論　文　内　容　要　旨

Single-cell DNA and RNA sequencing of circulating tumor cells

(循環腫瘍細胞の１細胞遺伝子解析)

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Recently, circulating tumor cells (CTCs) have been considered to represent the precise tumor heterogeneity and overall tumor characteristics. In previous studies, cellular heterogeneity in CTCs was shown to reflect the spectrum of mutations in tumor tissues, including primary and metastatic sites, and the existence of small subpopulations with different malignant profiles. CTC profiling at the single-cell level most accurately represents tumor heterogeneity and provides useful information for cancer treatment and research. After isolating the CTCs, their DNA and mRNA is amplified by whole-genome or whole-transcriptome amplification (WGA, WTA, respectively). WGA is classified as PCR-based, isothermal multiple displacement amplification (MDA)-based, and PCR with MDA hybridized methods such as multiple annealing and loop-based amplification cycling (MALBAC). Similar to WGA, many WTA methods can be used for low-input RNA samples. These methods use different priming strategies (polyT priming or random priming with ribosomal RNA depletion), second strand cDNA synthesis (polyA tailing or template switching), and cDNA amplification (PCR or isothermal in vitro transcription) approaches. Variations in these methods can affect amplification efficiency and accuracy, as well as introduce unwanted bias. In this study, we simulated single-cell sequencing of CTCs and used a TGW neuroblastoma cell line.

After spiking TGW neuroblastoma cells into blood derived from healthy volunteer, the cells were isolated by fluorescence-activated cell sorting using GD2 antibody. DNA and mRNA were amplified by four different WGA and three WTA methods, followed by single-cell DNA and RNA sequencing. In WGA methods, we examined one PCR-based, two MDA-based, one PCR with MDA hybridized WGA methods as follows. In the PCR-based method, fragmented DNA after enzymatic digestion is ligated to linker adaptors with universal sequences and amplified by linker adaptor-specific primers. The one MDA-based method uses the DNA primase Thermus thermophilus (Tth) PrimPol without artificial primers. TthPrimPol randomly synthesizes short DNA primers, and phi29 DNA polymerase begins processive polymerization using these primers. The other MDA-based method uses artificial random primers for processive polymerization by phi29 DNA polymerase. The PCR with MDA hybridized method generates looped DNA molecules by eight cycles of multiple displacement preamplification using specifically designed MALBAC primers and Bst DNA polymerase with strand displacement activity. The looped amplicons are further amplified by PCR. We evaluated the depth and uniformity of coverage using Hiseq 2500 (Illumina) for comprehensive genetic profiling of CTCs at the single-cell level. Multiple displacement amplification. In WTA methods, we examined three different WTA methods consisting of two PCR-based methods and one MDA-based method. The PCR-based methods use oligo-dT primers to reverse-transcribe polyA mRNA and template switching for second-strand cDNA

synthesis. The one PCR-based WTA method uses locked nucleic acid (LNA) technology with template-switching oligonucleotides containing modified guanosine and locks the first-strand cDNA, contributing to efficient second-strand cDNA synthesis. The MDA-based WTA method uses oligo-dT primers to reverse-transcribe polyA mRNA and phi29 DNA polymerase for cDNA amplification. We evaluated the number of sequenced reads from transcripts and gene expression patterns using Hiseq 2500 and Miseq for comprehensive transcriptomic profiling at the single-cell level. We also compared these parameters between WGA, WTA samples, and the bulk samples of 1×10^6 TGW cells.

In results, MDA-based WGA methods showed higher amplification efficiency than other methods with a comparable depth of coverage as the bulk sample. The uniformity of coverage greatly differed among samples (12.5–89.2%), with some samples evaluated by the MDA-based WGA method using phi29 DNA polymerase and random primers showing a high (>80%) uniformity of coverage. On the other hand, the MDA-based WTA method less effectively amplified mRNA and showed non-specific gene expression patterns. This possibly occurr because of amplification bias to shorter or fragmented low-quality mRNA caused by phi29 polymerase and linear cDNA amplification. The PCR-based WTA using template switching with locked nucleic acid technology accurately amplified mRNA from a single cell.

In conclusion, we performed single-cell sequencing of CTCs using a combination of FACS and various WGA and WTA methods. The MDA-based WGA method using the combination of phi29 DNA polymerase and random primers showed high performance for single-cell DNA sequencing, whereas the PCR-based WTA method using the combination of template switching and LNA technology showed high performance for single-cell RNA sequencing. Although we present a more reliable and adaptable approach for CTC profiling at the single-cell level, further investigation of other metrics such as the accuracy of variant calling or tools of QC of WTA samples to perform single-cell sequencing of CTCs derived from clinical patients should be performed. Such molecular information on CTCs derived from clinical patients will promote cancer treatment and research.