Original Article

Dissection of Cancer Heterogeneity by Single Cell Sequencing

Feng Tian[1], Haochuan Chen[2], Jin Hao[3], Songlin Zhou[4], Xu Luo[5,*], Xinghan Li[6,*]

[1]Department of Neurology, Harvard Medical School, F. M. Kirby Neurobiology Research Center, Boston Children's Hospital, Boston, MA 02115; [2]Department of Biochemistry，Duke Medical School, Durham, NC 27705; [3]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138; [4]Key Laboratory of Neuroregeneration of Jiangsu and Ministry of Education, Co-innovation Center of Neuroregeneration, Nantong University, Nantong, Jiangsu, China; [5]Department of Wounds and Burns, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, China; [6]Department of Stomatology, Shenzhen University General Hospital, Xueyuan AVE 1098, Xili University Town, Shenzhen, Guangdong, China

*Corresponding authors: Xu Luo, Department of Wounds and Burns, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, China, E-mail: doctorrosh2018@gmail.com; Xinghan Li, Department of Stomatology, Shenzhen University General Hospital, Xueyuan AVE 1098, Xili University Town, Shenzhen, Guangdong, China, E-mail: drlixh89@foxmail.com

Cancer is one leading cause of death worldwide. Major hurdles of efficacious cancer therapy stem from its intrinsic molecular and cellular heterogeneity. The emerging single cell sequencing technology have already shed light upon the fundamental pathology of caner and could provide clinical promises with crucial subpopulation information. In contrast to bulk sequencing methods which do not take intercellular heterogeneity into account, single cell sequencing can capture the genetic and epigenetic changes between cells originating from the same tumor, in hope of understanding the underlying carcinogenic mechanisms with a lot more dimensions. In this review, we first describe the heterogeneous characteristics of cancer, including the concept of cancer stem cells. Then we outline modern single cell sequencing techniques at different molecular scales, from the genome to epigenome, and perhaps higher chromatin structures. Their uses and drawbacks in the basic cancer research and clinical application are discussed. We also proposed that other basic research topics or novel biotechnologies, including cancer organoids, 3D printing, and gut microbiome, are possible to be combined with the current single cell sequencing approach for broader research and clinical applications.

Key Words: cancer; cancer stem cell; pathological heterogeneity; single cell sequencing; gut microbiota; fecal microbiota transplantation

INTRODUCTION

According to the demographic data in recent two decades, cancer accounts for the second leading cause of natural death worldwide.[1-3] In general, cancerous cells exhibit uncontrolled mitotic divisions, which results in an erratic cellular organization.[2,3] Their altered intercellular adhesive and motive properties also render them metastatic, being able to migrate via the circulation system and spread the malignant growth in other parts of the human body.[2,3] Multiple factors could contribute to the development of cancer, and changes in the genome and epigenome during this process have been widely appreciated.[2,3] Cancerous cells form when normal cells are exposed to genotoxic stresses such as short wavelength radiation, reactive chemicals, or microbial infection.[4] Under the influence of those carcinogens, genetic materials in normal cells may be mutated. Although these mutations seem to occur randomly, malicious mutations are more likely to be accumulated by selection, especially when the patients are exposed to cancer treatment such as chemotherapy.[3] Furthermore, second, third and higher order of novel mutations would evolve in the tumor population.[5] In combination with the microenvironmental variabilities, such as hypoxia, these genotypical differences render the *in vivo* cancer population highly heterogeneous.[5-7]

On the other hand, from a clinical perspective, there are several readily available and well-practiced technologies that can mitigate the effects of cancer, including surgery removal of tumors, radiotherapy, chemotherapy, and immunotherapy.[8] However, these approaches do not guarantee the complete eradication of cancer, and recurrence do occur in a proportion of patients where new genetic mutations may have evolved.[5] With currently implemented technologies, only 30% to 50% of all cancer can be properly prevented, not to mention the more challenging issue of metastatic cancer management.[4] In addition, even in the condition of many well managed cancer cases, the financial burden produced to the patients and their families have been manifested over the past few decades.[9] Therefore, dissecting the genetic and cellular basis of cancer recurrence would be critical to improve the current anti-cancer therapeutics.

One major hurdle to more effectively eradicate cancer is the described fact that the tumor population is highly heterogeneous.[5] Due to the unpredictable nature of carcinogenic mutations occur at the single cell level, there are usually distinct genetic and epigenetic variations between each individual cancer cell.[10] This cancer cell heterogeneity allows some minor strains of cancer cells to survive due to the random formation of genes that lead to resistance towards a particular type of treatment, and such resistance will be inherited by the next generation clone of cancer cells.[11] Albeit the odds may be very small, those rare mutations that possibly render the malignant cells prone to survival and proliferation, hence accumulating themselves over time. Intuitively, understanding the effects and dynamics of tumor heterogeneity is essential to treatment success in the foreseeable future, so the treatments can then be designed to target the particular subpopulations in the tumor microenvironment.[5]

In order to overcome the caveats caused by the intrinsic tumor heterogeneity, one possibility is to employ the emerging technologies at the single cell level. The currently available single cell analyses are primary sequencing based, which could have both applications in basic cancer mechanism studies as well as diagnostic related clinical uses.[12] In general, single cell sequencing is used to observe cellular genomic differences of high fidelity, which in turn allows scientists to investigate each cellular function exclusively and to better predict cellular behavior.[13] This series of high precision and high throughput technologies have provided promises to deconvolute the individual cell genome, transcriptome, epigenome or even higher order chromatin changes in various disease scenarios.[14,15] Nevertheless, difficulties may be found when analyzing the miniscule amount of genetic material there is in a single cell, but amplification protocols have been proved to be useful in making single cell sequencing feasible.[16] When single cell sequencing technology is applied to cancer cells, it allows for the individualized cancer cell behavior and development predictions, which can be useful for further understanding the dynamics of a tumor hence designing a specialized treatment for each type of mutated cells.

In this review, we discuss the molecular and cellular features of cancer heterogeneity from multiple levels. Accordingly, single cell sequencing can then be employed at genomic, epigenomic and transcriptomic levels. Current applications of single cell sequencing methods are mostly limited to basic research and proof of principle studies for liquid biopsy diagnosis. We also propose several aspects by which single cell sequencing approaches may be further improved in cancer research and clinical diagnosis. With more single cell analysis dataset available, it is highly possible that therapeutics could be developed to overcome the recurrent malignancy resulted from heterogeneity.

MULTIPLE LEVEL HETEROGENEITY OF CANCER

The heterogeneity of tumor has been documented for a few decades.[17] Subclones of cancer cells derived from the primary tumor show different karyotypes and phenotypes (such as responsiveness to chemotherapy drugs).[17,18] This feature partly contributes to the many recurrence of cancer after the primary tumors being treated by surgery, radiotherapy or chemotherapy.[18] Even a very tiny proportion of cancer cells could survive those treatments, the survivors may be the most resilient subpopulation to the same therapeutic approach. In fact, the so-called heterogeneity of tumor or cancer involves the multiple aspects of sub-populational differences, from molecular level to cellular level.[5]

Genomic heterogeneity

It has been widely accepted that cancer cells mainly arise from genetic mutations.[2] Those mutations could happen in a stochastic manner, and abnormal cells with growth favorable mutations could also expand and evolve during the process of tumor development.[19] Genomic instability renders the tumor population an intrinsic heterogeneity, which may also determine the cancer disease progression in different cases.[2,3,19] For instance, gastric cancers can be classified into genomically stable and unstable subtypes, primarily based on aneuploidy.[20,21] Genomically stable gastric cancer exhibits a diffused pattern pathology. Perhaps due to undifferentiated and invasive feature of this subtype of gastric cancer, the tumor develops quickly, not allowing a large amount of mutations to accumulate.[20] Another deep sequencing study for over one hundred types of triple-negative breast cancers have suggested very distinct patterns of genetic mutation frequencies in different patients.[19,22]

Although conventional genetic approaches have helped us identify multiple cancer related mutations, including tumor suppressor and oncogenes, most of these studies were done at a population level.[5] Only genetic alterations that happen at a large frequency in the populational level could be captured by conventional tissue analysis approaches, such as bulk sequencing or Western blotting.[21,22] This phenomenon might partly explain why several targeted cancer therapy approaches failed and that recurrent tumors may carry novel mutations.[23] Low frequencies genetic alterations include rare mutations, copy number variations (CNV) and single cell aneuploidy. Currently, very limited number of studies have systematically addressed those genetic subtleties at the single cell level.[24] Even though high throughput sequencing could offer better depth of whole tumor genomic data, low frequency mutations that might render the long-term cancer cell survival advantage would have to be overlooked in those bulk tissue analyses.

Epigenomic heterogeneity

In a general sense, the epigenome can be regarded as any add-on parts to the eukaryotic genomic DNA, and those add-ons could promote or inhibit the expression of particular genes in certain spatial and temporal manners.[25] The epigenetic components include DNA methylation, histone modification, non-coding

RNAs and higher order chromatin structures such as the formation of chromatin loop and boundaries.[25,26] Strikingly, the altered epigenomic characteristics, such as gene imprinting or histone markers, could be inherited from either of the patients. On the other hand, probably unlike the relatively stable genomic DNA, the epigenome could be more prone to alterations induced by physical environmental factors.[26]

In addition to well-known genomic heterogeneity in the tumor population, it is natural to ask if the altered epigenome in cancer cells could also exhibit individual difference.[20] First of all, genomic DNA hypermethylation is a phenomenon that have been reported in various types of cancers.[27] Those methylation sites are usually on cytosine, while other bases may also be involved in some cases.[28] The hypermethylation normally inhibits the expression of tumor suppressor genes, thus inactivating the anti-tumorigenesis machinery and leading to uncontrollable cell proliferation.[25,28] The conventional method for methylome dissection is usually bisulfate sequencing, which selectively transfer the unmethylated cytosine to 5-methylcytosine.[25,28] One recent study using the regional methylome profiling has suggested heterogeneous hypomethylation pattern of quiescence-associated genes, such as ALDH1L1, HOPX, WNT5A and SOX9.[29] Single cell methylation sequencing may be plausible, but the currently accessible techniques, such as single cell reduced representation bisulfide sequencing and post bisulfide adapter tagging, may not offer enough coverage to delineate heterogeneous intra-tumor subpopulations.[28,30]

The histone is the backbone structure that DNA winds on.[31] As a part of the epigenome, histone can be modified via several chemical modifications including methylation, acetylation, phosphorylation and so forth.[31] These so-called histone marks could also effectively change the expression of a subgroup of genes, possibly proto-oncogenes or tumor suppressors.[27,31] Such modification might not cause all-out cell proliferation, but it does support abnormal cell renewal and block mechanisms, an essential part to carcinogenesis.[31,32] Compared with the well-known heterogeneous cancer genome, it has been proved to be much more challenging to deconvolute the histone modification variations at the individual cell level.[27] Nevertheless, indirect evidence such as the differentially changed gene expression and non-coding RNAs in a number of well-studied cancers, including gastric cancer and breast cancer may suggest a similar heterogeneity pattern inside the tumor.[19,22,27]

Epigenetic coding is often influenced by environmental factors. Cancer cells, just like any other cells, can fine tune their epigenome to ensure optimal survival and proliferation in the given tumor microenvironment.[5] Epigenetic analyses on a single cell level may allow for the understanding of pro-growth microenvironmental differences, and for linking up epigenomic modifications to cellular behavior and ultimately cancer symptoms.

Transcriptome and proteome heterogeneity

The transcriptome and proteome are two major elements from the central dogma in molecular biology, which can direct influence the phenotype of normal and tumor tissue. In particular, the transcriptome describes the collective set of all RNA molecules in one individual cell or a cell culture, while proteome includes all proteins that satisfies the same conditions.[33] In the scenarios of malignant cell, even the regular cellular metabolism would translate the faulty DNA into irregular transcriptomes (aberrant genome), or the normally functional genes are expressed in an abnormal spatial or temporal manner. The genomic difference between cells can be represented by the transcriptome or proteome differences, as transcriptome and proteome are both metabolic results of the original copy of DNA down the line according to the central dogma.[33-35] Certain transcriptomic alterations could be biomarkers that pinpoint cancer in a biopsy setting (aberrant epigenome).[20] Those transcriptomes may occur more or less numerously when a tumor present, and the cancer can be identified by detecting which and by how much a particular transcriptome is overly abundant or is in short supply.[5] Moreover, regular sampling of circulating transcriptomes allows for consistent monitoring of cancer progression by measuring the shifts in concentration, which may also be analyzed at a subpopulation or single cell level. For instance, in breast cancer, the CCND1 mRNA has a significantly higher count of 129 than in a control group only exhibiting counts of 30 in a liquid biopsy of plasma.[36] This correlation can then be applied clinically where the oncologists look for CCND1 biomarker count anomalies to diagnose breast cancer. In addition, RNA profiling analysis at subpopulation level would identify unique cellular transcriptomic features in mouse models of prostate cells.[37] Given the pathological differences between individual cells in the cancer population, it is not surprising that heterogeneous transcriptomic patterns can be observed. Moreover, these traits might be more easily accessible for better diagnosis and classification of cancer by liquid biopsy.[38]

Proteomes, in the case of liquid biopsy, follows similar procedures in monitoring cancer.[38] Generally, proteome is the direct translated products of transcriptome in the cellular metabolic processes. Like transcriptome, proteome concentrations can be analyzed to search for tumor biomarkers, although some of which may be misleading for the time being.[5,16] Unlike transcriptome, proteome concentration and heterogeneity analyses have been proved to be somewhat difficult in practice and less feasible in a large scale.[39]

Cancer stem cell

Given the stochastic and heterogeneous nature of the cancer at the single cell level, one straightforward question to ask, which may be directly related to clinical applications, is whether some specific subpopulation of cancer cells may be more proliferative and render the drug resistance features to their daughters. The theory of cancer stem cell (CSC) is developed based on such ideas.[40] This concept is probably the key to address cancer population heterogeneity. Like the definition of stem cells, cancer stem cells possess two stem-cell like features, self-renewal and differentiation.[40] On one hand, they may stay dormant in order to evade the therapeutic insult.[40,41] On the other hand, they could also "differentiate" in an aberrant manner that render the cancer high metastatic potential.[5] Despite some controversies on this theory, it has been widely accepted that this hierarchical composition of the tumor does exist.[40] One convincing piece of evidence is the identification of cancer stem cell markers in acute myeloid leukemia (AML).[42] Lapidot et al. have demonstrated that the CD34+CD38− subpopulation has the cancer initiating property and can lead to leukemia when engrafted into the severe combined immune-deficient (SCID) mice.[42] In addition, subpopulation of tumor initiating cells, or CSCs, can also be observed in solid tumors, such as breast cancer and colon cancer.[43,44] The dissemination pattern of cancer cells in the microenvironment probably also determines many properties how cancer drug

resistance would occur.[5]

On the other hand, however, the CSC theory bears even more controversies as the promises it may provide.[40] For instance, how different or similar are CSCs and non-CSCs? Most conventional studies based on CSCs would utilize cell surface markers, while several variabilities may challenge the reliability of these markers.[45] Indeed, these identified CSC markers do not seem to be cell type specific, as the real stem cells during embryonic development.[46] It is also noteworthy that the mouse models or *in vitro* culture models of various cancer types might not well recapitulate the *in situ* tumor cell hierarchy in the patients.[5] The early failures of a number of therapeutic approaches targeting the so-called CSCs really render the strategy to target CSC a "gambling".[47]

Overall, it is still largely unknown for many solid tumors and leukemia, how the hierarchy of cancer cells, including the so-called CSCs, is arranged in the microenvironment.[5] Without the genetic and molecular dissection tools of cancer at single cell level, it would remain difficult to answer this question.

## SINGLE CELL SEQUENCING APPROACHES IN CANCER RESEARCH

To fully understand the holistic image of the tumor, the genomic DNA, RNA (usually transcriptome), and epigenomes of each individual cancer cell need to be deconvoluted at single cell level. In general, due to the minuscule amount of genetic and epigenetic materials present in single cells, some forms of barcoding and amplification would be required for each type of biomacromolecules.[23] In bulk genetic sequencing where there is a large number of cells available, conventional polymerase chain reaction (PCR) amplification is already sufficient. On the other hand, on a single cell scale, specific considerations need to be taken into account in order to capture the subtle differences from individual cells.[16] Single cell barcoding steps and various tricky amplification methods would be involved. Even if some of these single cell analyses are still technically demanding, the additional information that would be offered could potentially open new avenues for the next generation cancer research and more precise cancer target shooting. As mentioned previously, those readily available single cell analysis technologies can also be discussed based on different molecular levels of caner heterogeneity.

Single cell genomic sequencing in cancer research

Genomic DNA is the main genetic information storage medium in cells, which is also the primary material source for the so-called (genomic) DNA sequencing.[48] Since genomic DNA basically determines the phenotypes of the cells, including cancerous cells which carry mutations on the genome, DNA sequencing can be useful for both basic research and medical diagnosis.[16] For example, by examining the circulating tumor cells (CTCs) in the liquid biopsy, potential risks of cancer metastasis might be captured at a relatively early stage, allowing longer time window of further therapeutic intervention.[10]

The conventional genomic sequencing approach identifies signals at the cell population or tissue level, which is often termed as bulk DNA sequencing.[49] In general, bulk DNA sequencing involves steps including base calling, contig assembly and scaffold alignment. Besides, particular techniques to acquire sequencing reads have also been evolving during the past few decades (without taking the single cell level into account).[10,16] Before any genome can

be analyzed, an enormous amount of sequencing data first have to be generated. The vectors, artificial DNA that are only responsible to carry out a piece of DNA from a cell, are trimmed so it does not interfere with the main DNA. Low quality data without definitive base type is also skimmed. With those done, what's left are fragments of DNA yet to be assembled. The fully constructed DNA will then be displayed. Bulk DNA sequencing can be useful in the identification of genetic diseases, especially those that leads to cancer. There has already been success in finding cancer risks among particular family pedigrees by using DNA sequencing to discover mutations.[50] Genomic DNA sequencing can also be used to identify cancerous cell groups by contrasting it with cells known to be original.[49] On the other hand, bulk DNA sequencing fails to identify the inherent variations of cancer cells within a single tumor.[5] This may prove to be problematic as benign tumor cells can be intermixed with trace amounts highly malignant ones (or perhaps CSCs), and bulk sequencing does not take those malicious cancer cells into account, resulting in a false sense of safety for the patient who's tumor will turn malicious after a prolonged period of time.[16]
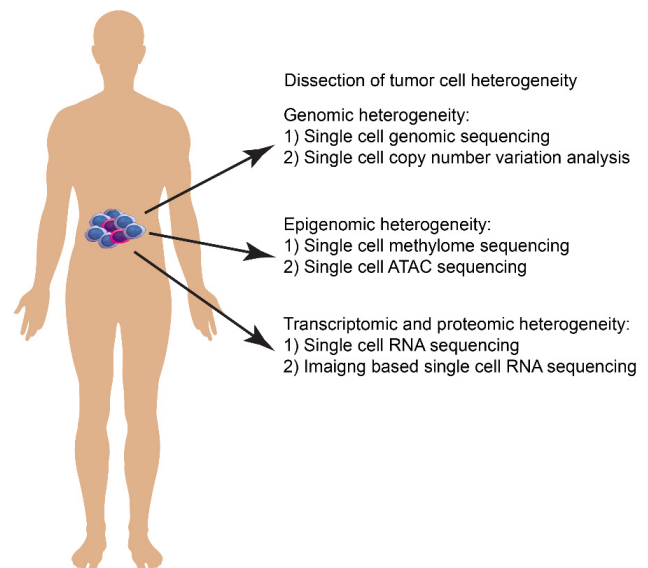
Figure. 1 Single cell techniques used to dissect tumor cell heterogeneity.

The emerging single cell genomic sequencing technology assesses the main genomic differences between cancer cells, hence the different types of mutation that occurred between different cells under genotoxic stresses.[51] This method allows for cataloging each cancer cell individually and for dissecting more complex cellular behavior. Apparently, before any high throughput sequencing is performed, the individual cells first need to be separated from the bulk tumor tissue.[30] Except for sorting the fluorescently labeled cancer cells one by one, one canonical way to achieve this is to use a microfluidic system. By adjusting the flow rate between trapped cells and oil droplets, each droplet will in principle present one barcoded cell or no cell.[52] This method could be readily employed in the detection of CTCs or cell free DNA.[30] One recent study has shown that several tumor-related mutations can be detected in the liquid biopsies of patients with high grade glioma.[53] Detection of circulating tumor material by deep sequencing is particularly important for types of cancers that are not apparent until a late stage.

Another critical step of single cell genome sequencing the signal amplification. This step requires not only sensitivity but also the separation of signals from different individual cells. One example of amplification is just based on PCR, the well-known DNA fragment amplification method. PCR is not rare for various uses in molecular biology, including the traditional bulk sequencing.[16] In PCR, a sample of DNA is inserted alongside with deoxynucleotide triphosphates (dNTPs) and oligonucleotide primers.[54] Then the DNA polymerase is added to duplicate, thus amplify, a single piece of DNA.[54] One variation of traditional PCR that has been found its use in single cell sequencing is the degenerate oligonucleotide primed PCR (DOP-PCR).[16] DOP-PCR ensures detection of single copy genomic DNA.[10,16] This method uses primers linked with random hexanucleotide sequence sequences for barcoding during amplification.[16] This methodology not only amplifies the genes themselves, but it also amplifies subtle changes present in the genes, and these alterations may cause some part of the genes to be overamplified or under-amplified to an unrecognizable extent, due to the differentiating effects of an exponential increase.[16] Not only is the coverage narrow, the lack of control in the specific position of such narrow windows may devastate DOP-PCR in cancer diagnosis. The results of DOP-PCR are usually random fragments of a single genome. Nevertheless, DOP-PCR is still useful in detecting CNVs.

Alternatively, a completely new method, multiple displacement amplification (MDA), can be opted instead. MDA uses more complex multibranch primers which also have higher reactivities.[55] It also ensures higher total coverage than DOP-PCR. The exponential amplification nature of MDA results in extreme amplification or lacked amplification just like those observed in DOP-PCR, but to a lesser extent, hence higher coverage.[16,55] On the other hand, the frowned-upon reproducibility of MDA renders CNV attempts useless, so MDA has to be used in other clinical and especially forensic applications instead.[56,57]

Multiple annealing and looping based amplification cycles (MALBAC) is another approach at whole genome sequencing.[58] It can be applied to single cell genomic DNA sequencing. Instead of the exponentially multiplying approach of amplification, it allows a more controlled "quasi-linear" amplification.[16,35] MALBAC protects the original piece of genomic DNA, making it the only DNA to be copied rather than mis-amplification of the existing copies of the original genomic DNA.[16] Such more controlled amplification curve allows for the eradication of sequencing dependent bias, which is present in both generic PCR and MDA sequencing both exhibiting an exponential amplification curve. Because of that, MALBAC is especially useful in detecting CNVs. Moreover, MALBAC tends to show less false negative SNV detection attempts, but it does display higher false positive detection of SNVs than MDA due to the lower fidelity DNA polymerase used in MALBAC.[35] The MALBAC method towards WGA is proven to be useful in sequencing CTCs in lung cancer patients.[59] The observed mutations in the CTCs from these lung tumors could be further used to design personalized treatments in order to avoid drug resistance.

Generally speaking, the utilization of single cell (genomic) DNA sequencing seems to increasingly more concentrate on relatively clinical applications, such as the detection of CTCs or cell free tumor DNA fragments.[5] This is probably because the genomic heterogeneity has already been revealed by other methods, while fundamental investigation of cancer biology would further explore how the mutation frequencies (especially rare mutations) would evolve over the course of tumor development and progression.

Indeed, the cancer cell evolution question can perhaps only be sufficiently addressed with high throughput single cell genomic sequencing.[10]

Single cell RNA sequencing in cancer research

In contrast to DNA sequencing, the currently available RNA sequencing studies or datasets are more relevant to basic research of caner, including the cellular behaviors of the so-called CSCs.[5] Single cell RNA sequencing can be used to assess transcriptome differences in a population of cells, and subsequently detect rare populations of cells that would otherwise go unobserved. Although this can be very important in cancer diagnosis and treatments, the intrinsic instability of RNA molecules in general might have limited its application mostly to basic cancer research.[60] The RNA purification step before the single cell sequencing analysis is then performed with more caution than genomic DNA sequencing.[61]

RNA is commonly amplified by pairing it with it is complementary DNA (cDNA) when there are sufficient cell samples, but RNA, just like any other genetic material present in a single cell, is of small quantity, on the pictogram scale of magnitude.[62] Therefore, some form of amplification is required for single cell RNA sequencing library construction.[63] In addition, most of the expressed human genes are actually expressed at a low copy number (less than 100).[64] This renders the amplification steps even more critical for the high fidelity of single cell transcriptome data analysis. Just as single cell DNA sequencing, amplification is to capture the subtle changes in the individual transcriptomes. Except for randomly primer PCR, unique molecular identification (UMI) methods are commonly used to tag the transcripts in the individual cells.[61] This technique is important to avoid amplification bias and ensure whole transcriptome amplification accuracy.[65] Because the sequenced genetic material in question is exclusively the RNA, a genomic DNA removal step is also required. To enhance the RNA yield, artificially synthesized cDNA library can then be generated by reverse transcription. DNAs and only DNAs can then undergo MDA, a non-PCR based DNA amplification method. MDA is commonly used in place of PCR when the DNA sample is miniscule, especially so in single cell sequencing.[35] This reverse transcribed, amplified RNA can now just undergo sequencing just like regular genomic DNA single cell sequencing, and it allows a modular approach towards sequencing different types of genetic materials, thus greatly reducing the need of a completely new and convoluted method to sequence RNA. It is also possible to sequence RNA directly using MALBAC.[16,35] It can be applied to single cell transcriptome sequencing more effectively than generic genomic DNA sequencing.[35]

Although direct utilization of single cell RNA sequencing is difficult to replace pathology for the reliable diagnosis of cancer, basic oncology research could be significantly facilitated with this technology.[61] One striking example published recently is a single cell transcriptome analysis for head and neck squamous cell carcinoma (HNSCC) patients.[66] This study has not only subclassified the tumor population into malignant and non-malignant group but also provided compelling evidence on how the heterogenous landscape within the tumor has been shaped. These finding would aid to match the primary HNSCC to the metastatic tumors through the lymph nodes. In addition, single cell transcriptome analysis could also be employed to probe how the immune system, as part of the non-malignant tissues, has been blocked in various cancers.[67,68]

Single cell epigenetic profiling and chromatin structure analysis in cancer research

As briefly discussed above, the heterogeneous epigenome of cancer has not been intensively studied due to certain technical difficulties.[25,27] Most of the published cancer epigenome studies on the single cell scale concentrate on the DNA methylome, which is not particularly more complicated than conventional deep sequencing of the genomic DNA. The key issue to be addressed in single cell methylome is still the sensitivity of detection and coverage. Nevertheless, a few studies have been reported recently to utilize this method for better revealing the cancer heterogeneity in a more global view. For instance, Hou et al. have combined genomic sequencing, RNA sequencing and methylome sequencing (termed as Trio-seq) in several hepatocellular carcinoma (HCC) samples. Remarkably, they were able to reveal subpopulation of HCC as well as how CNVs or hypermethylated genome regions affect gene expression in those cancer cells.[69]

Although genomic and epigenomic sequencing could offer countless pieces of useful information about specific tumor characteristics at bulk or single cell level, there is still a missing gap from the one dimensional sequence information to *in situ* three dimensional higher structures of the chromosome.[70] It is widely appreciated that the animal chromosomes do not appear as a linear form.[70,71] Instead, chromosomal loops and domains do exist, which can contribute to the regulation of gene expression.[71,72] Remarkably, a series of novel technologies have been developed to capture the higher order chromatin structures.[72] For instance, chemical crosslinking and pulldown can be used to capture spatially adjacent regions of the genome.[72] After this capture, PCR or high throughput sequencing can then be used to amplify the spatial information of the chromosome. Strikingly, this chromosome conformation capture (Hi-C) based deep sequencing method can now be accessible at single cell level.[73] Single cell Hi-C could potentially serve as a powerful tool to address whether and how chromatin structural changes may influence malignancy. Besides Hi-C, an alternative strategy that might require less signal input is assay for transposase accessible chromatin (ATAC) sequencing, which is also available on single cell scale now.[74] It is not difficult to imagine that combing with other single cell analysis tools, such as the case of Trio-seq, the missing link between one dimensional DNA or RNA sequence and higher dimensional chromatin structures might be filled, in the context of cancer development and progression.

CHALLENGES AND FUTURE DIRECTIONS

Currently, single cell sequencing technology is probably the optimal method to shed light upon the heterogeneity in the cancer cell population. This would broaden our knowledge in tumor development, evolution, and intercellular interactions. For instance, currently available methodologies of single cell genome amplification such as conventional PCR, MDA, and MALBAC do promise some degree of utility, but future improvements based on them are crucial for potential clinical applications.[16,35] Major issues to improve include sensitivity, fidelity and financial advantages. All these factors allow for not only broader usage in basic cancer research but a smoother clinical transition as well. In addition to these possibilities of technical improvement, several other approaches could be combined with single cell sequencing, allowing more fundamental questions of cancer biology to be

address and, consequently, a broader usage of clinical single cell analysis.

One very promising direction is probably the emerging high and super imaging technology.[75-77] If combined with single cell sequencing, imaging at cancer cell subpopulation at a deeper penetrance and a higher resolution may revolutionize the cancer pathology. Specifically, precision tools such as laser assisted tissue processing would greatly facilitate the scale that we could possibly analyze.[77] Isolating cells from each other is important before performing single cell sequencing. Laser cellular capture could be integrated into single cell sequencing during the precision tissue processing part. Super resolution imaging would also guide lasers around the outlines of cells on a cancer tissue sample, and the high energy lasers, laser capture microdissection (LCM), can then pierce through the tissue.[77] With the cells detached from the rest of the tissue, photonic force microscopy (PFM) can then be used to transfer the cells onto a collection tube.[78] This method of obtaining isolated cells may be applied if sequencing interests lies in a region of any particular cancer tissue. Lasers operate with more precision hence allowing higher fidelity regional modification[77]. Indeed, the combination of high-resolution live imaging has been applied in some scenarios that would be readily available for the real clinical use. One such striking example is the use of a label-free minimally invasive imaging technique based on the non-linear optic effect: stimulated Raman scattering (SRS).[75,76] In SRS, two coherent lasers of different frequencies are shone at a sample such that the frequency difference between the two lasers matches the resonate vibrational frequency of the molecule under observation. SRS imaging can visualize tissues with abundant lipid or protein level, though no specificity, at high resolution without chemical or immunohistochemical staining. This imaging has been reported to mark the tumor boundary or the myelin sheath structure in alive and anesthetized animals.[34,75,76,79] Assuming this single cell level technology would be combined with sequencing in neurosurgery, then the genetic and epigenetic information acquired from a tiny piece of tumor would be greatly enhanced.

Another possibility is to further expand the single cell analyses to the very end of the central dogma, namely the high throughput proteomic analysis on individual cells. As described before, single cell analysis of the cancer proteomes may be particularly difficult, even reading through the one-dimensional protein sequences without understanding the higher order protein confirmation. To combat this difficulty, a mass spectrometry based system can be set up to look for the subtle changes in proteome and metabolome concentration.[80] MS imaging (MSI) takes the pursuit of high resolution one step further, and MSI seems to be very clinically and pharmacologically applicable by visualizing molecular spatial organizations.[39] MSI also appears to function during a surgery, further fortifying MSI's clinical viability. For example, observations of the ADSV-TGM4 combination in the urinary track may indicate benign tumor formation in the prostates. Not only are specific transcriptomic and/or proteomic patterns important biomarkers on their own, heterogeneity between each RNA or protein molecule also seems to provide valuable information.

Current cancer treatments do not start with cancer cell sequencing. The determination of the treatment scheme may largely depend on the classification and subtyping of cancers based on histology.[3] In the near future, oncologists and surgeons could opt to study patient specific cancer organoids first.[79] Organoids are miniature artificial organs formed by a few cells in three dimension.[79,81] Unlike regular organoids, cancer organoids are

small cancer-like or tumor-like organs formed solely from cancer cells.[79,82] The genetic makeup of cancer cells in different patients may differ even for the same disease, and such difference results in treatment effectiveness variations in chemotherapy.[83] By experimenting with various treatments on cancer organoids, oncologists may tailor patients their own personalized medicine. Strikingly, 3D printing may be employed by scientists or doctors to reconstruct the solid tumor itself, perhaps also including its microenvironment. For example, when mixing the cells with matrices such as hydrogel, *in vitro* cultured cancer cells may be assembled into designated shaped by 3D printing.[82] Furthermore, if patient specific cancer organoid can be reconstructed *in vitro*, it would become a more easily accessible way to conduct single cell sequencing analysis. For example, to further enhance this process, single cell genomic and epigenomic sequencing can be utilized to study individual cells in the cancer organoids.[27,79] This will provide more insight into the structural formation of those organoids in relation to their genetic makeup and their spatial position.

Interestingly, although single cell sequencing methods are mainly concerned in analyzing human tissues, normal or malignant, they may also be used to dissect the microbiome in the context of cancer. The gut microbiome, otherwise known as the human gastrointestinal microbiota, is one of the frontiers of microbiology that can have a significant impact on human health, including several types of cancers.[84] The gut microbiome includes the colonies of microorganisms that lives in digestive system of humans, and it may combat cancer passively via tumor immunosurveillance.[84] It may serve to stimulate the endogenous human immune system to form an antitumoral response. For instance, certain bacteria families are enriched in the melanoma patients responding to anti-PD-1 immunotherapy.[85] In addition, the change of diet habit may shape the gut microbiome and influence cancer incidence. In first world countries, increasing amounts of people are shifting towards an excessively high energy diet. Disproportionate fat intake over prolonged periods of time may result in non-alcoholic fatty liver disease (NAFLD), which has a high potential to turn malicious and develop into non-alcoholic steatohepatitis (NASH), the perfect breeding ground for HCC.[86] In such cases, a fecal microbiota transplant (FMT) can be administrated. A healthy microbiota could aid to eliminate residue lipid droplets into short chain fatty acids which can be efficiently cleansed by the human intestines itself via absorption.[86] Although more FMT studies should be carried out to verify its anti-cancer effects, successful fecal transplants could be sequenced via deep sequencing (with or without single cell separation) to identify the microbial makeup to allow for artificial bacterial culture developments without the need of consistent fecal extraction.

Encompassing all, current single cell sequencing methods do promise a bright future for cancer basic research and clinical applications. Indeed, present hurdles from the acquisition of samples to the amplification of single cell signals would await further technical advances. It is very likely that the combination of other emerging cutting-edge technologies would complement the drawbacks of single cell analysis.[33,81,87]

Closing remarks

In closing, single cell sequencing can serve as an effective approach towards the more accurate and reliable genetic and epigenetic analysis of tumors, especially to reveal their heterogeneous cell composition and behavior. This could be critical important to address several issues related to cancer metastasis, drug resistance and recurrence. For basic cancer biology research, single cell sequencing analysis could also facilitate the investigation on how favorable mutations are evolving during the time course of cancer development. For clinical uses, sequencing the CTCs may aid to provide early diagnostic information for better and more effective therapeutic interventions.

Conflicts of interests

The authors declare no competing financial interests.

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