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Review article

Single-cell multi-omics in the study of digestive system cancers

Shuang Zhou [a,b,1], Nanfei Lin [b,1], Liying Yu [b], Xiaoshan Su [c], Zhenlong Liu [d], Xiaowan Yu [e], Hongzhi Gao [b,*], Shu Lin [f,g], Yiming Zeng [c,h,i,**]

[a] The Second Clinical Medical School of Fujian Medical University, Quanzhou, Fujian Province, China
[b] The Clinical Center of Molecular Diagnosis and Therapy, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian Province, China
[c] Department of Pulmonary and Critical Care Medicine, The Second Affiliated Hospital of Fujian Medical University, Respirology Medicine Centre of Fujian Province, Quanzhou, China
[d] Lady Davis Institute for Medical Research, Jewish General Hospital, & Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, QC, Canada
[e] Clinical Laboratory, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian Province, China
[f] Centre of Neurological and Metabolic Research, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian Province, China
[g] Diabetes and Metabolism Division, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia
[h] Fujian Provincial Key Laboratory of Lung Stem Cells, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian Province, China
[i] Jinan Microecological Biomedicine Shandong Laboratory, Jinan, Shandong Province, China

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ABSTRACT

Digestive system cancers are prevalent diseases with a high mortality rate, posing a significant threat to public health and economic burden. The diagnosis and treatment of digestive system cancer confront conventional cancer problems, such as tumor heterogeneity and drug resistance. Single-cell sequencing (SCS) emerged at times required and has developed from single-cell RNA-seq (scRNA-seq) to the single-cell multi-omics era represented by single-cell spatial transcriptomics (ST). This article comprehensively reviews the advances of single-cell omics technology in the study of digestive system tumors. While analyzing and summarizing the research cases, vital details on the sequencing platform, sample information, sampling method, and key findings are provided. Meanwhile, we summarize the commonly used SCS platforms and their features, as well as the advantages of multi-omics technologies in combination. Finally, the development trends and prospects of the application of single-cell multi-omics technology in digestive system cancer research are prospected.

1. Introduction

The digestive system, consisting of the digestive tract and organs, is prone to cancer, contributing to the most commonly diagnosed cancers [1]. Five of the top 10 fatal tumors are digestive system cancers, including esophageal, gastric, colorectal, liver, and pancreatic cancers. Because these tumors have high morbidity and mortality rates, oncologists have struggled with the lack of early detection and precise

Abbreviations: CAFs, cancer-associated fibroblasts; CITE-Seq, Cellular Indexing of Transcriptomes and Epitopes by Sequencing; CNV, Copy number variation; CRC, Colorectal cancer; CSCs, Cancer stem cells; CTCs, Circulating tumor cells; DOP-PCR, Degenerate oligonucleotide primer PCR; EAC, Esophageal cancer; ESCC, Esophageal squamous cell carcinoma; FC, Flow cytometry; GAC, Gastric adenocarcinoma; GC, Gastric cancer; HCC, Hepatocellular carcinoma; HSCs, Hepatic stellate cells; IPMNs, Intraductal papillary mucinous neoplasms; MALBAC, Multiple annealing, and cyclic amplification; MDA, Multiple displacement amplification; MMLV, Moloney Murine Leukemia Virus; MS, Mass spectrometry; Nanopore-seq, Nanopore sequencing; PaCa, Pancreatic cancer; PDAC, Pancreatic ductal adenocarcinoma; scBS-seq, single-cell whole-genome bisulfite sequencing; ScDNA-seq, Single-cell DNA sequencing; SCNAs, Somatic copy number changes; ScRNA-seq, Single-cell RNA sequencing; SCS, Single-cell sequencing; scWB, Single-cell western blotting; scWES, Single cell whole exon sequencing; SNV, Single nucleotide variation; SV, Structural variation; TAMs, Tumor-associated macrophages; TCR, T cell receptor; TME, Tumor microenvironment; UMIs, Unique molecular identifiers; WES, Whole exome sequencing; WGA, Whole genome amplification; WGS, Whole genome sequencing; WTA, Whole transcriptome amplification.

* Corresponding author.
** Corresponding authors at: Department of Pulmonary and Critical Care Medicine, The Second Affiliated Hospital of Fujian Medical University, Respirology Medicine Centre of Fujian Province, Quanzhou, China.
E-mail addresses: 1564747628@qq.com (H. Gao), ymzeng08@163.com (Y. Zeng).
[1] Shuang Zhou and Nanfei Lin contribute to this article equally as the first author

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treatment. The main reason for the lack of treatment is the heterogeneity and complexity of the tumor, coupled with the naive ambiguity of the immunosuppressive microenvironment. Cancer research, including cancer of the digestive system, is under unprecedented pressure.

Single-cell sequencing (SCS) has undergone a significant shift in the past decade as these technologies moved from experts' laboratories to other cancer research groups across the globe [2]. The methods of SCS have become more accessible due to the democratization process. The SCS methods have advantages over the traditional "bulk" DNA-seq and RNA-seq approaches. The latter can only provide mixed signals of multiple cell types and fusion of tumor clones with different genotypes [3]. In contrast, SCS technologies can realize the sequencing of a single cell due to their high resolution, which can analyze the cell polymorphism in tumors and solve the problem of tumor heterogeneity [2,4].

From single-cell transcriptome sequencing to genome sequencing, from epigenetics to proteomics, and then to SCS combined with spatial transcriptomics (ST), the development and application of this field have made great progress. Researchers have attempted to apply multi-omics SCS technologies to the study of the same tumor, realized the integration of multi-omics sequencing, and achieved surprising results, which will greatly promote the development of oncology and even overturn the original oncology research results [5,6].

This review highlights the research and application of multiple SCS technologies in five of the most common gastrointestinal cancers, which will help to introduce single-cell technologies into the clinical arena better. We believe that the clinical application of single-cell multi-omics binding to traditional omics will contribute to the diagnosis and treatment of digestive system cancers and ultimately improve patient survival.

1.1. The overall development of SCS technology

It has been more than 10 years since the earliest experiments in single-cell mRNA sequencing, DNA sequencing, and exon sequencing, but this field has rapidly developed [7,8]. In particular, the development of microdroplets, microfluidic, and nanopore technologies has made it possible to sequence thousands of cells simultaneously and greatly reduced sequencing costs [9–11]. The advent of commercial SCS products, represented by 10 ×Genomics, has further advanced research in this area. Unique molecular identifiers (UMIs) have also helped. These tag many molecules, such as the DNA genome, transcriptome, proteome, and immunological profiles, based on different sequencing targets [2,12]. These techniques are based on cell separation methods, and various single-cell isolation techniques have been fully developed. For single-cell preparation, the specimen should be as fresh as possible, and once it has been separated, it should be put right away in RPMI-1640 or DMEM medium with 20% fetal bovine serum on ice. The work should be finished within 10 h, and it should include washing the tissues in PBS, breaking them down with collagenase and hyaluronidase, and sorting the cells, among other things. The specimen should then be used right away for transcription or amplification steps [13].

Another critical step in obtaining genetic information from single cells is the amplification of the single-cell genome and transcriptome [3]. Amplification requires sensitivity, accuracy, and unbiasedness [14,15]. Single-cell epigenomics, single-cell proteomics, and integration with spatial omics have also received much attention [2,16]. This section will illustrate the relevant histological techniques of single-cell genomics, transcriptomics, epigenomics, proteomics, and ST. Fig. 1 summarizes the common technology platforms used for single-cell omics and their features.

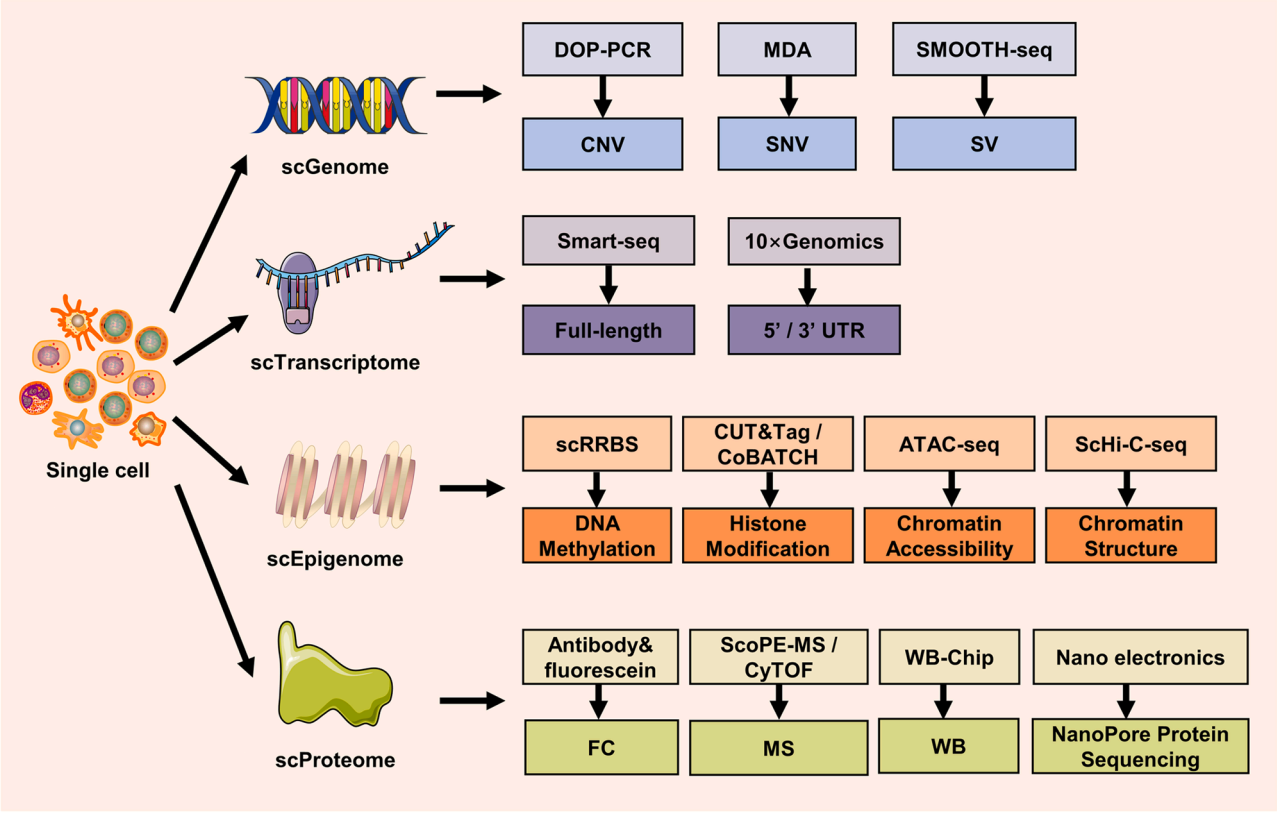

Fig. 1. The SCS technology platforms and features. Common SCS technology platforms and their various features are now available for you. For instance, scEpigenomics makes use of ATAC-seq to investigate chromatin accessibility, and DOP-PCR is mostly employed for discovering CNVs in individual cells.

1.2. Comparison of amplification methods used for single-cell genomic sequencing

Single-cell DNA sequencing (scDNA-seq) reveals unresolved biological questions in different fields, such as organism development, somatic mutations, genome function, and lineage evolution [17]. Cancer researchers mostly use whole genome sequencing (WGS) or whole exome sequencing (WES) to find structural variants (SV), copy number variants (CNV), and specific single nucleotide variants (SNV) [2]. Among them, CNV mapping analysis is more widely used [14]. Normal human cells contain two gene copies; the gene content is only at the pg level. Therefore, before sequencing, hundreds of thousands of amplifications of DNA must be performed using whole genome amplification (WGA) technology [5,18]. At the moment, the most common ways to copy the genome of a single cell are Degenerate oligonucleotide primer PCR (DOP-PCR) [19], Multiple displacement amplification (MDA) [20], and Multiple Annealing and Looping Based Amplification Cycles (MALBAC) [21]. Among them, DOP-PCR and DMA are the two most commonly used methods [16]. Overall, scDNA-seq is more challenging than scRNA-seq.

1.3. Single-cell RNA-seq—the most frequently applied SCS

Single-cell RNA sequencing (scRNA-seq) has many limitations, such as a low capture rate and amplification bias. However, with the great development in the past 10 years, scRNA-seq is undoubtedly the most mature SCS method [2]. It finds unique molecular features by looking at data on gene expression, exon splicing, and allele expression. This shows how different cell types work, as well as certain signals and cancer processes [14]. As with single-cell genomes, whole transcriptome amplification (WTA) is required before sequencing [22]. There are two ways to do WTA: quantitative reverse transcription PCR (RT-qPCR) for a small group of target genes, like 3 or 5′ sequencing, or full RNA sequencing of the whole transcriptome, like SMART-Seq2. It has been tried by scientists to get rid of amplification bias by using UMIs, high-fidelity enzymes, digital PCR instead of regular PCR, and better computer methods to fix amplification bias [23]. In terms of improving capture efficiency, it has been helpful to use high-viability single cells, improve cell lysis methods, use high-quality RNA, and tweak PCR conditions [24].

Thanks to the development of microfluidics, microwells, droplet technology, and technologies such as in situ barcoding and spatial transcriptome analysis, the throughput of scRNA-seq has increased from a few cells to hundreds of thousands of cells, and the cost has been greatly reduced [25]. Commercial sequencing products have made scRNA-seq simpler and more convenient. Currently, the two dominant technologies on the market are plate-based SMART-Seq2 and microdroplet-based 10 × Chromium 3′ sequencing, both of which have been successfully applied to the study of many diseases. In practice, which method is more suitable depends on the purpose of the study. For example, SMART-Seq2 captures full-length and is needed to study isoforms or gene fusions, which are more suitable for low-throughput situations. In contrast, 10 × Chromium 3′ sequencing captures a larger number of cells, allowing a comprehensive understanding of the transcriptional heterogeneity of cell populations [26].

Single-cell transcriptome sequencing has been widely used in immunology, developmental biology, oncology, and other fields in recent years, but the spatial structure information of tissues is lost during tissue dissociation. By integrating with scRNA-seq, ST fills this gap, allowing for spatio-temporal analyses of tissue function and pathological changes, as well as studying cellular component heterogeneity and transcriptional levels at the single-cell resolution level [27]. A specific description of spatial transcriptome technologies will be presented in Chapter 1.5. At present, single-cell transcriptome sequencing combined with ST is the most rapidly developing new technology in the field of single-cell omics combined with spatial omics, leading a new trend in the development of spatiotemporal genomics, which is promoting the development of cancer and tumor research, developmental biology, and other fields [28].

1.4. Single-cell sequencing in epigenomics

Epigenetics is the total number of genetic changes in DNA that do not alter the DNA sequence. It includes DNA methylation, histone modifications, chromatin accessibility, the spatial structure of chromosomes, etc. [29]. Researchers realize that single-cell epigenomics is crucial for the study of cancer and other diseases. However, single-cell epigenomics remains one of the most important technical challenges for SCS [16].

Despite the obvious technical hurdles, several new techniques have been developed (Table 3), such as scHi-C, scBS-seq, scRRBS, and scATAC-seq (10 ×Genomics) [15,30,31]. Among them, scATAC-seq has become the most widely used assay to measure single-cell chromatin accessibility. Open chromatin regions are labeled with Tn5 transposase as sequencing aptamers, amplified, and sequenced by PCR. Early ATAC technologies relied on FACS and labeling chemistry or microfluidic platforms. Later, a microdroplet platform (10 ×Genomics) for scATAC-seq was developed, which increased the cell throughput from hundreds to 10,000 cells in a single experiment. Subsequently, scATAC-seq was applied to T-cell receptor (TCR) studies, and the transcriptional marker ATAC-SEQ was reported. The technology has been widely promoted and recognized in cancer and immunology research [14,32,33].

1.5. Challenges in single-cell proteomics

Proteins are key performers in biological processes and cellular functions, making proteomics at single-cell resolution of particular interest to researchers [34]. Technological advances have made it possible to measure single-cell proteins so that we can analyze the cell types and states present in complex tissues. However, there are three major challenges in analyzing single-cell proteomics. (1) Mass spectrometry (MS) can only analyze the most abundant proteins and can miss low-abundance proteins [35]. (2) Amino acids and proteins are much more complex in type and structure than nucleic acids. (3) Proteins cannot be amplified, and sample loss during preparation needs to be minimized [36].

Four technologies are presently in existence. (1) The most well-known single-cell protein analysis method based on fluorescein-labeled antibodies is flow cytometry (FC), which has a high throughput and multiplicity but can only identify a small number of predefined proteins [37]. (2) MS-based, unlabeled proteomics techniques are more suitable for identifying proteomes in an untargeted mode. It includes label-free and multi-tagging methods. The label-free method is simple and easy to use, but only one single-cell sample can be analyzed at a time, with low throughput; the multi-tagging method can analyze multiple single-cell samples at the same time, with high throughput but requires the use of special chemical tags, among which the mass spectrometry flow-through (CyTOF) technology developed in 2002 uses lanthanide metal-tagged antibodies, which can detect as many as 51 proteins at a single time and is now widely applied. Later in 2019, the MS flow-based ScopE2-MS technology was developed, which

Table 3
Differences between scRNA-seq and ST used in combination and alone.

Feature/ Function	scRNA-seq	ST	scRNA-seq + ST
Spatial resolution	No	Yes	Yes
Cell type identification	Yes	Yes	Yes
Gene expression profiling	Yes	Yes	Yes
Regulatory network analysis	Yes	No	Yes
Cell type development analysis	Yes	Yes	Yes
Tissue microenvironment analysis	No	Yes	Yes
Mechanism of tissue development analysis	Yes	Yes	Yes
Therapy development	Yes	Yes	Yes

can detect up to 100 proteins simultaneously. It uses ScopE2 dyes for multi-tagging, which has higher sensitivity and accuracy, and it is a single-cell protein sequencing technology with a promising application that is still in the developmental stage and has already been used in immunology, oncology, and neuroscience research [38,39]. (3) NGS-based single-cell proteomics using antibody-oligonucleotide couplings (e.g., CITE-seq, RAID, etc.) for the detection of proteins on the cell surface or inside the cell [34,40]. (4) Imaging-based technologies that combine single-cell proteomics with spatial imaging for mapping the location of proteins in cells and tissues, such as multiplexed immunofluorescence mIF, spatial mass spectrometry imaging (SMSI), and imaging mass spectrometry flow-through (IMC), in which mIF is typically used for cell samples in cell culture dishes or sections and is widely used in clinical diagnostics. SMSI is typically used for tissue samples, focusing on disease pathology. IMC, on the other hand, combines flow cytometry and MS with high throughput, high sensitivity, and high resolution and is commonly used in cell and tissue samples to simultaneously detect the expression levels and subcellular localization of multiple proteins, making it a very beneficial tool in the fields of immunology, cancer research, and histology [41,42].

1.6. Spatial transcriptome

Spatial transcriptomics (ST) technology is an emerging technology that has been developing rapidly in recent years. It is capable of simultaneously obtaining information about the gene expression of a cell and the specific location of the cell in tissue space [43]. The application of ST can help researchers better understand the structure and function of cells within tissues, as well as the interactions between cells [44].

ST techniques can be categorized according to how the spatial information is acquired, and there are four main categories: (1) Microdissection techniques: these use lasers or microfluidic devices to separate and capture individual cells or small areas of interest from tissue sections, such as LCM and Tomo-seq [45]. (2) In-situ hybridization methods: use of fluorescent or colorimetric probes in fixed tissue sections to detect specific RNA molecules. The probes can be pre-designed or generated in situ by enzymatic reactions. Imaging platforms or microscopy can show the spatial distribution of the probes. Examples of in situ hybridization methods include MERFISH, seqFISH, and ST [46]. (3) In situ sequencing methods: These methods use synthetic sequencing or ligation reactions to sequence RNA molecules directly in fixed tissue sections. Sequencing reactions can be performed on the tissue surface or within the tissue matrix. The spatial coordinates of the sequenced molecules can be recorded through a microscope or imaging platform. Examples of in situ sequencing (ISS) methods include FISSEQ and Slide-seq [47]. (4) In situ capture methods: these methods use spatially barcoded primers or probes to capture RNA molecules in fixed tissue sections. The captured RNA molecules can then be released and subjected to RNA sequencing or other molecular analysis. By using spatial barcoding, it is possible to retrieve the spatial information of the captured molecules. Examples of in situ capture methods include Slide-seqV2, 10 ×Visium, and STARmap [45]. In conclusion, various ST technologies have been updated and upgraded in recent years and integrated with single-cell transcriptome technologies to vigorously promote the development of developmental biology, immunology, cancer research and other fields [47].

1.7. Integration of multiple SCS technologies offers advantages over use alone

SCS technologies, while powerful, all have limitations, and to be more useful, researchers have endeavored to integrate different SCS tools effectively [48]. A few examples are briefly described below to illustrate the advantages of such integration.

(1) Integration of scRNA-seq with bulk RNA-seq. The use of bulk RNA-seq to assist scRNA-seq is particularly common for multi-omics studies. When used alone, bulk RNA-seq does not provide information about gene expression in individual cells, and scRNA-seq does not directly correspond to cell populations for specific phenotypes. By combining scRNA-seq and bulk RNA-seq, researchers can obtain a complete picture of a sample's transcriptomic landscape, which helps identify subpopulations of cells with different gene expression profiles and phenotypes, which can help in understanding disease progression and developing targeted therapies [49,50]. The advantages of using the two together over using them alone are shown in Table 1.

(2) Combination of scRNA-seq and scDNA-seq. To link gene expression to genotype, researchers usually use single-cell transcriptomes and single-cell genomes in conjunction, and Table 2 shows the advantages of combining the two. First, cell lineage and evolution can be determined. By analyzing cell expression patterns and genetic alterations, researchers can reconstruct lineage relationships between different cell types and track the evolution of cell populations over time. This information is critical to understanding the development of cancer and other diseases caused by cellular heterogeneity [51]. Second, subpopulations can be identified and characterized: through combined analysis, researchers can identify and characterize cellular subpopulations defined by a combination of gene expression patterns and genetic alterations. This information can be used to study the heterogeneity of complex tissues and identify new drug targets [52]. Overall, combining scRNA-seq and scDNA-seq provides a powerful tool for understanding cellular landscapes and unraveling disease mechanisms.

(3) Combination of scRNA-seq and scATAC-seq. scRNA-seq is particularly useful for studying gene expression patterns in heterogeneous cell populations (e.g., those found in tissues or tumors). However, scRNA-seq is unable to distinguish between the presence or absence of genes, and it is difficult to accurately quantify the expression of genes with low or highly variable levels of expression. scATAC-seq measures the accessibility of chromatin, the physical state of DNA that determines the accessibility of transcription factors and other regulatory proteins. This information is critical to understanding how chromatin regulates gene expression. However, ATAC-seq can only measure open chromatin

Table 1
Differences between scRNA-seq and bulk RNA-seq used in combination and alone.

Feature/ Function	scRNA-seq	bulk scRNA-seq	scRNA-seq + bulk scRNA-seq
Resolution	Single-cell	Population of cells	The mixture of single cells and the population of cells
Applications	Identifying and characterizing rare cell populations, studying cell interactions, tracking cell differentiation, and developing new therapeutic targets	Studying overall trends and differences in gene expression between conditions and identifying differentially expressed genes	Comprehensive study of cellular heterogeneity, identification of cell-type-specific gene expression patterns, and validation of scRNA-seq results
Cost	More expensive	Less expensive	Reasonable use, cost-effective
Advantages	High-resolution view of gene expression, ability to identify rare cell populations, and study cell interactions	It can be performed on a large number of samples and provides a population-level view of gene expression	It combines the strengths of both scRNA-seq and bulk scRNA-seq
Disadvantages	More expensive and time-consuming than bulk scRNA-seq, it can be difficult to analyze data from large numbers of cells	Can mask heterogeneity in gene expression but may not be able to identify rare cell populations	Requires more complex computational analysis

Table 2
Differences between scRNA-seq and scDNA-seq used in combination and alone.

Feature/ Function	scRNA-seq	scDNA-seq	scRNA-seq + scDNA-seq
Measures gene expression	Yes	No	Yes
Measures genetic variation (CNVs, SNPs, structural variants)	No	Yes	Yes
Can identify different cell types	Yes	No	Yes
Can identify genetic variants	No	Yes	Yes
Can study gene regulation	Yes	No	Yes
Can track cell differentiation and development	Yes	No	Yes
Can identify genetic variants associated with cell types	No	No	Yes

and cannot distinguish between active and inactive chromatin. By combining scRNA-seq and ATAC-seq, researchers can gain a more comprehensive understanding of cellular heterogeneity and regulatory mechanisms. scRNA-seq can provide information about the expression of actively transcribed genes, while ATAC-seq can provide information about the regulatory elements that control gene expression. This combined information can be used to identify new regulatory mechanisms and develop new therapeutic strategies [53].

(4) Combination of scRNA-seq and ST. ScRNA-seq, in conjunction with ST, provides a powerful tool for studying tissue structure and function. ScRNA-seq can be used to identify cell types and subtypes, while ST can be used to study the distribution of these cell types in tissues [54]. This information can be used to study a wide range of biological questions, including:

Understanding tissue structure: by combining scRNA-seq and ST data, researchers can identify the different cell types that make up a tissue and how they are arranged [54].

Identifying cellular interactions: By studying the co-localization of different cell types, researchers can identify which cells interact with each other [55].

Understanding disease progression: By studying spatial and temporal changes in gene expression patterns in diseased tissues, researchers can identify the molecular mechanisms and targets behind disease progression [56].

In summary, by combining the two technologies, scRNA-seq and ST, researchers can obtain a complete picture of the structure and function of tissues, which is important for biology, medicine, and biotechnology. Table 3 below summarizes the advantages of combining scRNA-seq and ST.

2. The application of SCS in the digestive system cancer study

SCS technology is developing particularly rapidly in digestive system cancer research, and the extent of its application depends on the ease of obtaining samples from disease species. For example, the explosion of articles on single-cell analysis of liver cancers and CRCs has provided additional opportunities for growth in this field. To better understand the research ideas and technical details, we will describe in detail in some sections the number of cases, sequencing platform, key clinical patient information, sampling method, and key findings provided for specific studies. Fig. 2 is a snapshot of the second part of the article.

2.1. SCS in esophageal cancer (ESCC) research

There are two main subtypes of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC accounts for approximately 90% of esophageal cancers [57]. ESCC is a common aggressive malignant tumor with typical intratumoral and intertumoral heterogeneity, which is one of the main reasons for the limited therapeutic efficacy. SCS helps to decipher the mechanisms governing tumor heterogeneity and has the potential to develop precise and effective therapeutic approaches for ESCC patients. Among them, the most commonly used SCS technology is scRNA-seq. scRNA-seq based on the SMART-seq2 platform is widely used for drug-resistant recurrence in ESCC, with the most applications in radiotherapy resistance [58,59]. Among these drug resistance studies, there are not only studies using patient specimens but also studies based on human ESCC cell lines treated with radiation to simulate human treatment [59]. There are also studies using mouse models to simulate the different stages of human ESCC development, which have well-mapped the development of ESCC [60].

It is easy to see from Table 4 that 10 ×Genomics is also a popular scRNA-seq platform for researchers. These SCS studies for patients with cancerous species usually fall into two categories: cancerous tissue from the same patient, diseased tissue at different distances from the cancerous species, and so-called normal tissue 5 cm away. Either cancer specimens from patients with different stages of ESCC [61]. Instead of using SCS technology, some studies used data from databases to analyze to get the experimental results they wanted [62,63]. Spatial omics are commonly used with mIF technology [64]. ST, on the other hand, is more used by the 10 ×Genomics Visium platform, which has obvious advantages in revealing cellular interactions in ESCC [47,65]. Another study utilized the scRNA-seq database and Nano-String spatial whole-transcriptome analysis technology to effectively use biopsy tissue specimens easily accessible in the ESCC clinic, leading to the finding of early cancer markers.

These studies also made good use of the currently popular organoid technology for result validation [66]. It can be seen that scRNA-seq integrates multiple histological techniques and plays a significant role in revealing the molecular mechanisms of tumor heterogeneity and drug resistance in ESCC, and early cancer prediction markers.

2.2. SCS in gastric cancer (GC) research

According to the World Health Organization (WHO), gastric cancer (GC) ranks fifth in the global cancer incidence. It is one of the more common cancers worldwide [71]. GC usually has multiple stages, from no atrophic gastritis (NAG) to chronic atrophic gastritis (CAG) to gastrointestinal metaplasia (IM) to GC. At different stages, the cellular mapping, cellular functions, and interactions with other cells in gastric tissues change all the time and are full of complexity [60,65]. GC is characterized by difficulties in early diagnosis, insidious progression, and an extremely low survival rate in late stages, which is related to the intratumoral heterogeneity of tumors, a lack of good therapeutic targets, and factors such as tumor microenvironment (TME) and immune escape. To overcome these problems, researchers have fully utilized SCS for their studies. It includes the analysis of cellular heterogeneity and the subpopulation of GC tissues, which helps to understand the cellular diversity within the tumor [72]. It can also be used to construct the developmental trajectory of tumor cells, tracking the development of cells from origin to maturity and helping to identify key events that may affect tumor development [59]. Of course, it can also be used to analyze the gene expression patterns of different subpopulations, including the detection of differentially expressed genes, to discover potential therapeutic targets [59]. The TME is very important for therapeutic sensitivity. Using SCS, we can study the distribution and interactions of immune and non-immune cells in the TME, which can help us gain insights into the impact of the microenvironment on tumor development and drug resistance, thus facilitating the development of new drugs [73,74]. Organoid technology has also been fully applied in this field, and this technology provides a great help for GC research [67,75].

In addition, there have been a series of novel studies in the field of ST. One of them is a spatial metabolomics study of 362 GC patients using high-resolution imaging mass spectrometry. This study provides valuable information for the molecular classification system of GC, which helps doctors choose the appropriate therapeutic approaches [76]. Another study on intratumor heterogeneity in GC, which analyzed

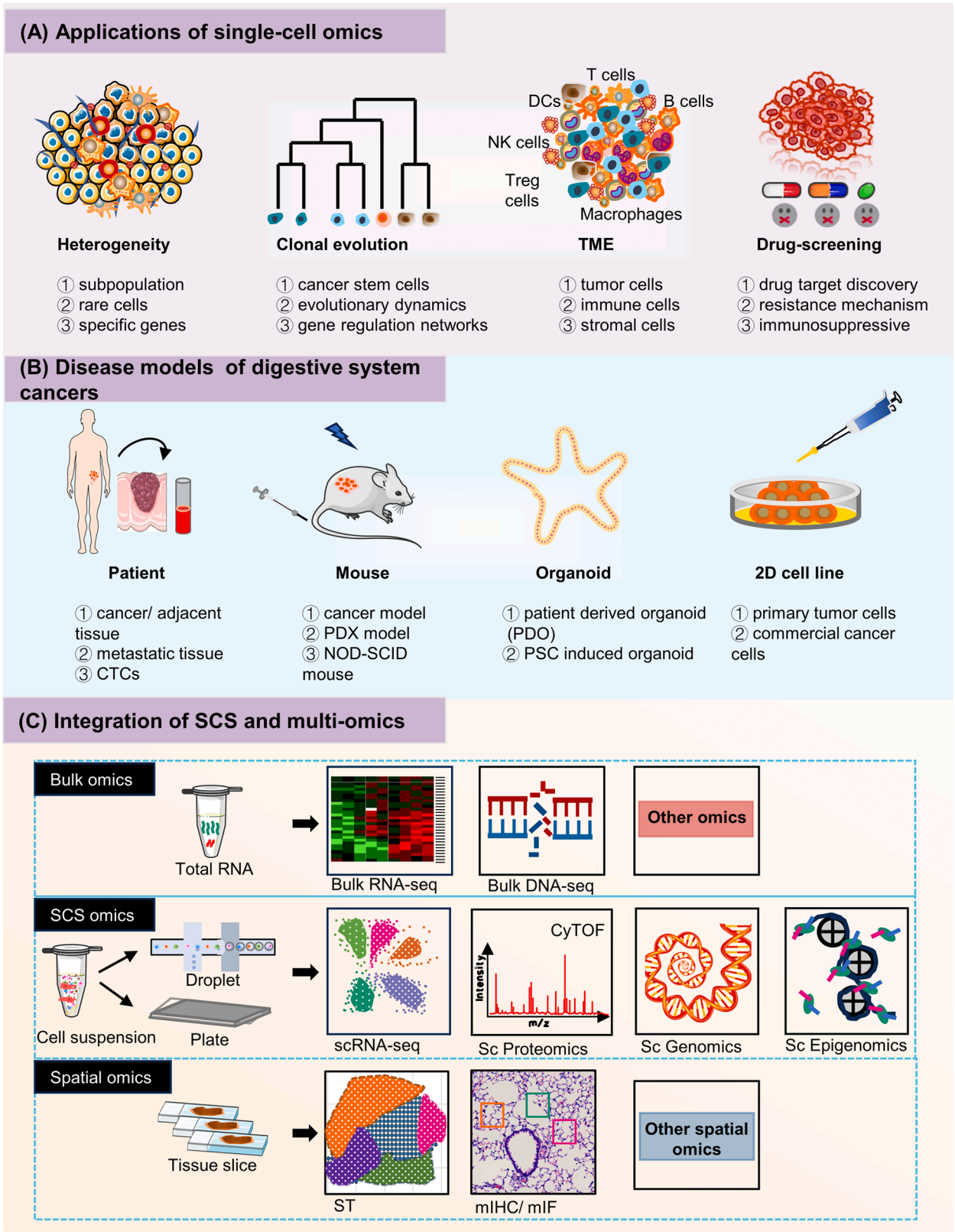

Fig. 2. Single-cell omics technology is employed to investigate digestive system cancers in several disease models. This diagram provides an overview of the applications of single-cell multi-omics, the different disease models employed in the research, and the current integration of the most often utilized single-cell multi-omics technologies. For instance, SCS is usually combined with bulk-seq, whereas scRNA-seq is frequently applied with CyTOF (scProteomics), and the widely used ST technologies are mostly employed with scRNA-seq as well. Indeed, the integration of traditional omics techniques is prevalent, and it is rather common to combine two or even many omics methods.

Table 4
Summary of the application of SCS technology in ESCC research.

ScSeq-method/ platform	Sampling method	Sample information	Key findings and novelties	Ref
scRNA-seq: SMART-seq2	cell experiment	37 KYSE-30 cells; 73 paclitaxel-resistant KYSE-30 cells	Paclitaxel resistance mechanisms	[58]
scRNA-seq: SMART-seq2	biopsy and/or surgery	3 primary ESCC patients; 2 primary EAC patients	Intratumoral heterogeneity and genetic characteristics of ESCC and EAC	[67]
scRNA-seq: SMART-seq2	cell experiment	human ESCC cell line KYSE-180 were irradiated with 2 Gy X-rays using a linear accelerator	Transcriptional change; radioresistance in ESCC	[68]
scRNA-seq: SMART-seq2	cell experiment	ESCC cell lines KYSE180 with and without fractionated irradiation (FIR)	radiation resistance in ESCC	[69]
scWES: MDA + bulk WES	biopsy and/or surgery	74 longitudinal biopsy samples collected from 40 patients; 2 surgical samples (carcinoma and adjacent normal tissues) from 2 ESCC patients	Sensitivity and resistance gene mutations; radiation therapy relapse	[59]
scRNA-seq: 10 × Genomics	animal experiment	Mice were treated with 4NQO (100 μg/ml) in drinking water for 16 weeks to induce multi-staged ESCC carcinogen	A mouse model mimicking human ESCC development; single-cell ESCC development map	[60]
scRNA-seq: 10 × Genomics	surgery	Five ESCC and five corresponding non-malignant samples from 10ESCC patients	Heterogeneity; gene expression and CNV status	[70]
scRNA-seq: 10 × Genomics	surgery	tumors and adjacent normal tissues from 60 ESCC patients (stage I, stage II, and stage III)	ecosystem and TME of ESCC; patient survival markers	[61]
scRNA-seq: 10 × Genomics + Spatial transcriptomics: 10x Genomics Visium	surgery	ScRNA-seq: 79 surgically removed esophageal samples including NOR, LGIN, HGIN, and tumors from 29 patients with ESCC; ST: 20 samples from 5 patients	ANXA1/FPR2 signaling is an important crosstalk mechanism between epithelial cells and fibroblasts in promoting ESCC	[65]
ST: Nano-String spatial whole-transcriptome analysis + scRNA-seq data from database	biopsy	4 slides were employed for spatial WTA sequencing: 5 micrometers slices of ESPL and ESCC patient FFPE tissue	Using biopsy tissue specimens; ST technology combined with scRNA-seq data; predictors of ESCC risk that could help prevent and early intervene in ESCC	[66]

cancer tissues from 64 GC patients by transcriptome, third-generation sequencing technology (Nanostring), DNA copy number, and histo-morphometric phenotypes, found that gene expression and mutation profiles differed significantly between superficial and deep tumor regions and metastatic lymph nodes, which suggests that future clinical trials of targeted therapies must consider evaluating deep regions of primary tumors and/or metastases [77]. The last article is about the spatial expression profile and gene regulatory network of the human gastric body epithelium, which explains the epigenetic regulation of key genes in the human gastric body epithelium through the integrated analysis of scRNA-seq, ST, and single-cell transposase-accessible chromatin sequencing (scATAC-seq) technologies and also identifies a stem/progenitor cell population, which systematically understands the gastric body epithelial cellular New insights into diversity and homeostasis [78]. To allow for better differentiation and emulation, we have taken a categorized approach to presenting these studies, as detailed in Table 5.

As summarized in Table 5, most of the studies on gastric adenocarcinoma are dominated by scRNA-Seq, and more than 80% of scRNA-seq is based on the 10 × Genomics platform, which tends to be stable and maneuverable, as well as having mature data analysis methods. However, fewer use a particular technique alone, and more are combined with bulk RNA-seq, qPCR, immunofluorescence, immunohistochemistry, and other techniques. In the last 5 years, a large number of studies based on SCS technologies have been published, and more than 60% of them have combined bulk RNA-seq tools. Interestingly, after 2022, more than 30% of the articles do not collect samples for sequencing but instead rely on data from existing databases to re-mine or develop algorithms to create new models to predict GC prognosis or disease-specific targets. In terms of sample collection, with the widespread use of endoscopic technology, fresh tissue specimens can be obtained through upper gastrointestinal endoscopy in addition to surgical specimens, allowing more SCS studies to be conducted. For single-cell preparation of tissue samples, traditional collagenase IV-based combinations are widely used, and solid tumor-based single-cell isolation kits have also been used more frequently in recent years due to the rapid advancement of technologies. The integration and application of the latest spatial histology and related technologies based on organoid culture techniques have also promoted the development of the GC field. Organoids derived from patient samples are a good alternative to in situ cancer tissues. These organoids can be preserved in a deep cryogenic refrigerator for a certain period of time, compensating for the dependence on fresh specimens for SCS procedures [78].

SCS-based research has brought many new ideas and thoughts in the direction of cancer cell evolution law, microenvironment analysis, immune characterization, drug resistance, and immunotherapy effect prediction of GC, which will ultimately open up new therapeutic methods and drugs for GC.

2.3. SCS in colorectal cancer (CRC) research

Colorectal cancer (CRC) is the third-most deadly and fourth-most common cancer worldwide. CRC is characterized by a variety of genomic, epigenomic, and transcriptomic changes, as well as multiple cellular processes that promote tumorigenesis [97]. SCS techniques are more widely used in CRC than in other gastrointestinal tumors, which is related to the high rate of CRC surgeries and easier sample acquisition. The research of SCS applied to CRC focuses on the following issues: first, tumor heterogeneity; second, tumor immune microenvironment; third, the mechanism of CRC metastasis and recurrence; and lastly, the discovery of new drug targets. We organize some application cases of SCS technology in CRC research in Table 6 for your reference and study.

Technology-wise, we find that scRNA-seq has been the mainstay of SCS technology for the last 5 years, but its actual platforms and methods are more colorful, although 10 ×Genomics is still dominant. The coupling of more than two SCS technologies also appears to be very

Table 5
Summary of the application of SCS technology in GC research.

ScSeq-method/platform	Sampling method	Sample information	Key findings and novelties	Ref
scRNA-seq (10 ×Genomics) + scCNVs: (10 ×Genomics)	cell experiment	GC cell lines (9 species)	Heterogeneity; GC evolution in vitro	[79]
scRNA-seq (10 ×Genomics)	animal experiment	gastric tissues from mice (Healthy/ inflammatory)	Lineage-Specific Epithelial Responses; gastric corpus metaplasia	[80]
	gastric organoids by air-liquid culture	Gastric organoids (stomach bodies and antrum of P53-/- mice)	Heterogeneity; gastric tissue niche	[81]
		Gastric organoids (*CD44-Cre/Cdh1*$^{loxP/loxP}$*/tdTomato* mice)	Mechanism of HDGC triggered by CDH1 deletion; Specific markers of early tumor lesions	[82]
	surgery	GC and paired paracancerous tissues	Heterogeneity of T cells; Immunity and immunotherapy	[83]
	surgery	GC and paired paracancerous tissues; Intestinal metaplasia tissues	TME; cellular reprogramming	[84]
	biopsy	biopsy specimen excised by upper gastrointestinal endoscopic from GC or non-GC patients	Heterogeneity; Malignant epithelial cell features; Rare subtype of GC	[85]
	surgery	tumor tissues and matched normal tissues from untreated non-metastatic GC patients	active cell subtypes and interaction; TME; Heterogeneity; Immunity and immunotherapy	[86]
	surgery	tumor samples and adjacent mucosal samples were resected during surgery from GC patients	CAF affecting the prognosis of GC; TME; Heterogeneity; Immune cell subpopulation analysis	[87]
	surgery	GC and paired paracancerous tissues; metastatic organs or tissues (liver, peritoneum, ovaries, lymph nodes)	Heterogeneity; TME; organ-specific metastatic features	[88]
	biopsy and/or surgery	tissues from GC patients (4 pre-treatment samples, 4 post-treatment samples and 3 pre-post pairs) through biopsy and/or surgery	TME after neoadjuvant chemotherapy; Therapeutic mechanism of NACT in GC	[89]
	biopsy	Antrum mucosa biopsy specimen from GC, chronic atrophic gastritis and intestinal metaplasia patients	Single-cell network of premalignant lesions and early GC; biomarkers of gastric early-malignant cells	[90]
	therapeutic peritoneal lavage and/or puncture	Peritoneal lavage fluid from early/ advanced GC patients; ascites from untreated/systemically treated GC patients	Developmental trajectories of cancer/ immune cells; Discovery of prognostic genes; Application of PDOs in validation experiments	[91]
	therapeutic puncture	GC patients with malignant ascites, peritoneal cancer cells were obtained during therapeutic puncture	Intratumoral heterogeneity of metastatic GAC; Prognostic markers	[92]
	surgery	Primary tumor tissues and corresponding positive peri gastric lymph node samples after surgical resections	Lymph node metastasis mechanism; TME; Heterogeneity	[93]
scRNA-seq (Smart-seq2)	surgery	primary cancer and paired metastatic lymph node cancer tissues from	GC lymph node metastasis marker gene; GC evolutionary driver gene	[94]
scDNA-seq (SNV: MDA)	surgery	GC and paired paracancerous tissues from GC patients	Heterogeneity; somatic mutation spectrum	[95]
scRNA-seq (10 ×Genomics) + ST (NanoString) + RNAscope ISH	biopsy and/or surgery	surgical resection and biopsy samples from GC patients	TME; Heterogeneity; Cell Fate Trajectories; Rare Cell Populations; Application of PDOs in validation experiments	[96]

common. Particularly worth introducing is the use of SCS technology combined with ST to explore the immune spectrum of CRC liver metastases, which reveals the impact of neoadjuvant chemotherapy on the immune microenvironment of liver metastases and provides new ideas for the treatment of liver metastases [120]. Some studies have investigated the interactions between stromal cells (e.g., CAF) and immune cells in the CRC TME using SCS-integrated spatial omics technology, providing good clues for elucidating the mechanisms of cancer progression and metastasis. Firstly, CRC tissues were analyzed by ST in combination with public scRNA-seq mapping to identify cell-cell interactions at the invasion front, and it was found that CRC cells are exclusively located at the invasion front, and these CRC cells are specific to the immune cell environment [122]. Another similar study used single-cell and spatial analyses of cells from tumors and adjacent tissues to characterize the cellular composition and elucidate the potential origin and regulation of tumor-enriched cell types in CRC, demonstrating the interaction of FAP^+ fibroblasts and $SPP1^+$ macrophages in CRC. These two studies provide a potential therapeutic strategy to improve immunotherapy by disrupting the interaction of associated fibroblasts and $SPP1^+$ macrophages. Two studies in 2023 on liver metastasis in CRC both used ST binding RNA-seq to map cellular profiles of CRCs and matched liver metastatic CRCs. One of these compared cellular transcriptome differences between primary and liver metastatic tumors [123]. While another focused on heterogeneity between patients, between paired lesions in the same patient, and within individual lesions, and identified a reactive and suppressive immune microenvironment as a driver of CRC metastatic progression [124].

In terms of sample use, most studies still use surgical or biopsied cancerous and paracancerous tissues based on patient sources, and if metastasis-related issues are being studied, patient peripheral blood is used for immune cell isolation or specific immune cells are isolated from tumor tissues for immune microenvironment analysis. It is well known that the intestine is the first structure to be cultured organoids. As organoid technology has improved, many articles have used organoids from crypt structures in the intestine for studies related to colorectal cancer cell lines or tumor tissues [125]. Some of them are used as sequencing samples, and some of them are used as models for validation experiments. In general, organoids are becoming more and more popular in CRC research [99,117]. Additionally, a part of the research focuses on circulating tumor cell (CTCs). Analysis of the heterogeneity of CTCs in patients' peripheral blood will help to understand the metastasis mechanism of CRC [100]. Another part of the research focuses on the study of tumor-infiltrating immune cells, which requires the separation of cancerous or paracancerous tissues into single-cell suspensions and then the use of sorting and other methods to isolate the immune cells of interest [98,105]. The actual research process requires a rational sample grouping design according to the research purpose.

Similar to GC, after 2021, a considerable number of studies no longer directly collect cancer specimens for sequencing, but based on SCS data in the existing GEO database and bulk RNA-seq data in the TCGA database for data mining and algorithm development, which has yielded good results in several aspects. For example, the construction of

Table 6
Summary of the application of SCS technology in CRC research.

ScSeq-method/platform	Sampling method	Sample information	Key findings and novelties	Ref
scRNA-seq (10 ×Genomics)	animal experiment	mouse tissues from 3 groups (control, AB680-treated, PD-1-blocked-treated)	Immunotherapy mechanism for targeting CD73 and PD-1 in CRC	[13]
scRNA-seq: scWTA (BD Rhapsody WTA amplification kit)	animal experiment + T Cell Isolation Kit	CRC tissues from Humanized PD-1/PD-L1 Knock-In CRC Mouse Models	Photodynamic therapy mechanism; tumor-infiltrating T cell; humanized PD-1 mice	[98]
scRNA-seq (Fluidigm C1) + scDNA-seq (Fluidigm C1)	organoids	mouse CRC model; Organoid culture of small intestinal cells	Tumorigenesis; Intra-tumor heterogeneity; cancer evolutionary dynamics	[99]
scRNA-seq (Smart-seq V4)	CTCs from peripheral blood	27 patients with mCRC	Circulating tumor cell (CTC); metastasis mechanism	[100]
scRNA-seq (DNBelab Single-Cell Kit [MGI, Shenzhen, China])	surgery	14 untreated CRC tumor samples (7 young-CRC: age <50 years; 7 old-CRC: age ≥50 years)	Heterogeneity; TME between Young-Onset and Old-Onset CRC	[101]
scRNA-seq (10 ×Genomics)	surgery	16 samples (5 colon tumor, 3 colons matched normal, 3 liver metastasis, 5 liver matched normal)	Heterogeneity of CSC in CRC; CSC marker genes	[102]
scRNA-seq (10 ×Genomics)	therapeutic puncture	ascites from a CRC patient (before/after chemotherapy)	Heterogeneity; ascites derived cells; chemotherapy susceptibility; resistance mechanisms	[103]
scDNA-seq: scWES (KAPA Biosystem)	surgery	tumors and adjacent normal colon of CRC patients	Genetic characterization and clonal evolution of CRC initiation cells (CRCICs)	[104]
scDNA-seq: scWES	surgery	liver metastasis cancer tissues and adjacent non-cancerous tissues	TME; heterogenicity; granulocytes enrichment in CRC liver metastases	[105]
scRNA-seq (Smart-seq2)	surgery	T cells isolated from peripheral blood, adjacent normal and tumor tissues of 12 CRC patients	Basic characteristics of tumor-infiltrating T cells (TILs); A web-based application was developed for querying and customizing T cell datasets	[106]
Parallel Single-Cell Genome and Transcriptome Sequencing (KAPA Biosystem)	surgery; animal experiment	cancer and para-cancer tissues, peripheral blood, and lymph nodes of CRC patients with microsatellite stability; Peripheral blood of the elderly without cancer; transgenic (CRC) mouse model	A five-gene fibroblast specific prognostic marker; Prevalent Genomic Alterations in tumor stromal cells	[107]
scDNA-seq: scWGA (Ampli1 Kit from Silicon Biosystems)	warm autopsy and/or surgery	multiple tumoral and normal tissue samples	Heterogeneity of mtDNA	[108]
scDNA-seq: scWGA (Ampli1 Kit from Silicon Biosystems)	surgery	60 cells collected from a liver metastasis and a recurrent liver lesion	Cancer evolution, Chemotherapy, Mutational signatures	[109]
scRNA-seq (10 ×Genomics)	surgery	tissues from a CRC patient (73 years old, female, Stage III)	Heterogeneity; cell markers	[110]
scRNA-seq (10 ×Genomics)	surgery	tissues from 6 CRC patients (3 left-sided and 3 right-sided CRC patients)	Resolving the difference between left-sided and right-sided CRC	[111]
scRNA-seq (Smart-seq2)	surgery	11 CRC patients (7 males and 4 females)	Traditional Chinese medicine syndromes classification is associated with tumor cell and microenvironment heterogeneity	[112]
scRNA-seq (10 ×Genomics) + CITE-seq	surgery	tumors and adjacent normal colon of 16 CRC patients	prognostic and non-prognostic T cell types	[113]
scRNA-seq (10 ×Genomics) + scATAC-seq + WES	biopsy and/or surgery	12 CRC patients	Heterogeneity; phenotypic and functional diversity of tumor-associated macrophages and T cells	[114]
scDNA-seq: scWES (BGI, Shenzhen, China)	surgery	7 patients with liver metastatic CRC (primary tumor, liver metastatic tumor, normal colonic tissue)	Rare mutation; Phylogenetic tree of the mutant spectrum	[115]
scRNA-seq (10 ×Genomics) + scT&R-seq	surgery	tissues from 8 patients with untreated primary CRC	Features of CSC: telomeres are short, highly heterogeneous and plastic	[116]
scRNA-seq (STRT) + scWGS (MALBAC)	surgery; organoids	tumor tissue, normal tissue and corresponding organoids cultured in vitro from 7 patients with CRC	Culture conditions and biological characteristics of organoids in patients with CRC were evaluated	[117]
scRNA-seq (Smart-seq2 + DNBelab C4)	surgery	18 CRC patients (primary tumor, adjacent noncancerous tissue and the brim of matched tumor)	Immune microenvironment; Transcriptome map; B cell heterogeneity and function	[118]
scRNA-seq (10 ×Genomics)	surgery	tumor tissues from MSI-H/ dMMR mCRC patients (3 sensitive, 3 resistant)	Mechanism of anti-PD-1 resistance in MSI-H or dMMR mCRC	[119]
scRNA-seq (10 ×Genomics) + ST (10 ×Genomics Visium)	surgery	tumor tissues and/or paired adjacent colon, liver metastasis, adjacent liver, colon lymph nodes and PBMC	Immune; TME; liver metastasis; R package; scMetabolism Effects of neoadjuvant chemotherapy	[120]
scRNA-seq (10 ×Genomics) + mIF	surgery	tumors and adjacent normal colon of 18 CRC patients	Mechanism of bone marrow targeted therapy; TME	[121]

prognostic models, the prognostic value analysis of certain genes, heterogeneity analysis, the prognostic value of CAF or immune cells, etc. [126–128]. It is no exaggeration to say that all these discoveries have promoted the development of cancer cell mapping, recurrence and metastasis mechanisms, target prediction, and new drug development.

2.4. SCS in liver cancer research (including intrahepatic bile duct tumors)

Liver cancer is one of the most common malignant tumors, with the highest morbidity and mortality rates in the world. According to the origin of cancer cells, it is divided into primary and secondary liver cancer. Primary liver cancer is further divided into hepatocellular carcinoma (HCC) and cholangiocarcinoma. Among them, HCC is the most common liver cancer, accounting for about 90% [129]. Currently, there are several major problems in liver cancer, such as difficulty in early diagnosis, easy of metastasizing, and limited and ineffective treatment methods. Among the reasons for poor therapeutic outcomes are the following: the heterogeneity and complexity of HCC; the susceptibility of HCC to drug resistance; the difficulty of early diagnosis; and the poor therapeutic outcomes in advanced patients. The emergence of SCS technology represents a powerful strategy to characterize the complex molecular landscape of cancer [130–132].

Researchers have fully utilized various SCS technologies to investigate the pathogenesis and drug resistance mechanisms of HCC, including the clonal evolution and developmental trajectory of cancer cells. The heterogeneous immune microenvironment of HCC is an important factor in drug resistance and recurrence, and single-cell histology methods, in combination with other methods, including flow

cytometry, multicolor immunofluorescence (mIF) or multicolor immunohistochemistry (mIHC), and spatial histology, have provided in-depth analysis of the immune microenvironment, thus providing more evidence for the effectiveness of immunotherapy, targeted therapy, and other therapies, providing more evidence for the effectiveness of immunotherapy, targeted therapy and other therapies. Another part of the research is targeting a particular type of cell, such as immune cells in the tissue microenvironment, including T cells [133], macrophages [134], innate lymphoid cells [135], as well as a series of studies on hepatic stellate cells (HSCs) and cancer-associated fibroblasts (CAFs) [136–138]. Whether using liver cancer tissue samples, paired paraneoplastic and metastatic tissues, liver cancer model animals, transgenic mice, or even directly using SCS data from databases, thus analyzing the efficacy of liver cancer treatment and predicting the sensitivity of chemotherapy or targeted therapy, all of these are very valuable.

In this section, we will not discuss in detail the application scenarios of scRNA-seq represented by 10 ×Genomics and SMART-seq2 but focus on how to integrate scRNA-seq technology with proteomics, ST and other technologies for research. Among them, single-cell proteomic technology represented by mass spectrometry flow (CyTOF) technology is combined with scRNA-seq to achieve multidimensional characterization of specific cell subpopulations, which is especially suitable for the study of peripheral blood immune cells and tumor immune cells in the microenvironment [139–142]. Multicolor immunohistochemistry belongs to both proteomics and spatial histology techniques, and since cellular communication in the TME is particularly important for the immunosuppressive environment of tumors, a large number of studies have used multicolor immunohistochemistry or multicolor immunofluorescence staining to confirm new cell subpopulations and targets identified by SCS omics. A recent study based on scRNA-seq and the coupling of imaging mass spectrometry (IMC) flow and multicolor immunofluorescence (mIF) constructed spatially resolved single-cell profiles from formalin-fixed and paraffin-embedded tissue sections from patients with nonalcoholic steatohepatitis-associated hepatocellular carcinoma (NASH-HCC), viral HCC (HBV-HCC, and HCV-HCC), and demonstrated that myeloid-derived suppressor cells (MDSCs) and interactions between tumor-associated macrophages (TAMs) and effector T cells underlie NASH-HCC immunosuppression and are a viable therapeutic target [143].

In another study, using scRNA-seq in conjunction with ST and mIF techniques, they identified a specific immune barrier structure, TIB, in the HCC microenvironment, which is formed by the interaction of $SPP1^{+}$ macrophages and CAF that can limit the efficacy of immunotherapy by restricting immune cell infiltration into malignant areas. They then tested their conclusions in mice and found that blocking SPP1 to disrupt the TIB structure increased the sensitivity of HCC cells to immunotherapy [138].

HCC organoids have attracted much attention because they can be used as in vitro models to understand the pathogenesis of cancer well and the accessibility of the methodology. Several studies have attempted to generate HCC organoids from human HCC specimens and maintain the growth of HCC spheroids in a three-dimensional culture system, which can be used for the construction of HCC organoid libraries, drug toxicity analysis, and drug screening and precision medicine research [144]. In addition, xenografts from patients with HCC are popular, as well as the NOD-SCID mouse model, which mimics the human immune system and allows for better research on the relationship between tumor cells and the immune system. Cai et al. (2023) used organoids and xenografts from liver cancer patients to test how well cilengitide worked when combined with the γ-secretase inhibitor LY3039478. They found that this combination worked much better than cilengitide alone [145]. Another CRISPR-based genome-wide screening found that the combination of DOCK1 inhibition and metformin provided effective targeted therapy for metformin-resistant patients, using not only patient-derived HCC organoids and in vivo xenografted hepatocytes, but also HCC models from immune-activated mice [146]. Both liver cancer carcinoid organs and SCS-related technologies have become powerful tools applied in the screening of early diagnostic markers and prognostic markers, as well as drug resistance mechanisms and new drug discovery and development for HCC [147,148].

2.5. SCS in pancreatic cancer (PaCa) research

Pancreatic cancer (PaCa) is the fourth-leading cause of cancer-related deaths in the world and is regarded as the deadliest cancer, characterized by a poor prognosis and the lowest 5-year survival rate of all solid tumors, mainly due to a late diagnosis and poor treatment. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of PaCa, accounting for 90% of PaCa cases [149].

As single-cell resolution technology becomes more widely available, there is a clearer understanding of the genetic landscape of PDAC, such that there are only a few major hard-to-treat driver lesions such as Kras and TP53 and a variety of additional genetic alterations, including low-frequency copy number variants that contribute to the genetic diversity of the disease [150]. The PDAC scenario involves a diverse range of immunosuppressive microenvironments, which consist of various types of immunosuppressive cells like tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and other stromal cells. Additionally, the interactions between these immune cells, stromal cells and the extracellular matrix have significant impacts on the patients' prognosis and their resistance to drugs. The SCS has revealed, at an unprecedented resolution, the complexity of the PDAC microenvironment heterogeneity [151]. Several findings have often overturned initial perceptions. For instance, ductal cells are not a major component of the PDAC TME, while tumor-infiltrating immune cells and pancreatic stellate cells accumulate during tumor progression [151,152].

In the pathogenesis of early-stage cancer and precancerous lesions, surgical or biopsy specimens at different levels of IPMN (Intraductal papillary mucinous neoplasms) have been used to perform SCS, single-cell genomics sequencing to uncover genetic heterogeneity of driver mutations, and single-cell transcriptomes to identify heterogeneity of epithelial and vesicular chemotaxis cells [153–155]. During the concomitant progression of PDAC, epithelial cells, stromal cells in the microenvironment, and fibroblasts undergo dynamic changes on which a large number of studies have focused [156]. Since most PDACs are detected at advanced stages, surgical specimens are quite limited, and samples for SCS analysis are no longer as readily available as those for CRCs and HCCs, a considerable number of studies have been based on biopsy tissue specimens as well as model animals such as genetically engineered mice [151,157,158]. These studies have utilized SCS analysis of pancreatic cancer tissue specimens from patients or mice at different disease stages to uncover important markers and dynamic changes associated with disease progression. To study pancreatic cancer metastasis, several studies have used single-cell resolution analysis of primary and metastatic cancer tissues in combination with proteomic and spatial data analysis to obtain metastasis-related prognostic and therapeutic information [157,159].

More studies have been conducted to address intratumor heterogeneity than intertumoral heterogeneity, which is also inseparable from the fact that surgical specimens for pancreatic cancer are more difficult to access. To compensate for the limitation of surgical specimens, the patient's cancer tissue-derived organoids have also become a good medium for study. Some of these studies have utilized PDO as a target for functional and phenotypic analyses [152,159], while others have directly utilized PDO as a substitute for in situ tumors for single-cell analyses [156,160,161]. A study in 2022 on patient-specific modeling of stroma-mediated chemoresistance in PaCa utilized primary PDAC-derived organoids and patient-matched CAFs in three-dimensional (3D) co-culture and then used image-based drug assays to determine the sensitivity of different chemotherapeutic drugs. The findings demonstrated that the co-culture led to a rise in the growth of PDAC-like organoids and a reduction in cell death caused by

chemotherapy. Additionally, it induced a pro-inflammatory state in CAFs. Consequently, the co-culture system proves to be a valuable tool for analyzing personalized drug responses. [162].

PDAC is susceptible to resistance to both chemotherapy and immunotherapy, mainly caused by an immunologically heterogeneous microenvironment and dense extracellular matrix. Newer therapies, such as lysoviruses, can be effective in treating PDAC by remodeling the TME and damaging tumor cells by either directly killing tumor cells or augmenting the tumor immune response. In 2023, Liu et al.'s study on OX40L-armed lysosomal viruses enhanced T-cell responses and remodeled the TME using flow cytometric and single-cell RNA-seq analyses of tumor-infiltrating immune cells and stromal cells to gain insights into the stroma of lysosomal viral (OV) therapies, which demonstrated that OV-mOX40L in combination with anti-IL6 and anti-PD-1 significantly prolonged the lifespan of PDAC mice. This study provides a new therapeutic strategy for the treatment of pancreatic cancer [163].

To study immune dysfunction, researchers have made full use of spatially resolved multi-omics. scRNA-seq was combined with the ST, mIHC, and CyTOF to analyze untreated PDAC tumors and matched adjacent normal pancreatic tissue and immune cells in the systemic circulation. They delineated depletion phenotypes of CD8 + T cells, immunosuppressive signatures of bone marrow cells, and maps of immune landscapes at spatial resolution. All of these provide comprehensive resources for the functional study of PDAC and the exploration of therapeutic targets. [164]. Similarly, another study analyzed untreated and chemo-resistant pancreatic cancer tissues by single-cell/nucleus RNA sequencing, holistic proteomics and phosphorylated proteomics, ST, and cellular imaging, was able to identify tumors and overgrowth subpopulations with different histological features. The chemo-resistant samples contained triple-enriched inflammatory CAFs, all of which will help to understand the organization of PDAC contributing to improved treatment of the disease [165]. In addition to ST, flight mass cytometry is also widely used as a single-cell proteomics method. One of them is the use of CyTOF to analyze tumor growth, survival and immune function in mice. In conjunction with scRNA-seq, we analyzed whether combined inhibition of MEK and STAT3 can reprogram CAFs and the immune microenvironment to overcome PDAC resistance to immune checkpoint inhibitors [166]. In another study in metastatic pancreatic cancer, 38 cell surface or intracellular markers in peripheral blood mononuclear cells were simultaneously detected using single-cell MS flow cytometry, providing potential prognostic features from immune correlations that could be used for patient stratification [167]. CyTOF, as a representative of single-cell proteomics technology, is a great tool for studying immune cells as well as the TME. Yi et al. (2023) analyzed and clustered multiple immune cells from normal liver and pancreatic cancer liver metastases (mice) by using scRNA-seq, followed by ICM to analyze epithelial-mesenchymal transition markers in pancreatic cancer liver metastases, and finally explained the iNKT cell-mediated anti-tumor immunity in PaCa liver metastases [168].

In conclusion, with more refined tissue imaging capabilities, single-cell analysis, and disease modeling, the mechanisms of PDAC resistance, as well as the structure of complex tumor mechanisms, will be more fully resolved, which will provide a rich resource for the effective treatment of PaCa.

3. Conclusion and perspective

According to the WHO, digestive system cancers account for about 22% of all cancers worldwide and are the most common type of cancer. Various digestive system cancers differ in their pathogenesis and treatment approaches, but they also share many commonalities, including the prevalence of early diagnostic difficulties, tumor heterogeneity, and drug resistance, which in turn are very much related to the clonal evolution of cancer cells and the microenvironment in which they reside. In addition to the cancer cells, the TME also includes a variety of immune cells, stromal cells (including various subtypes of CAFs), extracellular matrix, etc. These cells are not single but rather quite complex and diverse, and cell-cell interactions also play an important role in tumor development, metastasis and drug resistance.

Although SCS technology has unique advantages in addressing such heterogeneous diseases as cancer, it also has certain limitations, such as the inability to independently correlate genotypes and phenotypes, which indicates the need for high-throughput, low-cost multi-omics technology to map the overall tumor tissue landscape. There are also technical costs, timeliness, and the difficulty of bioinformatics analysis. We provide the following outlook for the development of single-cell omics in digestive system cancers.

First, each SCS technology needs to be improved. For scRNA-seq, it is critical to improve assay throughput and develop targeted amplification techniques suitable for the detection of non-polyadenylation RNA species [169]. In single-cell epigenomics, nanopore-sequencing (nanopore-seq) technology allows the detection of DNA and protein modifications as well as the direct detection of RNAs. In addition, nanopore sequencing with longer sequencing read lengths is a promising new technology for SCS [170]. MS-based single-cell proteome analysis will be the future direction of development, which requires breakthroughs and improvements in sample preparation, data analysis, and standardization [171]. Meanwhile, MS based on imaging technology is also an important development direction in the field of single-cell multi-omics.

Second, innovations must be made in upstream and downstream technologies for common SCS steps, such as tissue dissociation, upstream single-cell suspension preparation techniques, and downstream sequencing data analysis. Packaging the homogenization, reproducibility, and visualization of SCS data analysis into software and integrating it into the instrumentation in the workflow is especially critical to determine whether SCS can truly serve clinical applications.

Third, to promote the convergence of multiple SCS technologies and the development of single-cell multi-omics. This issue is of great significance, and at the same time, the challenge is enormous. Integrating 2D or multidimensional data depends on breakthroughs in cell labeling and bioinformatics, both in terms of experiments and data analysis. ScRNA-seq has been integrated with single-cell genomics and single-cell proteomics in many cases. On the other hand, the integration of SCS with in situ sequencing and spatial imaging technologies has also attracted much attention because the localization of cells in tumor tissues and their roles in TME are critical for cellular function and identity, which is the driving force behind the rapid development of related technologies such as in situ sequencing and ST.

Finally, SCS product packages are being developed to enable higher throughput, more automated, more convenient, and lower cost applications of SCS technology in digestive system tumors. Two points are worth mentioning: (1) Cell sponge products can be developed to obtain cells or secretions from the digestive tract through minimally invasive techniques for SCS analysis of some digestive system tumors. (2) Develop a method for isolating single cells from formalin-fixed paraffin-embedded (FFPE) tissue to make solid tumor specimens more convenient. In addition, FFPE-based in situ sequencing technology and spatial multi-omics technology represented by ST are also development areas that need to be actively promoted. However, ST technology is not very mature; there are some limitations, and more breakthroughs need to be made to improve spatial resolution and reduce cost.

There is still a long way to go for the clinical application of single-cell technology in digestive system cancers, but it has already made a fruitful contribution to early diagnosis of the disease, staging and typing of cancers, prediction of patient prognosis, prediction of the efficacy of therapeutic methods, and the development of new drugs. In these areas, more novel discoveries are being made now and in the future, as shown in Fig. 3. Tumors of the digestive system can be diagnosed with the help of endoscopic techniques, and their biopsy specimens are easy to obtain. A significant portion of the research is based on these biopsy specimens. It is believed that with the development of single-cell multi-omics

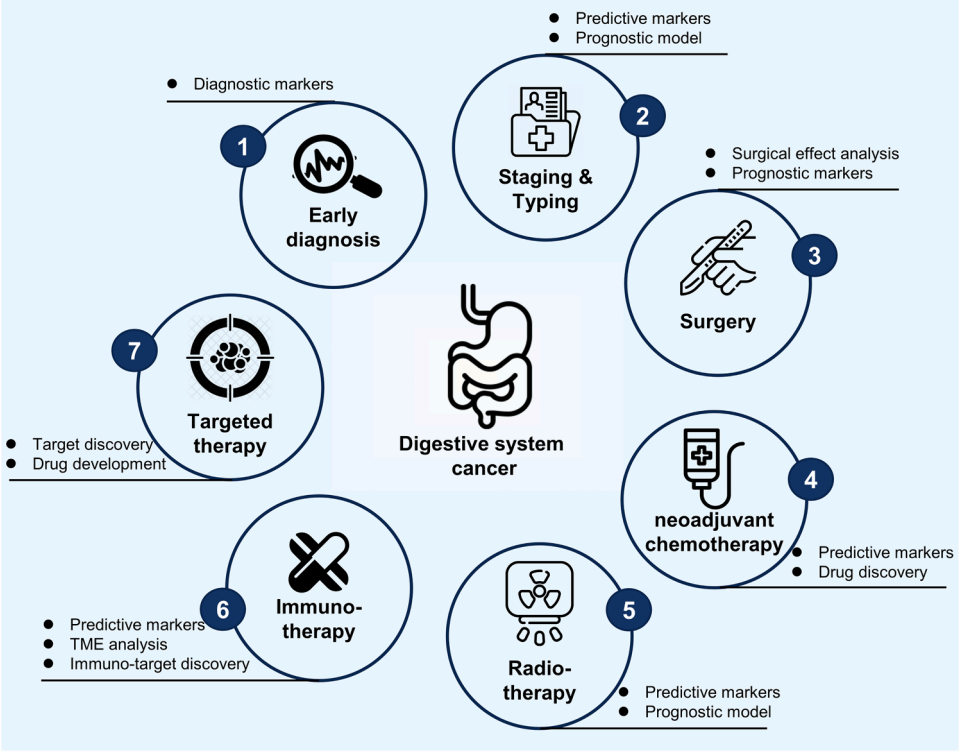

Fig. 3. Prospects for the advancement of single-cell multi-omics in the study of digestive system cancers. Single-cell multi-omics is an exceptionally powerful tool in the field of digestive system cancer research. It enables early detection, accurate staging, and precise classification of cancers. Additionally, it can predict patient prognosis, predict the effectiveness of various treatments (such as targeted therapies and immunotherapies), and encourage the development of novel drugs.

technology, the diagnosis and treatment of digestive system cancers can break through the limitations of traditional cancer research in the future, and obtain more medical research and clinical translational results that will benefit patients.

Ethics approval and consent to participate

Not applicable.

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Author statement

In compliance with the guidelines regarding authorship contribution, the authors of this manuscript declare their contributions as follows:

SZ and NL conducted the literature review and wrote the draft. LY and XS created the figures and tables. XY, HG, and YZ edited the paper. YZ conceived the study. SL and ZL directed the writing and revised the paper. All authors read and approved the final manuscript.

This declaration confirms that each author listed above has contributed significantly to the conception, design, data analysis, drafting, or critical revision of the manuscript. All authors have reviewed and approved the final version submitted for publication.

CRediT authorship contribution statement

SZ and NL conducted the literature review and wrote the draft. LY and XS created the figures and tables. XY, HG, and YZ edited the paper. YZ conceived the study. SL and ZL directed the writing and revised the paper. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Consent for publication

Not applicable.

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