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Advancements in Single-Cell Sequencing Techniques: Implications for Understanding Tumor Heterogeneity and Personalized Cancer Therapy

[1]-Amal Aqeel Alawfi ,[2]-Mohammed Ibrahim A. Hamadi,[3]-Sultan Mousa J. Wasili,[4]-Wejdan Ali Alwan Fadhel,[5]- Majed Ahmed Mohamed Badwialan,[6]-Abdulrahman Fadhi Dakheen Alanazi,[7]-Mohammed Saleem Aljizani,[8]-Gassim Yazeed Yahia Ghzwani,[9]-Amal Saad Ahmad Alqarani,[10]-Khalid Ahmed Ahmed Hothan,[11] - Ahmad Mohamed Khawaji,[12]- Faleh Obaid Zabbar Al Harithi,[13]-Ali Murdi Mohsen Alqarni,[14]-Nouf Ahmad Eprahim Shahhar,[15]-Abdullah Mohammed Abdullah Alomi

[1]KSA, Ministry Of Health, King Salman Bin Abdulaziz Medical City In Madinah

[2]KSA, Ministry Of Health, Abuarish General Hospital

[3]KSA, Ministry Of Health, Abu Arish General Hospital

[4]KSA, Ministry Of Health, Abu Arish General Hospital

[5]KSA, Ministry Of Health, Jeddah

[6]KSA, Ministry Of Health

[7]KSA, Ministry Of Health, Abdullaziz Hospital, Jeddah

[8]KSA, Ministry Of Health, Bani Malek General Hospital, Jizan

[9]KSA, Ministry Of Health, Tabuk Health Cluster Duba General Hospital

[10]KSA, Ministry Of Health, King Khalid University Hospital

[11]KSA, Ministry Of Health, Endemic Disease Vector Control Center Sabya

[12]KSA, Ministry Of Health, Al-Muzahmiya General Hospital

[13]KSA, Ministry Of Health, King Khalid University Hospital

[14]KSA, Ministry Of Health, Jazan General Hospital

[15]KSA, Ministry Of Health, King Khalid University Hospital

Abstract

Background: The advent of single-cell sequencing technologies has revolutionized cancer research by enabling the detailed examination of cellular heterogeneity and molecular characteristics at the individual cell level. This approach provides insights into the complex dynamics of tumor biology, including identifying rare cell populations and the mechanisms underlying tumorigenesis, progression, and treatment resistance.

Methods: This review delineates the advancements in single-cell analysis techniques, particularly focusing on single-cell RNA sequencing (scRNA-seq), single-cell DNA sequencing, and emerging multiomic approaches. We systematically explore novel methodologies, including microfluidics, combinatorial indexing, and in situ sequencing. We also evaluate the application of these techniques in various cancer contexts, highlighting their capacity to uncover new biomarkers and therapeutic targets.

Results: Recent findings demonstrate that single-cell sequencing can effectively delineate the tumor microenvironment, reveal clonal evolution, and identify distinct cellular subpopulations. These

technologies have facilitated significant advancements in understanding the heterogeneity inherent in tumors, enabling the characterization of cancer stem cells, circulating tumor cells, and immune microenvironments. Comparative analyses of different sequencing platforms, such as 10X Genomics and SMART-Seq, underscore their strengths and limitations in capturing transcriptomic diversity.

Conclusion: Single-cell sequencing technologies are essential for advancing our understanding of cancer biology and improving clinical outcomes through personalized medicine. Despite certain limitations, including sensitivity issues and batch effects, ongoing technological enhancements promise to refine these methods further, paving the way for more comprehensive insights into tumor biology and therapeutic strategies.

Keywords: Single-cell sequencing, Cancer heterogeneity, Tumor microenvironment, Biomarkers, Personalized medicine

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1. Introduction

Single-cell sequencing, using next-generation sequencing, has advanced rapidly in recent years. The first single-cell mRNA sequencing experiment occurred in 2009, followed by the first single-cell DNA sequencing experiment in human cancer cells in 2011, and the initial single-cell exome sequencing experiment in 2012 [1, 2]. Single-cell sequencing markedly surpasses earlier sequencing technologies in elucidating human biology related to embryonic cells, intracranial neurons, malignant tumor cells, and immune cells, as it enables the examination of cellular and microenvironmental heterogeneity at single-cell resolution. It has transformed our capacity to examine the transcriptional, genomic, epigenomic, and metabolic attributes of hundreds of individual cells in detail, enabling an impartial investigation of the cells inside tumor lesions. It moreover offers molecular insights, encompassing single-nucleotide variations (SNVs), copy number variations (CNVs), and structural variations (SVs) [3, 4]. Nonetheless, the constraints of single-cell sequencing must not be overlooked; they include its restricted sensitivity, size, and accuracy; inadequacy in reconstructing clonal evolution in spatial and temporal sequences; and noise resulting from the preamplification of single-cell RNA. These constraints have mostly been addressed by technological advancements and the integration of other developing technologies, allowing researchers to examine multiomic data at a single-cell level.

Single-cell sequencing provides unparalleled resolution, allowing the investigation of optimal management and individualized therapies across several contexts, including developmental study, the creation of human cell atlases, and cancer research [5, 6]. Besides developmental biology, single-cell RNA sequencing (scRNA-seq) and its associated technologies may facilitate the discovery of new markers, uncommon subpopulations, and evolutionary trends, particularly in brain development [7, 8]. In cancer research, it can be utilized to identify or examine rare subpopulations; circulating tumor cells (CTCs); the tumor or immune microenvironment; tumor heterogeneity and molecular subtype; mechanisms related to tumorigenesis, progression, metastasis, evolution, relapse, and therapy resistance; and cancer stem cells (CSCs) [9-12].

This study encapsulates current advancements in single cell sequencing technology, including novel sequencing methods, data processing techniques, and applications within cancer research.

2. Advancing single-cell sequencing technologies

Advancements in single-cell isolation, sequencing, cDNA library preparation, and analytical techniques have significantly enhanced single-cell sequencing analysis capabilities. In recent years, single-cell transcriptomics, genomes, proteomics, epigenomics, and interactomics sequencing have advanced swiftly. scRNA-seq technologies include many standardized procedures: isolation of a single cell, RNA extraction, reverse transcription, preamplification, and detection [13]. The first operations are undoubtedly the most critical since they dictate the precision and quantity of the amplified material. Research shown that a reduced single-cell dissociation temperature (6 °C) mitigates the stress responses elicited at 37 °C,

including the activation of 512 heat shock proteins. Furthermore, the dissociation procedure influences transcription signatures [14].

Besides the need for single-cell isolation, the primary problem in single-cell sequencing is in interpreting the sequencing results at the single-cell level. Single-cell barcoding methods using plate microreaction systems and combinatorial indices have effectively addressed this limitation, hence enhancing the throughput of single-cell analysis by a minimum of 100-fold. Plate microreaction systems typically include individual cells, functional beads, and reverse transcriptomes. The surface of the functional beads is altered using oligonucleotides, including primers, cell barcodes, unique molecular identifiers (UMIs), and poly(dT) moieties from 5′ to 3′. The primer and poly(dT) components are consistent throughout the microreaction systems, but the cell barcode is distinct for each microreaction system, and the UMI is specific to each molecule inside an individual cell. Furthermore, according to specific sequencing objectives, the UMI may annotate diverse molecules, including the DNA genome, transcriptome, immunological profile, and proteome. These attributes provide the accurate identification of molecular characteristics of an individual cell. Drop-seq, Seq-Well, and inDrop are bead-based methodologies. Zhang et al. elucidated the distinctions between Drop-seq and inDrop comprehensively. The cell barcode length is 38–41 bp in inDrop and 12 bp in Drop-seq, whereas the cell barcode capacity is 147,456 (384*384) in inDrop and 16,777,216 (412) in Drop-seq [15]. Moreover, the same kind of bead is used in Seq-Well and Drop-seq [16].

A novel single-cell barcoding approach using combinatorial indexing addresses the challenges associated with the comparatively high expense of extracting individual cells for bead-based barcoding methods. Combinatorial indexing-based barcoding methods identify individual cells by the sequential insertion of cellular barcodes without the need for cell isolation; these technologies include Sci-Seq, Microwell-Seq, and Split-Seq [17]. In Sci-Seq, two iterations of transposase barcoding and PCR ligation labeled 9,216 (96x96) individual cells. Three split-pool rounds presented three segments of the oligonucleotide sequence to the magnetic beads in Microwell-Seq [18]. Nevertheless, an insufficient number of cells for further investigation are isolated in Sci-Seq and Microwell-Seq. Split-seq, derived from Sci-Seq, employs five rounds of barcoding, significantly enhancing sequencing across millions of single cells while reducing costs [8].

Following the barcode-targeted molecules, the following critical phase is preamplification, during which the transcripts undergo reverse transcription. The preamplification process in 10X Genomics technology transpires as follows. The transcripts are first attached to the 5′ poly(dT) in oligonucleotides via the polyA tail at the 3′ end. Secondly, the oligonucleotides undergo reverse transcription from the 5′ to the 3′ end, using the bound transcript as a template. The expansion of the oligonucleotide chain concludes with the addition of multiple cytosine bases, a process mediated by a specific terminal transferase. Third, the template switch oligo (TSO) is incorporated into the template by reverse transcriptase, subsequently followed by the insertion of the TSO to the complementary strand. The newly synthesized full-length cDNA, referred to as first-strand cDNA, serves as the template for further cDNA amplification, known as template switching. Fourth, the second cDNA strand is produced using the first cDNA strand as a template [19]. The whole procedure is referred to as in vitro transcription. Moreover, multiple displacement amplification (MDA) is a non-PCR DNA amplification technique that utilizes isothermal amplification. MDA employs a specialized DNA polymerase known as bacteriophage phi29 DNA polymerase, capable of amplifying minimal quantities of DNA, even from a single cell, with high binding affinity [20]. Moreover, the recently developed in situ 10-cell RNA sequencing, employing Taq and Phusion polymerases, has significantly enhanced the yield of approximately 500 bp preamplification products and permits a tenfold reduction in T24-containing primer concentration without any discernible loss in preamplification efficiency. Furthermore, in situ 10-cell RNA sequencing allows the concurrent sequencing of 10 microdissected cells within their native tissue environment [21].

Most single-cell sequencing approaches for detection depend on empirical factors or predetermined structures to assess complexity. A recent study shown that integrating nonnegative matrix factorization with Bayesian model comparison with contemporary techniques enables clear evaluations of the heterogeneity depth inside the tumor microenvironment (TME) [22]. Single-cell hierarchical Poisson factorization facilitates the identification of both continuous and discrete expression patterns from de novo

scRNA-seq data; moreover, the recently created super CT can train the expandable supervised classifier upon the input of RNA data [23,24]. The precise examination of cell subsets enhances comprehension of clonal composition and heterogeneity.

3. Transcriptomics

At now, scRNA-seq is extensively used to characterize the transcriptomes of individual cells. The droplet-based 10X Genomics Chromium and the plate-based Switching Mechanism at the 5′ End of RNA Template sequencing (SMART-Seq) are two commonly used systems. The 10X Genomics technology utilizes a microfluidics methodology to isolate, label, amplify, and prepare a cDNA library from 5,000 to 10,000 single cells rapidly. Nonetheless, it only identifies the 3′ or 5′ terminus of the transcript with a bias, necessitating a substantial quantity of cells in a single sample (preferably above 90%). Furthermore, the cell capture rate is inferior to that of SMART-Seq, rendering it unsuitable for identifying uncommon samples with a limited number of cells [25].

SMART-Seq, introduced in 2012, enables the identification of full-length transcripts [26]. SMART-Seq v2, introduced in 2013, omits purification steps, substitutes the terminal guanosine at the TSO 3′ end with locked nucleic acid (LNA), and employs betaine to enhance protein heat stability, thus significantly improving yield [27]. The core principle of full-length sequencing is the use of Moloney murine leukemia virus (MLV) reverse transcriptase, which favors full-length cDNAs as substrates for its terminal transferase activity. Additionally, SMART-Seq v4 exhibits enhanced efficiency in template swapping, hence reducing the duration of cDNA synthesis and library formation, while also demonstrating superior sensitivity for low input and increased repeatability [28]. SMART-Seq v2 and v4 are extensively used in oncological studies, including investigations on hepatocellular carcinoma (HCC) [29-31]. Furthermore, SMART-Seq does not need supplementary equipment, indicating that it mostly relies on proficient researchers.

Recent research analyzed sequencing data from CD45 cell samples produced by the 10X Genomics and SMART-Seq v2 platforms. Specifically, SMART-Seq v2 exhibited greater sensitivity and identified a higher number of genes inside a single cell, particularly low-abundance transcripts and alternatively spliced variants. The use of 10X Genomics resulted in heightened dropout rates and amplified noise in transcripts exhibiting low expression levels. Nonetheless, 10X Genomics identified a greater number of genes owing to its enhanced coverage of numerous cells, hence facilitating the detection of uncommon cell types. Subsequent examination of the sequencing data demonstrated that the two platforms identified unique differentially expressed genes (DEGs) within cell clusters, suggesting the possibility of integrating complimentary findings to examine heterogeneity [32]. A separate investigation integrating 10X Genomics and SMART-Seq v2 elucidated the immune cell landscape and examined the dynamic migratory and status-switch characteristics in hepatocellular carcinoma (HCC) [33].

Spatial transcriptomics and temporal lineage tracing provide comprehensive examination of the local environment and dynamic interactions inside an individual cell. Temporal and geographic variability affect tumor heterogeneity and stress responses, making them essential for cancer diagnosis, subtyping, classification, and therapy in cancer research [34-36]. The systematic use of spatial barcodes enables the encoding and retrieval of locational data on individual cells, hence offering valuable insights for research and diagnostic purposes. A recent work elucidated a comprehensive geographic map of single-cell phenotypes and cellular communities, revealing phenotypic variability in the breast tumor microenvironment [37]. Moreover, single-molecule RNA fluorescence in situ hybridization (smFISH) provides accurate spatial data in pancreatic and breast cancers [38, 39].

The progression of cancer is a dynamic process including several stages, including oncogenesis, metastasis, and the emergence of treatment resistance. Establishing the temporal and molecular characteristics of each phase in this process is essential for understanding cancer biology and formulating successful treatment methods. The recently established technology, lineage tracing by nuclease-activated editing of ubiquitous sequences (LINNAEUS), was effectively used to rebuild lineage trees in zebrafish [40]. Single-cell analysis using CRISPR-Cas9 technology was used to delineate a comprehensive malignant cell spectrum in a KRAS mutant murine model. This approach addresses the challenge of poor mutation sensitivity and the

difficulties in elucidating the evolving characteristics of tumor subtypes, hence aiding in the monitoring of dissemination patterns and critical genes in lung cancer. The recent results suggest the possibility of creating tailored therapies and enhancing the clinical treatment of lung cancer in individuals with KRAS mutations [41]. A separate work that integrated scRNA-seq with high-confidence clonal tracing provided the first comprehensive characterisation of leukemic stem cells, enhancing the understanding of leukemia oncogenesis and therapy [42].

Single-nucleus RNA sequencing (snRNA-seq) originated in 2013, when Grindberg discovered the challenges of dissociating integrated cells from brain tissue [43]. Div-seq and DroNc-seq have been used to address the challenge of detecting uncommon brain cells and acquiring integrated cell types [44, 45]. Simultaneously, researchers have performed snRNA-seq analysis on brain tissues from autopsies to enhance the cellular landscape obtained from scRNA-seq data, indicating that frozen materials are amenable to snRNA-seq study [7]. At present, snRNA-seq has been extensively used across several tissues and cell types, including the kidney, heart, lung, pancreas, and notably, brain tissue [46-50]. In comparison to scRNA-seq, scRNA-seq offers several benefits. Initially, snRNA-seq may be used to examine valuable frozen materials, since the nuclear membrane may stay intact, in contrast to the cell membrane in frozen tissues [51]. Secondly, snRNA-seq does not generate artifactual transcriptional stress responses or biases, which may arise from single-cell isolation in scRNA-seq, therefore accurately representing the true transcriptional state. Third, snRNA-seq may prevent the loss of certain cell types attributable to varying susceptibilities to proteases. The manufacture of a single nucleus is more straightforward than that of a single-cell suspension, hence reducing the formation of pseudocell populations caused by enzymatic hydrolysis and mechanical pressure.

SnRNA-seq has been extensively used to investigate brain cancers because of the challenges associated with acquiring fresh brain tissue for study, since most samples are cryopreserved. In 2020, MIT researchers performed scRNA-seq and snRNA-seq studies on fresh or frozen tumor samples and assessed the sequencing outcomes concerning cell and nuclear quality, cellular makeup, and other metrics. The comparison revealed that the two sequencing approaches identified analogous cell types with varying cellular proportions across various tissues. For instance, the ratio of immune cells was elevated, but the parenchymal cells (such as neural crest and neuroendocrine cells) were markedly reduced in neuroblastoma, as shown by scRNA-seq analysis. Nonetheless, the ratio of considerable cells, particularly malignant cells, was elevated, while specific immune cells were markedly reduced or entirely missing in the snRNA-seq data. Notably, scRNA-seq detected far fewer neurons than snRNA-seq, highlighting the considerable potential of snRNA-seq in elucidating the landscape of brain malignancies [52]. SnRNA-seq acquires sequencing data from intron and intergene regions, facilitating greater resolution cell type identification and yielding more comprehensive gene information. Nonetheless, the quantity of RNA inside a single nucleus remains much less than that seen in whole cells, indicating that snRNA-seq may be unsuitable for investigations involving immune cells.

4. Genomics

Single-cell DNA sequencing has not yet advanced to high-dimensional analysis owing to its prohibitive cost. Consequently, the most cost-effective approach is to first implement bulk sequencing, followed by targeted single-cell DNA sequencing of the specific mutations or variants of interest. TARGET-seq, which combines genomic DNA and coding DNA genotyping, provides extensive coverage of critical mutation hotspots and allows the very sensitive examination of mutations in individual cells. Nonetheless, TARGET-seq depends on the study of established mutations and does not facilitate the detection of novel changes [53]. The computational method Cardelino incorporates clone tree data from bulk exome sequencing and rare variant alleles from scRNA-seq, distinguishes phenotypic differences among clones, and has identified differentially expressed genes (DEGs) related to the cell cycle and proliferation pathways between cancerous and healthy skin tissues in [54].

Single-cell sequencing is extensively used to identify differentially expressed genes and may reveal critical hallmark genes during tumor growth. A variety of bioinformatics tools exist to analyze scRNA-seq data and provide significant discoveries. Mixed isolated cells may be hierarchically classified into distinct subsets

according to differentially expressed genes along the pseudotime or copy number variations, facilitating the construction of clonality trees, as shown in medulloblastoma [55]. Secondly, SCENIC analysis using gene expression levels may elucidate the dynamic relationships among genes inside individual cells, facilitate the construction of a gene regulatory network, and uncover regulatory alterations during phenotypic transitions. Third, the analysis of enriched gene ontology terms and pathway analysis utilizing the Kyoto Encyclopedia of Genes and Genomes could elucidate the expression of genes implicated in specific signaling pathways related to tumorigenesis, the cell cycle, epithelial-to-mesenchymal transition (EMT), and immune responses across various subclusters. Significantly, by integrating expression data with specific cell types, we can elucidate the roles of these cell types in tumor growth processes, including angiogenesis and tissue remodeling [56].

5. Utilization of single-cell sequencing in oncology

Alongside the Human Cell Atlas, single-cell sequencing technology offers a unique chance to elucidate the functional states of individual cancer cells. Microwell-Seq facilitated the elucidation of cellular hierarchy and clonal heterogeneity in normal bone marrow and acute myeloid leukemia [57]. Integrating clinical pathology information with single-cell sequencing data may reveal new diagnostic and prognostic biomarkers, as well as potentially therapeutically useful cell types or states [58]. In conclusion, The Cancer Cell Atlas offers reference material for neoadjuvant treatment, particularly in cases when baseline advice is missing [59].

The tumor microenvironment (TME), consisting of cellular and acellular elements, is essential to carcinogenesis, progression, invasion, metastasis, and medication resistance. These components coordinate a microenvironment that both promotes and inhibits tumors, so modulating tumor development and influencing tumor evolution, with heterogeneity also playing a role. The molecular foundation for heterogeneity is evident in diverse malignant subpopulations characterized by structural changes, chromosomal rearrangements, epigenetic alterations, and gene expression profiles [60-63]. Furthermore, nongenetic intratumor heterogeneity is a significant predictor of phenotypic heterogeneity and evolutionary dynamics in lung cancer, beyond the influence of genomic characteristics alone [64]. Nonetheless, heterogeneity is the primary barrier to the advancement of successful medicines; thus, elucidating tumor tissue heterogeneity would significantly enhance our comprehension of the fundamental processes and facilitate the development of targeted therapies in clinical trials. Recent studies indicate that heterogeneity remains constant over time between replicates of the same culture, strongly implying a controlled process rather than a random one, as previously indicated [65].

6. Conclusions

Single-cell sequencing has progressed rapidly, and forthcoming sophisticated technologies will surely enhance precision and accuracy in molecular cancer research, including the tumor microenvironment and heterogeneity. Consequently, it greatly enhanced our comprehension of cancer diagnostic stratification, biomarkers, targeted therapy, and prognostic prediction.

Nonetheless, the constraints of single-cell sequencing must be acknowledged. Initially, scRNA-seq inherently demonstrates that not all eukaryotic cells exhibit transcription at a uniform basal rate. Transcription happens in pulses; hence, instantaneous sequencing cannot fully elucidate the transcription map [66,67]. Furthermore, the majority of sequencing techniques are optimized for 3′ or 5′ reads and lack sensitivity to low-abundance transcripts. Secondly, scRNA-seq in isolation cannot establish a correlation between genotype and phenotype, indicating the need for high-throughput, cost-effective multiomic technologies to elucidate the whole tumor tissue landscape. Third, batch effects may arise from the use of various platforms, processing methodologies, and analyses conducted on different days. Such batch effects are obviously evident when analyzing data from various sequencing projects. This sort of sequencing study is difficult to execute in bigger populations; thus, current results should be utilized cautiously and validated in clinical trials. Furthermore, the variability across patients and the many platforms used in various studies undermine the trustworthiness of the aforementioned findings.

Multiomics amalgamates high-throughput biomolecular data pertaining to several elements, including the genome, transcriptome, proteome, interactome, epigenome, and metabolism, with the transcriptome being pivotal. The multiomics data facilitate the examination of intricate interactions and relationships within tumors across various states and phenotypes, while also offering supplementary detailed information on the dynamic processes of tumor initiation, progression, growth, immune evasion, metastasis, relapse, and therapeutic resistance. The use of multiomics techniques enhances per-cell technical dependability and sensitivity, leading to improved accuracy, robustness, compatibility, and expandability, as well as advancements in the preamplification process. These data facilitate the development of a sophisticated computational framework that allows for the de novo identification of both continuous and discrete expression patterns from single-cell sequencing data.

In conclusion, the amalgamation of sequencing methodologies can produce intricate, high-throughput data encompassing genomic, epigenomic, transcriptomic, proteomic, spatial, and temporal information, thereby requiring the formulation of robust and accurate models or algorithms to devise novel strategies for diagnosis, classification, targeted therapy, and prognostic prediction. Significantly, the precision and sensitivity of nascent technologies and computational analyses need enhancement, and expenses should become more economical in the imminent future.

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التطورات في تقنيات تسلسل الخلية الواحدة: تأثيرها على فهم تغاير الأورام والعلاج الشخصي للسرطان

الملخص

الخلفية :أحدثت تقنيات تسلسل الخلية الواحدة ثورة في أبحاث السرطان من خلال تمكين فحص دقيق للتغاير الخلوي والخصائص الجزيئية على مستوى الخلية الفردية. يوفر هذا النهج رؤى متعمقة حول الديناميكيات المعقدة لبيولوجيا الأورام، بما في ذلك تحديد التجمعات الخلوية النادرة والآليات الكامنة وراء التسرطن، وتطور المرض، ومقاومة العلاج

الطرق :تستعرض هذه المراجعة التطورات في تقنيات تحليل الخلية الواحدة، مع التركيز بشكل خاص على تقنيات تسلسل الحمض النووي الريبي للخلية الواحدة(scRNA-seq) ، وتسلسل الحمض النووي للخلية الواحدة، والنهج متعددة الأوميكس الناشئة. نستكشف المنهجيات الحديثة بشكل منهجي، بما في ذلك تقنيات الميكروفلويدكس(microfluidics) ، الفهرسة التركيبية، وتقنيات التسلسل في الموقع. كما نقيم تطبيق هذه التقنيات في سياقات السرطان المختلفة، مسلطين الضوء على قدرتها على الكشف عن مؤشرات حيوية وأهداف علاجية جديدة.

النتائج :تشير النتائج الحديثة إلى أن تسلسل الخلية الواحدة يمكنه بشكل فعال تحليل البيئة الدقيقة للورم، وكشف تطور المستنسخات الخلوية، وتحديد التجمعات الخلوية المميزة. وقد ساهمت هذه التقنيات بشكل كبير في فهم التغاير الموجود داخل الأورام، مما أتاح توصيف الخلايا الجذعية السرطانية والخلايا الورمية المنتشرة والبيئات المناعية الدقيقة. تُظهر التحليلات المقارنة لمنصات التسلسل المختلفة، مثل 10 Genomics X وSMART-Seq، نقاط قوتها وقيودها في التقاط تنوع النصوص الوراثية.

الخلاصة :تُعد تقنيات تسلسل الخلية الواحدة أساسية لتعزيز فهمنا لبيولوجيا السرطان وتحسين النتائج السريرية من خلال الطب الشخصي. على الرغم من بعض القيود، مثل مشكلات الحساسية وآثار الدفعات، فإن التحسينات التكنولوجية المستمرة تعد بمزيد من التطوير لهذه الأساليب، مما يمهد الطريق لرؤى أكثر شمولاً في بيولوجيا الأورام واستراتيجيات العلاج.

الكلمات المفتاحية :تسلسل الخلية الواحدة، تغاير السرطان، البيئة الدقيقة للورم، المؤشرات الحيوية، الطب الشخصي