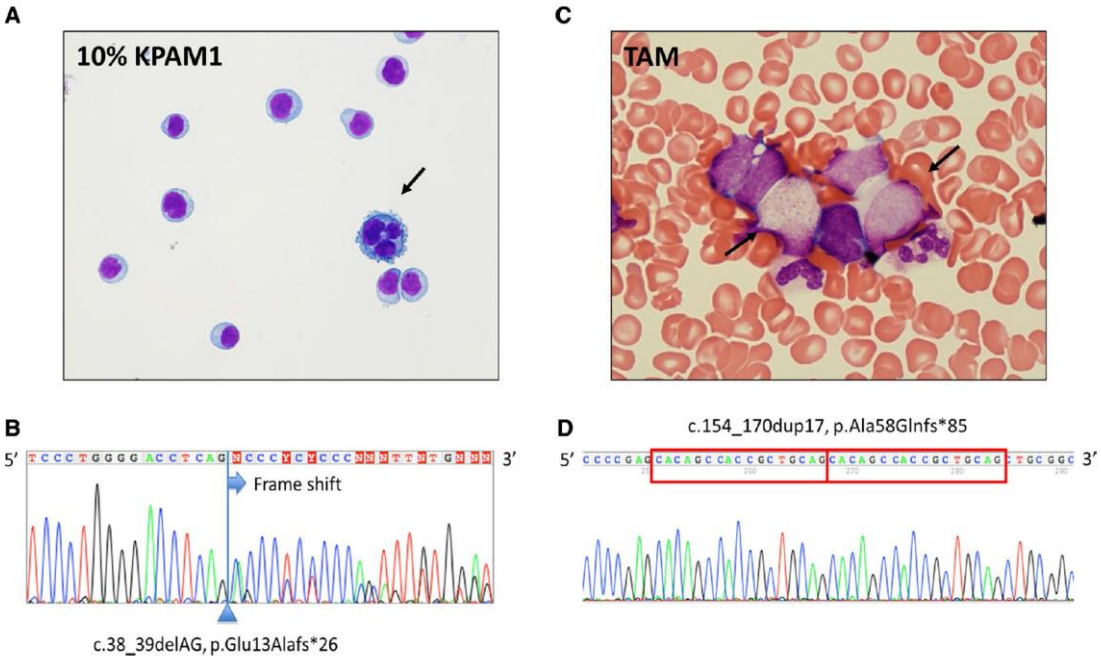

Fig. 2 GATA-1 mutation analysis of KPAM1 cell line and peripheral blood obtained from TAM patient. **a** A microscopic image of the smear sample of 10% KPAM1 cell suspension obtained by mixing KPAM1 with KO52 at a rate of 1:9. The black arrow indicates KPAM1 cell. **b** GATA-1 mutation analysis of dissected KPAM1 cells. **c** A microscopic image of the smear sample of peripheral blood obtained from TAM patient. The black arrows indicate TAM blast cells. **d** GATA-1 mutation analysis of dissected TAM blast cells

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