단일세포 전사체학 분석을 통한 간암 발병기작의 이해

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Translational Application of Single-cell Transcriptomic Analysis in Hepatocellular Carcinoma

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The emergence of single-cell technology in recent years has made remarkable progress for understanding of biological process in various diseases including cancers. In particular of cancer, single-cell transcriptome analysis provides a powerful tool for comprehensive characterization of cancer cell subpopulations within a heterogeneous bulk populations. Hepatocellular carcinoma (HCC) is a highly heterogeneous cancer and its molecular mechanism is extremely complex associated with a poor prognosis. To date, the molecular mechanisms of hepatocarcinogenesis remain unclear. Here, I review current status of single-cell transcriptome analysis for HCC, focusing on their application for cancer genomics, circulating tumor cells, cancer stem cells and tumor infiltrating cells in HCC. (**J Liver Cancer 2018;18:1-8**)

Keywords: Sequencing; Hepatocellular carcinoma; Transcriptome; RNA sequencing; Circulating tumor cells

Received Feb. 16, 2018
Revised Feb. 16, 2018
Accepted Mar. 7, 2018

INTRODUCTION

Precision medicine is a medical service that provides the individual patients with the prediction, diagnosis and prevention of disease, and the most optimized tailored treatment on the basis of the unique characteristics of each person in terms of genetics, environments and life style. In recent date, advances

in genomics with available big data in the field of health have ushered in a new era of precision medicine. In particular, therapeutic paradigm for cancer has shifted from nonspecific cytotoxic treatment strategy toward precision medicine which takes into account individual genetic features. The human genome map has been constructed through the Human Genome Project, which was started in 1990 and completed in 2003, and the genetic information of the individual is the most important factor to realize such precision medical care, and can be used for various diseases. DNA sequencing was the basis for the human genome project, but since the next generation sequencing (NGS) has been introduced due to the development of molecular biology techniques, full-length genome sequencing analy-

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sis, Whole Genome Sequencing, and Whole Exome Sequencing (WES), which analyzes the entire exon region, are used for early diagnosis of specific diseases. With the development of genomics, gene analysis using Big Data will accelerate the realization of personalized treatment for cancer.

In the last decades cDNA microarray-based comparative geneomic hybridization and expression profiling methods based on proteomics have not only been used to investigate the molecular mechanism of carcinogenesis,^{1,2} but also been used in clinical settings for evaluating tumor staging and predicting prognosis of cancer.²⁻⁴ However, these expression profiling methods have limitations in detecting such as alternative splicing or gene fusions. Recently, with advancement in NGS technology, RNA-sequencing (RNA-seq) enables unpredictable sensitivity and transcriptomic profiling to discover new RNA species and to further develop a comprehensive transcriptome study.^{5,6} In general, it is well known that the development of cancer is caused by genetic alterations such as gene mutations or gene translocation and epigenetic regulations including DNA methylation, histone modification or non-coding RNA. In particular, NGS-based transcriptome analysis (RNA-seq) is a powerful tool for both defining the transcriptomes of cells and delineating alternative splicing variants associated with cell function.⁵ In general, conventional molecular profiling of a solid tumor is based on an average molecular signatures and phenotype from a bulk sample composed of in a variety of cells population. However, there is significant difference in transcriptional profiling between bulk RNA-seq and scRNA-seq. Because each cell in the tumor is constantly differentiated and proliferated, and heterogeneous in existing cells, there are many limitations in understanding the pathogenesis of cancer, and many obstacles in the development of diagnostic biomarkers and the development of target therapeutics for cancer.⁷ In order to overcome these problems, the emerging single cell RNA-seq (scRNA-Seq) is able to dynamically analyze the genetic and cytologic heterogeneity of each cell in specific tumor tissues, which it is possible to comprehensively study the molecular mechanism of carcinogenesis and the process of cancer evolution, and resistance to chemotherapy.⁸⁻¹⁰ The appropriate targets for studying the heterogeneity of single cells include heterogeneous cells in the

tumor tissue,¹¹ cancer stem cells (CSC),¹² circulating tumor DNA¹³ and cell-free DNA.¹⁴ The heterogeneity of single cells is manifested in cell morphology, cell phenotype and genomics and proteomics. This heterogeneity can be analyzed in a variety of ways: imaging for cell morphology, immunofluorescence for cell phenotype, single-cell sequencing including WES and single-cell Western blotting or single-cell proteomics.

1. Workflow of single-cell RNA sequencing

Human primary tumors can be divided into two main categories: hematologic and solid tumor. Comprehensive characterization of different cell types from heterogeneous bulk tissues is essential for understanding of pathogenesis and progression of any cancer. However, profile data from bulk sample representing the average expression of all cells has limitations on characterizing individual transcriptional profiles. For this reason, scRNA-seq is generally performed on each cell isolated from complex tissues composed of multiple distinct cell types in cancer. In order to achieve single-cell sequencing data, the first step is mechanical dissociation into cell suspension from bulk samples including solid tumorous tissues and bone marrow. Next, single-cell isolation is generated by several methods such as Serial dilution, Mechanical micromanipulation, Laser capture microdissection, Fluorescence activated cell sorting, Microfluidics.¹⁵⁻¹⁷ After single-cell isolation, complementary DNA (cDNA) is synthesized from RNA via reverse transcription (RT). Subsequently, cDNA amplification is generated via polymerase chain reaction (PCR) of synthesized cDNA or RT-PCR of amplified RNA by *in vitro* transcription of synthesized cDNA. These double stranded cDNA fragments are further processed to mRNA sequencing library by random primers or attaching adapters. Lastly, single-cell DNA sequencing or single-cell RNA-seq is performed. Recently, single-cell RNA-seq is more established in a variety of methods than single-cell DNA sequencing. After single-cell sequencing, bioinformatic and statistical analyses are conducted to evaluate intra-tumor heterogeneity. In particular, single-cell RNA-seq data show significant discrepancy in distributional properties compared with average RNA-seq data de-

rived from conventional bulk samples because of the cell cycle effects.¹⁸ A schematic diagram of single-cell sequencing analysis is shown in Figure 1.

2. Single-cell transcriptomic analysis in HCC

RNA-seq is a very effective tool for comprehensive characterization of whole transcriptome at both gene and exon levels and for identifying novel splicing variants. Recently Huang et al.,¹⁹ firstly reported RNA-seq-based transcriptome analyses in tumor and non-tumorous tissues of 10 HBV-related hepatocellular carcinoma (HCC). They identified 1,378 significantly differently expressed genes (DEGs) and 24,338 differentially expressed exons (DEEs). Comprehensive functional analyses demonstrated that DEGs were most significantly enriched in cell growth-related, metabolism-related and immune-related pathways suggesting a very complicated mechanism for hepatocarcinogenesis. Furthermore, RNA-seq data analyses at exon level revealed a much complex landscape of transcript-specific differential expressions in HCC. In particular, a novel, highly up-regulated exon-exon junction was detected in ATAD2 gene.¹⁹ However, in recent date, NGS has

moved bulk tissue sample analysis to scRNA-Seq because by single-cell sequencing data is more specific and reliable for identifying heterogeneity within cell populations, defining rare cell populations and exploring dynamic interactions between cell groups. In particular, scRNA-seq is recognized as a very useful tool for investigating development and progression of in a variety of cancers including HCC. Advances of single-cell genome, transcriptome and DNA methylome sequencing technologies have greatly contributed to the study of intercellular and intracellular heterogeneities.²⁰ After that, combined genome and transcriptome analyses of a single-cell based on either microarray or NGS have been developed to execute in-depth research of tumor heterogeneity.²¹ This single-cell dual-omics sequencing technique can reveal the relationship between genome and transcriptome but has the limitation of not being able to unveil epigenetic regulation of gene expression.²² Consequently, in order to overcome this drawback, single-cell triple omics sequencing (scTrio-se) techniques including the genome, epigenome and transcriptome have been developed.²³ This technique will help explore the dynamic mechanisms which simultaneously occur in genetic and epigenetic regulation on a specific gene expression associated

Figure 1. Generation of single cell sequencing data from primary human cancer cells can be described through a set of fundamental process: (1) sample acquisition from patient; (2) creation of single cell suspension; (3) temporary storage; (4) isolation of single cells and library preparation; (5) sequencing; and (6) bioinformatics and statistical analysis. cDNA, complementary DNA; IVT, *in vitro* transcription; RT-PCR, reverse transcription polymerase chain reaction; PCR, polymerase chain reaction.

with heterogeneity in a single cell. Recently, Hou et al.²⁴ firstly revealed genetic, epigenetic, and transcriptomic heterogeneity in 25 single cancer cells derived from a human HCC tissue sample using scTrio-seq. In this study, they showed the correlations among genomic (copy-number variations, CNVs), transcriptomic, and methylomic data analyzed in the same individual cells of HCC. Also, they revealed that changes in the gene dosage of certain regions due to CNVs proportionally affect the RNA expression levels of the corresponding regions, whereas they do not significantly affect the DNA methylation levels in these regions. Moreover, they demonstrated subpopulations of cancer cells according to the genome (CNVs) information, and infer malignancy and metastasis potentials of the subpopulations based on triple-omic information.²⁴ This integrative omics technique can provide simultaneously comprehensive information about genomic, epigenomic and transcriptomic heterogeneities within a single-cell of HCC.

3. Single-cell analysis in cancer stem cell in HCC

Recent studies have described that a subpopulation of tumor cells harboring the ability to propagate, called cancer stem cells (CSCs), is responsible for tumor initiation, progression and metastasis. In addition, studies have demonstrated that CSCs in various human tumors play a key role in tumor recurrence, chemoresistance and radioresistance²⁵⁻²⁷ in the clinical aspect. There are two types of heterogeneity of cancer: intertumor and intratumor heterogeneity. In HCC, intertumor heterogeneity is known to be determined by various molecular subtypes^{28,29} but the factors that cause intratumor heterogeneity are not well understood yet. In particular, there have been few reports on the heterogeneity of liver CSCs at the single-cell level in HCC. However, recent study by Zheng et al.³⁰ demonstrated that CSC heterogeneity may contribute to a molecular and biological diversity of HCC cell groups and consequently, patient prognosis. In this study, the authors revealed that liver CSCs at the single-cell level exhibited phenotypic, functional and transcriptional heterogeneities. Also, different CSC subpopulations contain distinct molecular signatures, which are associated with prognosis of patients. Accordingly, heterogeneity at the single cell level of liver CSCs

may be critical for the tumor progression and prognosis in HCC and might be an important for the development of target agent for HCC.

4. Single-cell analysis of circulating tumor cells in HCC

Circulating tumor cells (CTCs) are cells that have shed into the blood stream from a primary tumor and give rise to metastasis to other organs in the body. The early detection of metastasis of an advanced cancer is essential for determining therapeutic strategy and predicting prognosis. CTCs can be useful for monitoring metastatic spread of cancer without invasive diagnostic procedure. With the advances of technology for isolation of CTCs, rare cancer cells within blood streams can be detected without conventional detection methods like a microscopic imaging. Conventional CTC staining criteria are as follows: the presence of cell surface epithelial cell adhesion molecule (EpCAM) and cytoplasmic epithelial cytokeratins but the absence of the hematopoietic CD45 marker.³¹ However, strict imaging criteria is required in order to select pure CTCs from surrounding leukocytes from blood samples. The microfluidic CTC isolation technologies could allow researcher to manipulate tumor cells without contamination of blood cells³² and preserve cell viability with high-quality RNA contents suitable scRNA-seq.^{12,33} Recent study of meta-analysis in the relationships between CTC positivity and clinical-pathological parameters showed that CTC positivity in the blood was significantly correlated with the tumour, node and metastasis (TNM) stage, tumor size, vascular invasion, portal vein tumor thrombus or serum AFP level, but not with number of tumor.³⁴ Also, CTC positivity was significantly increased risk of disease recurrence and death.³⁴ However, the characteristically low EpCAM expression in this HCC has limited the clinical utility of standard CTC measurements. As a strategy to solve this drawback, Kalinich et al.³⁵, developed a high-throughput microfluidic CTC-iChip which efficiently depletes hematopoietic cells from blood specimens and enriches for CTCs with well-preserved RNA to detect CTC-derived signatures. In this study, the HCC specific RNA-based digital PCR (dPCR) CTC-scoring assay was es-

established. To develop the dPCR assay, a total of 10 genes were selected: albumin, alpha fetoprotein (AFP), glypican 3, transferrin, alpha 2-HSglycoprotein, apolipoprotein H, fatty acid binding protein 1, fibrinogen beta chain, fibrinogen gamma chain and retinol binding protein 4. The authors established cross-validated logistic model using a digital signature of 10 liver-specific transcripts. The positivity of HCC-derived CTCs was observed in nine of 16 (56%) untreated HCC patients but in 1 of 31 (3%) patients with chronic liver disease at risk for developing HCC. Positive CTC scores declined in 9 of 32 (28%) patients receiving therapy, but RNA-based digital CTC scoring was not correlated with the standard HCC serum AFP level. This study suggest that digital RNA quantitation is useful tool as a sensitive and specific CTC readout which enable high-throughput clinical screening and monitoring applications for HCC patients and chronic liver diseases at high risk of HCC.³⁵

5. Single-cell analysis in T-cell in HCC

The tumor is a tissue expressing high antigenicity due to various mutations in terms of immunology and thus are subject to immune surveillance by the immune system. In this regards, development and progression of cancer is closely linked to failure of immune surveillance including elimination of cancer cell in initial stage or immune defense to prevent immune escape.³⁶ Recently, immunotherapy is the new hot-issue in cancer treatment. The main types of immunotherapy treating cancer include monoclonal antibodies, immune checkpoint inhibitors, cancer vaccines such as DNA or peptide vaccine. T cells play an important role in anticancer immune response. Studies have described that activated CD8⁺ effector T cells mediate tumor elimination³⁷ but regulatory T cells (Tregs) mediate significant tumor immune dysfunction.³⁸ However, anticancer immunity is declined owing to T cell exhaustion by several factors including decrease of effector T cell function caused by T cell dysfunction and abnormally increase of check point inhibitors such as programmed cell death protein 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA4), lymphocyte activation gene 3 protein, and killer cell lectin-like receptor G1.^{39,40} The response rates to targeted immuno-

therapy are not uniform among cancer patients⁴¹ and therefore identification of new biomarkers to predict response is required. The factors determining response to checkpoint inhibitors such as anti-PD1 or anti-CTLA4 include mutational loads, tumor-infiltrating lymphocytes (TILs) levels and the expression of drug targets.⁴¹ Moreover, tumor infiltrating Tregs in transcriptome analyses of several cancers are reported to suppress tumor-specific effector T cell.⁴² The T cell receptor (TCR) that is responsible for recognizing antigen as peptides bound to major histocompatibility complex molecules, plays an essential role in regulating the selection and activation of T cells.⁴³ The TCR repertoire for each person is so diverse because of TCR composed of α - and β -chain genes that are derived by somatic V(D)J recombination. The link T-cell function and TCR specificity will enable one to determine which functional subsets of T cells have undergone clonal expansion.⁴⁴

Currently, immunotherapy for various cancers is a potentially attractive option. Previous studies on immunotherapy for HCC have shown the feasibility and safety but the results are not satisfactory.^{45,46} Recent studies demonstrated that high numbers of CD8⁺ TILs predict better prognosis in colorectal cancer⁴⁷ and ovarian cancer⁴⁸ but predict worse prognosis in nasopharyngeal carcinoma.⁴⁹ TILs in HCC are significantly exist but their tumor killing effect is limited.⁵⁰ Recently Zheng et al.⁵¹, analyzed TCR sequences and transcriptomes using scRNA-seq of single T cell isolated from HCC patients and revealed distinct subtypes and clonal expansion of TILs. Using the spectral clustering technique, 11 unique T cell subsets including 5 clusters for CD8⁺ and 6 clusters for CD4⁺ cells, were identified. The key genes for each cluster for CD8⁺ cells are as follows: lymphoid enhancer-binding factor 1, CX3C chemokine receptor 1, solute carrier family 4 member 10, layilin (LAYN) and granzyme K whereas those for CD4⁺ T cell clusters include C-C chemokine receptor type 7, forkhead box P3 (FOXP3), CTLA4, Granzyme A, chemokine (C-X-C motif) ligand 13 (CXCL13) and granulysin. In particular, among a total of 401 genes highly expressed in tumor-infiltrating Tregs, FOXP3, CTLA4, tumor necrosis factor receptor superfamily member 18, tumor necrosis factor receptor superfamily member 4 and chemokine (C-C motif) receptor 8

were also identified in other cancers such as in colon and lung cancers.⁴² These findings suggest that Tregs are closely related to the development and progression of HCC. In addition to Tregs for hepatocarcinogenesis, the role of tumor-infiltrating exhausted CD8⁺ T cells is also important. The genes associated with tumor-infiltrating exhausted CD8⁺ T cells, such as CTLA4, hepatitis A virus cellular receptor 2, ectonucleoside triphosphate diphosphohydrolase-1, T-cell immunoreceptor with Ig and ITIM domains, programmed cell death 1 and tumor necrosis factor receptor superfamily member 9, and CD27, are previously identified.⁴² In HCC, several novel genes as novel exhaustion markers, such as myosin VIIA, tryptophanyl-TRNA synthetase, and CXCL13 LAYN, pleckstrin homology like domain family A member 1 (PHLDA1), synaptosome associated protein 47 (SNAP47) are also identified.⁵¹ Among them, high expression of PHLDA1 and SNAP47 were significantly associated with poor prognosis of the HCC patients. LAYN which encode layilin was highly expressed in Tregs isolated from lung and colon cancers.⁴² Zheng et al.⁵¹, demonstrated that LAYN was highly expressed in both tumor Tregs and exhausted CD8⁺ T cells from tumor tissue of HCC, and revealed that it is upregulated on activated CD8⁺ T cells Tregs and repressed the CD8⁺ T cell function *in vitro*. Furthermore, the transcriptional profiles by scRNA-seq analyses of individual T cells isolated from HCC, coupled with assembled TCR sequences, revealed 11 T cell subsets associated with immunopathogenesis for development of HCC, and thus these findings could be used for further understanding the role of TIL in a variety of tumors and for the development of potential immunotherapy for cancers.

CONCLUSION AND FUTURE PERSPECTIVES

Recent advances of single-cell transcriptomics have made remarkable progress for deeper understanding of biology by providing information on gene expression at the level of single cell. In particular of cancer, single-cell technology provides a powerful and comprehensive tool for the characterization of tumor profiles including intra-tumor heterogeneity and epigenetic alterations. Compared to conventional bulk average molecular phenotyping, single-cell technology has the advan-

tage of characterizing molecular phenotype with a higher resolution of cancer cells. Also, single-cell RNA sequencing allows detection of rare cancer cells and CTCs, elucidation of mechanism for drug resistance and prediction of prognosis of cancer patients.

HCC is a highly heterogeneous cancer and its molecular mechanism is extremely complex associated with a poor prognosis. To date, the molecular mechanisms of hepatocarcinogenesis remain unclear. Immune dysregulation as well as chronic inflammation induced by hepatitis virus B/C or alcohol plays a key role in hepatocarcinogenesis. For this reason, high-throughput analysis of single-cell transcriptome derived from tumor cell or immune cells is required for better understanding of molecular pathogenesis, developing novel therapeutic agents against HCC, and tailoring an individual's cancer therapy. Although only a few studies on scRNA-seq of HCC have reported, these studies provide new insights into mechanistic research of development and progression of HCC. Thus, applications of single-cell transcriptome analysis enable comprehensive characterization of tumor and immune cell in HCC can provide mechanistic insights into HCC progression and metastasis, which ultimately will contribute to the development of novel therapeutic strategies and improvement of clinical outcomes. However, current scRNA-seq still has several limitations such as low capture efficiency, a quantitative accuracy by complex expression distributions and highly technical noise, and a difficulty in validating data due to a complicated analytical network inference. Therefore, further investigation to minimize these shortcomings is required to achieve more potential application of single-cell transcriptome analysis in the clinical setting.

Conflicts of Interest

The authors have no conflicts to disclose.

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