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Brief Report

Advancements in minimal residual disease detection: a practical approach using single-cell droplet PCR for comprehensive monitoring in hematological malignancy

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Satoshi Uchiyama*, Kentaro Fukushima*, Seiichiro Katagiri, Junichi Tsuchiya, Tomohiro Kubo, SungGi Chi and Yosuke Minami

***Abstract*:** The identification of chromosomal abnormalities accompanied by copy number alterations is important for understanding tumor characteristics. Testing methodologies for copy number abnormality have limited sensitivity, resulting in their use only for the sample provided at the time of diagnosis or recurrence of malignancy, but not for the monitoring of minimal residual disease (MRD) during and after therapy. We developped the "DimShift" technology which enable to measure the copy number of target gene/chromosome in each cell, which is given by the single cell droplet PCR. Qualitative result of DimShift given by peripheral blood was perfectly concordant with that of bone marrow. These findings and performances are promising to be the new methodology for MRD detection in malignant diseases utilizing bone marrow as well as peripheral blood.

Keywords: chromosomal abnormalities, droplet digital PCR, minimal residual disease, myelodysplastic syndrome

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Correspondence to:
Yosuke Minami
Department of Hematology, National Cancer Center East Hospital, 2-7-1 Kashiwano-ha, Kashiwa, Chiba 277-8577, Japan
yominami@east.ncc.go.jp

Satoshi Uchiyama
SungGi Chi
Department of Hematology, National Cancer Center East Hospital, Kashiwa, Japan

Kentaro Fukushima
Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Japan

Seiichiro Katagiri
Department of Hematology, Tokyo Medical University Hospital, Shinjuku City, Japan

Junichi Tsuchiya
Tomohiro Kubo
Department of Research and Development, TL Genomics Inc., Koganei City, Japan

*These authors contributed equally.

Chromosomal abnormalities are frequently observed in hematological disorders. For example, myelodysplastic syndrome (MDS) is frequently accompanied by chromosomal aneuploidies, as in chromosomes 5−, 7−, 8+, 17−, or 20−.[1–3] These chromosomal aneuploidies are worth identifying and monitoring since they provide information regarding disease status or indicate poor prognosis.[4,5] Cytogenetic methods, such as fluorescence *in situ* hybridization or G-banding, are widely used to detect chromosomal copy numbers.[6] However, because they are low-throughput methods that require numerous steps and may not be welcomed in routine laboratory workflows. Therefore, in this study, we developed a novel high-throughput and sensitive assay for aneuploidy detection using droplet polymerase chain reaction (PCR). A PCR-based method would facilitate more rapid and more frequent monitoring of chromosomal status. Generally speaking, PCR is not suitable for counting just one or two copies, although this technique works as a powerful tool for detecting a novel genetic marker, such as BCR-ABL,[7] which is frequently present in cells of patients with chronic myelogenous leukemia and acute lymphoblastic leukemia, but not in cells from healthy patients. In a general real-time PCR, the information of the template molecules is retained during the exponential amplification phase and is reflected as the difference in Ct values. However, the sensitivity is not high enough to determine the ratio of abnormal cells if a sample is a mixture of normal karyotype and aneuploid cells. In digital PCR, where DNA is amplified in a nanoliter-sized compartment, the reaction is efficient enough for the amplification of only one molecule of DNA.[8] At the endpoint, however, the high efficiency makes it impossible to preserve information regarding the number of template molecules. The results only provide information as to whether or not the DNA of interest was amplified, that is, whether the target DNA was present or absent. We speculated that information on the number of template molecules might be retained before the reaction reaches its

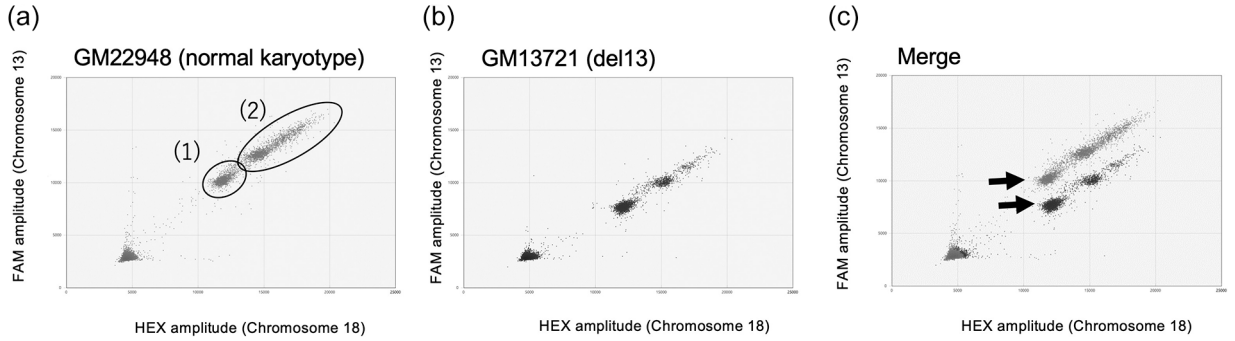

Figure 1. (a) GM22948 cells, which have a normal karyotype, were counted and the number of cells was adjusted to approximately 5000. The cells were then mixed with PCR enzymes, buffer (ddPCR Multiplex Supermix; Bio-Rad), and specific probes/primers (IDT), and subsequently loaded on a cartridge with oil to create droplets using a specialized instrument (QX200 Droplet Generator; Bio-Rad). In the 2D readout, (1) indicates the dots obtained from these droplets, which contained one cell with enzymes and probe/primers, and (2) indicates there were more than two cells in each droplet. The droplets were transferred to a PCR tube to conduct the PCR [85°C 60 min, 95°C 10 min, (94°C 30 s, and 62°C 2 min) × 23, 98°C 10 min, 10°C hold, ramp rate = 1°C/s] using a thermal cycler (C1000 Touch; Bio-Rad). The fluorescence was analyzed by the droplet reader, and the manufacturer's software displayed the 2D readout. (b) The same experiment was conducted using GM13721 cells with the deletion of chromosome 13. (c). The results from both cell line experiments were merged. FAM signals obtained from the GM13721 cells were reduced on the 2D readout (arrows).
PCR, polymerase chain reaction.

plateau in a compartmented droplet. Therefore, we attempted to convert the information on the amount of template molecules into other signals, such as fluorescence intensity.

First, we performed nanoliter-scale PCR using cells as templates. The number of cells was adjusted to approximately 5000 so that the simultaneously generated 20,000 droplets would contain single cells. The cells were then mixed with PCR enzymes, buffer (ddPCR Multiplex Supermix; Bio-Rad) and specific probes/primers (IDT), and subsequently loaded on a cartridge with oil to create droplets using a specialized instrument (QX200 Droplet Generator; Bio-Rad). The droplets were transferred to a PCR tube to conduct the PCR [85°C 60 min, 95°C 10 min, (94°C 30 s, and 62°C 2 min) × 23, 98°C 10 min, 10°C hold, ramp rate = 1°C/s] using a thermal cycler (C1000 Touch; Bio-Rad). The fluorescence was analyzed by the droplet reader, and the manufacturer's software displayed the 2D readout. The GM22948 normal karyotype without 1q deletion cell line and the GM13721 aneuploidy (deletion) of chromosome 13 cell line (Coriell Institute for Medical Research) were used in the experiments. A few dozen probes were designed and used in a multiplex reaction to cover a predominant region of a chromosome, with FAM probes targeting chromosome 13 and HEX probes for a reference chromosome. In the preliminary consideration, we examined the most suitable conditions for ddPCR. Samples were reacted with various cycles, and HEX/FAM fluorescence was measured to search for optimal conditions that could distinguish between positive and negative cells and copy number differences. Finally, positive droplets were selected by Ct 23 of ddPCR, as shown in Figure 1; the intensities of FAM on the 2D readouts differed, while HEX signals gave the same range of intensities. Compared to the FAM signal obtained from GM22948, GM13721 gave a reduced FAM signal. It should be noted that the signal dots were surely derived from cell templates and not from cell-free DNA because the dots appearing at the diagonal on the 2D readout were both positive for FAM and HEX. In our method, when cell-free DNA is amplified, it is depicted in the FAM-only and HEX-only positive populations, which means that it is theoretically not represented in the double positive HEX-positive FAM-positive cell population. Cell-free DNA contaminating a sample[9] might also give some nonspecific signals, but the possibility that one droplet contained cell-free DNA responding to both FAM and HEX probes simultaneously was considered to be quite low.[10]

Next, we applied the technology to patients' materials and compared the results to conventional methods as a pilot study. We examined the clinical availability by analyzing chromosomes 5, 7, 8, 17, and 20, using peripheral blood or bone marrow samples from 15 patients diagnosed with MDS (Supplemental Table 1). Patients with

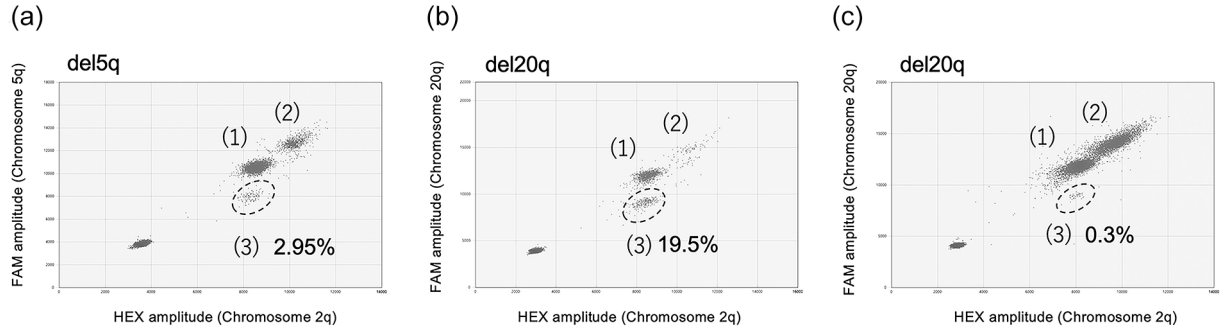

Figure 2. Peripheral blood was collected from a patient with MDS. The blood cells were hemolyzed and then used in the new assay to determine the copy number of chromosome 20. In the 2D readout, (1) indicates the signals from each droplet with one normal karyotype cell, (2) indicates more than two cells, and (3) indicates groups of deleted cells. The numbers of (3) dots were divided by the total values of (1), (2), and (3) to calculate the population of abnormal cells as 0.3%. The manufacturer's software compensated for the number of droplets with more than two cells using a Poisson distribution. (a), (b), (c): each result was shown with each sample from different patients.
MDS, myelodysplastic syndrome.

MDS often exhibit copy number abnormalities of these chromosomes. As with the results of cell lines, the new assay detected chromosomal copy number abnormalities in clinical samples (Figure 2). As summarized in Supplemental Table 2, the results obtained by the new assay showed a qualitative concordance with those obtained by cytogenetical methods [see also Figure 2(a) and (b)]. In one case, with regard to chromosome 20, the new assay detected a small population of cells (0.3%) with the deletion [Figure 2(c)], while G-Banding did not detect such an abnormality. Since G-Banding usually examines 20 cells in each test, a small population of less than 5% is theoretically overlooked in this test. Although the results obtained using the new assay were not verified by another method, it is possible that the discrepancy in detecting the small population was due to the higher sensitivity of the new assay.

In another case, the new assay did not detect the deletion of chromosome 20, which was detected by G-Banding. Considering that this patient had a very complicated karyotype with eight marker chromosomes[11] (58<2n>, XY, +2, der(3)t(1;3)(q25;p13)ins(3;?)(p13;?), +del(6)(q?), +8, add(9)(p11), +add(11)(p11.2), +14, −16, +18, +19, −20, −20, +8mar), some part of chromosome 20 could have been integrated into marker chromosomes to give the impression of a normal amount of that chromosome. It should be noted that, unlike G-Banding, the new assay does not provide visual information, but does provide objective molecular information. Taken together, the correspondence with the cytogenetic results demonstrates that the new assay could be clinically useful for the qualitative assessment of chromosome copy numbers.

PCR is reportedly unsuitable for counting a small number of template molecules. The newly developed assay could be a breakthrough in the field of chromosomal tests, where cytological methods have been predominantly applied for several decades. As a rapid, high-throughput method with high sensitivity, the new assay is expected to become a new standard for counting chromosomes in the near future. Considering the molecular mechanism, this new assay should not be applied for detecting translocations. For example, t(4;14) and t(14;16) are frequently observed in patients with multiple myeloma.[12] For many such translocation-type genetic abnormalities, specific primers for the responsible gene are used, and quantitative PCR is performed. The new test method we have developed is more suitable for detecting numerical chromosomal aberrations than for detecting translocation-type genetic abnormalities. It can also perform these chromosomal numerical aberrations more quickly and inexpensively than previous methods and can assist in clinical decision-making.

Declarations

Ethics approval and consent to participate

The clinical study was conducted at the National Cancer Center East Hospital, Osaka University Graduate School of Medicine, and Tokyo Medical University Hospital with the approval of

the Institutional Review Boards of each institution. Patient samples were collected after obtaining informed consent.

Consent for publication

Written consent was obtained for the publication of de-identified medical information.

Author contributions

Satoshi Uchiyama: Conceptualization; Resources; Writing – original draft.

Kentaro Fukushima: Investigation; Resources; Writing – review & editing.

Seiichiro Katagiri: Investigation; Resources.

Junichi Tsuchiya: Investigation; Methodology; Validation; Writing – original draft.

Tomohiro Kubo: Investigation; Methodology; Writing – original draft.

SungGi Chi: Conceptualization; Investigation; Resources.

Yosuke Minami: Conceptualization; Investigation; Resources; Supervision; Validation; Writing – review & editing.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

Contact the corresponding author for access to the original data.

ORCID iD

Kentaro Fukushima https://orcid.org/0000-0002-8003-2584

Supplemental material

Supplemental material for this article is available online.

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