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Review Article

Single-cell RNA Sequencing and Spatial Transcriptomic Technologies and Applications in Exploring Gastric Cancer: A Review

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Abstract

Global deaths attributed to gastric cancer (GC) are increasing, yet our understanding of it remains limited. Recently, single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics have provided important insights into the dissection of metastasis-related biological processes in subpopulations of cells in the GC tumor microenvironment, especially intratumoral cellular heterogeneity and cell-cell interactions. In this review, we discuss the mechanisms underlying GC metastasis and potential strategies for developing upcoming immunotherapies by combining advances in scRNA-seq and spatial transcriptomics.

Introduction

Gastric cancer (GC) is a major component of global cancer incidence and mortality, with a particularly high incidence in Eastern Europe, Asia, and Central America. Metastasis is a leading cause hindering the development of anticancer therapies,[1] leading to the spread of cancer cells across different tissues through a multistep biological cascade.[2] The occurrence of cancer cell metastasis depends on their own molecular alterations and the reprogramming of the tumor microenvironment (TME). GC is characterized by high epithelial-mesenchymal transition (EMT), high stromal cell and immune cell infiltration, and high T-cell exhaustion, indicating that tumor metastasis and an immunosuppressive TME may be responsible for the poor prognosis.[3] Explaining the heterogeneity and cell-cell interactions is critical to reveal the TME in GC and improve cancer immunotherapy.

While emerging bioinformatic programs have been developed to analyze sequencing data, bulk RNA sequencing (RNA-seq) has limitations in discerning complex cell populations and cell-cell interactions.[4–6] In recent years, single-cell RNA sequencing (scRNA-seq) has become an important method for analyzing gene expression at a single-cell resolution. There are several obvious advantages for scRNA-seq over bulk RNA-seq data, including analyzing cell populations and their interactions in the TME, thereby providing a comprehensive understanding of the mechanism underlying tumor cell metastasis.[7,8] However, the necessity for tissue dissociation leads to loss of spatial information, which is a limitation of scRNA-seq. To address this limitation, spatial transcriptomic platforms retaining spatial architecture have emerged, such as digital spatial profiling (DSP) and 10× Genomics Visium, allowing analysis of tumor-TME interactions at an unprecedented depth.[9–11]

GC metastasis cascade

GC is characterized by a poor prognosis and a low long-term survival rate, contributing significantly to the increased cancer mortality worldwide. Metastasis is the main cause of cancer mortality, presenting significant challenges in treatment.[7,12] As described in "The Seed Theory", metastatic tumor cells utilize nutrients from the new environment to grow in the new soil. GC commonly metastasizes to the peritoneum, bone, liver, and lymph nodes,[13,14]

Keywords: Gastric cancer; Tumor microenvironment; Metastasis, scRNA-seq; Spatial transcriptomics.

Abbreviations: CXCL12, C-X-C motif chemokine ligand 12; CAFs, cancer-associated fibroblasts; CNVs, copy number variants; CTLs, cytotoxic CD8+ T lymphocytes; DCs, dendritic cells; DEGs, differentially expressed genes; DSP, digital spatial profiling; EMT, epithelial–mesenchymal transition; ECM, extracellular matrix; FFPE, formalin-fixed paraffin-embedded; GC, gastric cancer; IL, interleukinLIF, leukemia inhibitory factor; MSCs, mesenchymal stem cells; MDSCs, myeloid-derived suppressor cells; NK, natural killer; PC, peritoneal carcinoma; PM, peritoneal metastasis; ROIs, regions of interest; scRNA-seq, single-cell RNA sequencing; SARIFAs, stroma AReactive invasion front areas; TME, tumor microenvironment; TAMs, tumor-associated macrophages; TILs, tumor-infiltrating lymphocytes; UMI, unique molecular identifier.

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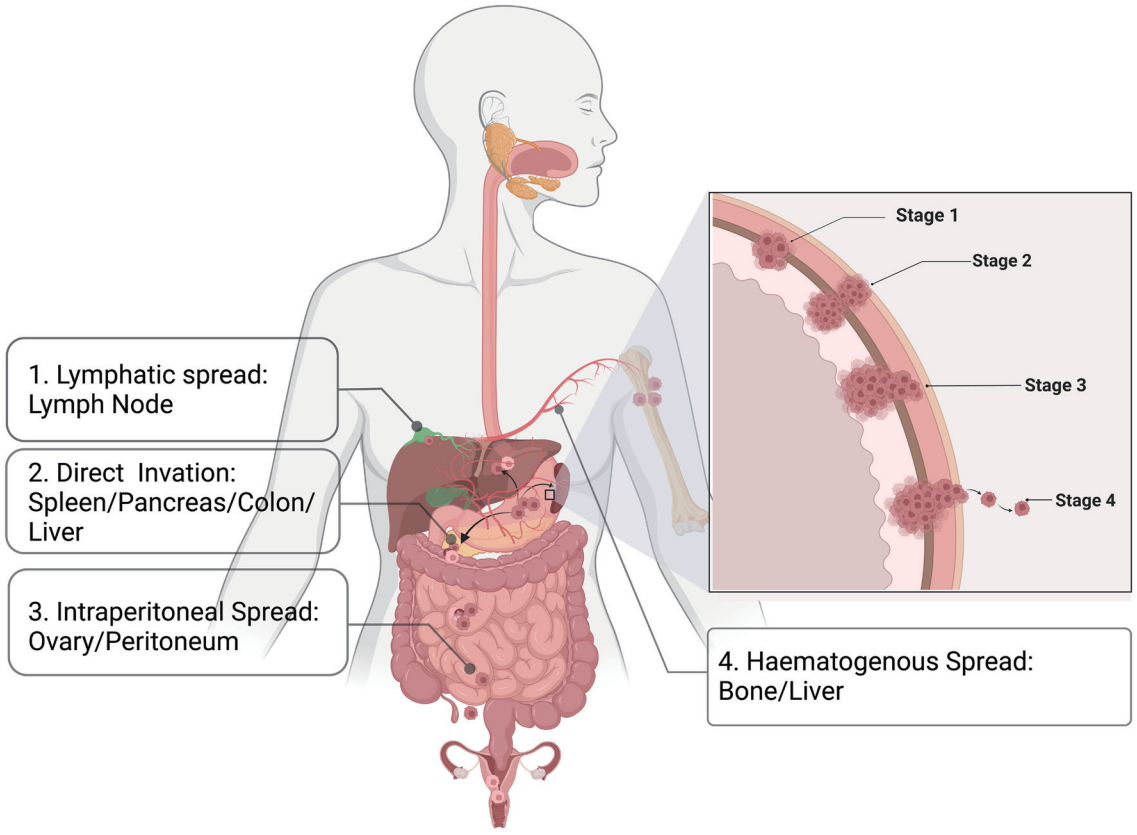

Fig. 1. Summary of the metastatic routes and sites in gastric cancer. The primary routes of gastric cancer metastasis involve intraperitoneal, lymphatic, and hematogenous dissemination as well as invasion into adjacent organs. Common metastatic sites comprise the spleen, pancreas, colon, liver, peritoneum, ovaries, lymph nodes, and bones. Figure adapted from BioRender.com.

as shown in Figure 1. Peritoneal metastasis (PM) accounts for 53–66% of distant metastatic GCs,[15] with a median survival time of only 4 months following the diagnosis.[13,14] GC patients with pleural metastasis also often have PMs (32%). Patients with neurological metastases also usually have lung metastases (21% of all cases) but very few PMs (9%). More than half of the patients with lung metastases also have liver metastases. Women with ovarian metastases often develop PMs (56%).[16]

Major routes of metastasis in GC include lymphatic, intraperitoneal, hematogenous spread, and direct invasion into adjacent organs.[17] Among them, the peritoneum is the most prevalent metastatic site for GC.[13,14] GC cells tend to seed in the abdominal and pelvic organs, such as the intestine, ovary, diaphragm, bile, and rectum surface, often forming localized tumors. Routine cytology has been used to assess the risk of PM in GC with serosal invasion. However, its sensitivity remains limited, as PM still occurs in many patients with negative cytologic results.[18] The growth of a primary tumor requires sufficient blood supply, resulting in angiogenesis that can support nutrition for tumor metastasis. These newly formed blood vessels can also serve as passages for cells to exit the tumor and enter the circulatory blood system. In addition, tumor cells may disseminate to other tissues via the lymphatic system. Once the cells are arrested in a new organ through the circulation, they may initiate the formation of a macroscopic tumor by the development of new blood vessels.[19] Ultimately, successful dissemination depends on both the molecular alterations in the cancer cells themselves and the microenvironment they encounter.

Classification and characteristics of scRNA-seq and spatial transcriptomics

The scRNA-seq technique

The TME consists of various cell types and extracellular components that surround human tumor cells and are nourished by a vascular network. The cellular populations within the TME are extremely complex, and recent advances in scRNA-seq have enabled identification and analysis among various malignant cell types.[20] For example, Wang *et al*. performed scRNA-seq on malignant peritoneal cells obtained from ascitic fluids of GC patients to find prognostic signatures.[21] The scRNA-seq program involves single-cell isolation, library construction, sequencing, and data analysis, as shown in Figure 2. Currently, scRNA-seq is mainly in two forms: plate-based and droplet-based.[22–24]

SMART-seq2

SMART-seq2 utilizes fluorescence-activated cell sorting to place individual cells, allowing for a flexible experimental set-up with optional pause points, particularly when time is limited. Once cells are lysed, reverse transcription and PCR are carried out. SMART-seq2 exhibits a low dropout rate compared to bulk RNA-seq, enabling comprehensive characterization of broader gene expression profiles. Additionally, SMART-seq2 provides full-length mRNA coverage, facilitating the quantification of high-abundance expression at the transcript level.[25,26] These platforms offer a fast and efficient means to analyze 50 to 500 single cells

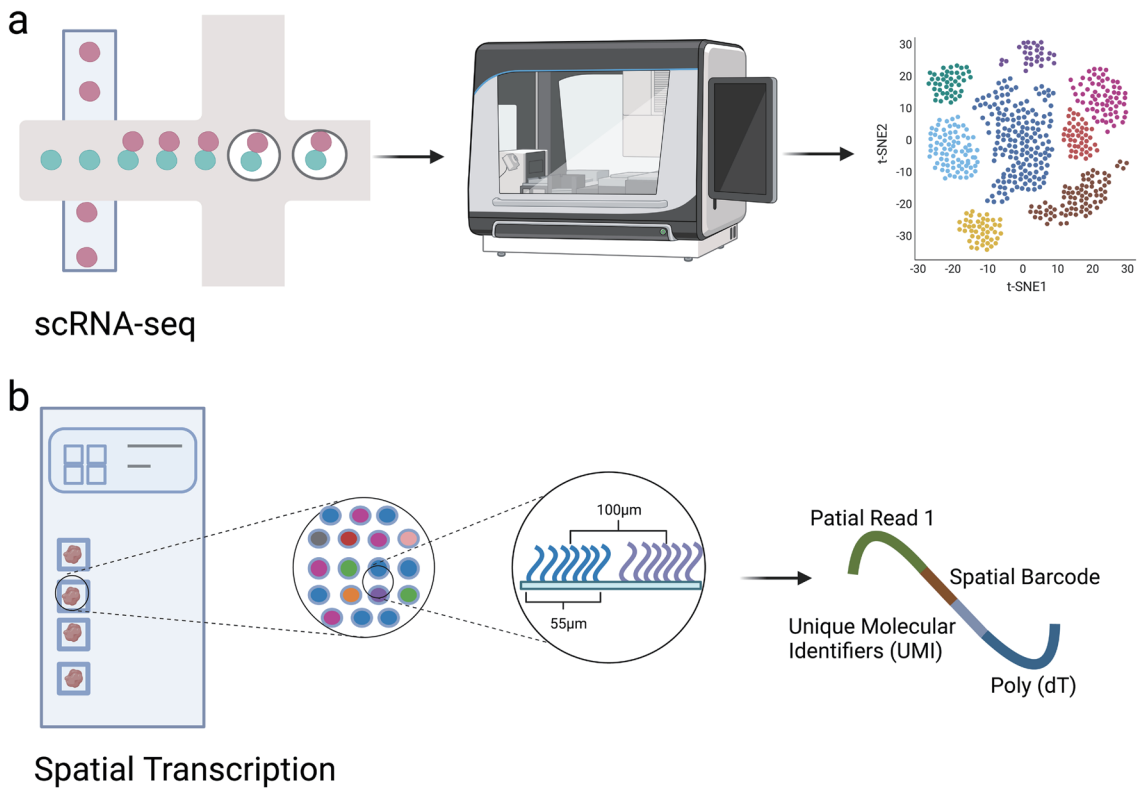

Fig. 2. Schematics of scRNA-seq and spatial transcription techniques. (a) The scRNA-seq workflow. Single cells are mixed with single gel microbeads, forming small droplets of oil-enclosed water. Next, the cell membrane is disrupted, releasing mRNA from the cells. mRNA binds to the DNA barcode on the gel microbeads, producing cDNA via reverse transcriptase. The final steps involve amplification and sequencing. (b) Structure of 10× Genomics Visium chips. All oligos in the same spot contain the same spatial barcode (16 nt), and the spatial barcode sequence information is obtained through sequencing analysis to determine the spatial location of the mRNA. The UMI (12 nt) of each oligo in the same spot is unique and is used for transcript quantification via sequencing analysis. Finally, a 30-nt poly (dT) is used to capture mRNA from the FFPE sample. Figure adapted from BioRender.com.

in a single experiment. However, plate-based scRNA-seq is more expensive and has lower cellular throughput compared to droplet-based scRNA-seq.[27]

10× Genomics

Advances in microfluidic technology have allowed individual cells to be separated into droplets containing lysis buffer and cell barcodes. Droplet-based scRNA-seq, such as 10× Genomics, can generate scRNA-seq data for thousands of cells in a single experiment, enabling a more comprehensive exploration of cell types in a tissue compared to plate-based techniques, which are typically limited to hundreds of cells.[28] However, droplet-based scRNA-seq has technical limitations. These methods sequence either the 3 or 5 ends of mRNAs, which poses challenges in assembling full-length mRNA transcripts.[29]

The above methods fail to capture the epigenetic heterogeneity that may drive cellular behavior, thus scRNA-seq results describe only a subset of the molecular phenotype of a cell. Moreover, these sequencing methods cannot recover splicing patterns or sequence variants. Despite their advantages and disadvantages, both techniques have been successfully applied in the analysis of the TME. Furthermore, combining complementary strengths has provided a more comprehensive understanding of the TME.[30]

Spatial transcriptional profiling

Because the tissues must be resolved before sequencing, both bulk RNA-seq and scRNA-seq fail to retain anatomical information. Fortunately, spatial transcriptional profiling provides gene expression information while conserving tissue architecture information. Employing high-resolution spatial transcriptomics to understand the heterogeneity and cell interactions within an intact tissue section marks the next major milestone. In particular, this approach effectively characterizes solid tumors composed of malignant cells, stromal cells, and immune cells.[31] Newer "spatial transcriptomic" platforms, such as DSP and 10× Genomics Visium, promise to yield a more comprehensive understanding of cell-cell variations within and between tumors.

10× Genomics Visium

The foundation of Visium technology lies in a slide containing four capture regions where tissue can be visualized and processed for gene expression analysis. Each capture region contains 5,000 spots with spatial barcodes unique to individual features on the slide. In particular, a unique molecular identifier (UMI) and a poly (dT) sequence within a probe enable transcript quantification and the capture of poly(A)-tailed mRNA diffusing toward the slide surface, followed by reverse-transcription as cDNA and library generation.[10] The structure of 10× Genomics Visium chips is shown in Figure 2. Fresh-frozen tissues are required for the Visium technique to ensure high-quality RNA. In addition, the spot diameter of 55 mm does not yet allow single-cell resolution.[9]

Table 1. Comparison of transcriptomic platforms

Name	Tissue size	Type of RNA captured	Cellular resolution	Amount of data generated	Ref.
The scRNA-seq technique					
SMART-seq2	No requirement	Poly-adenylated RNA	Single cell level	Full-length mRNA; tens to hundreds of cells	20,26
10× Genomics	No requirement	Poly-adenylated RNA	Single cell level	Full-length mRNA; thousands of cells	28,29, 35
Spatial transcriptional profiling					
10× Genomics Visium	Maximum of 6.5 × 6.5 mm per capture area (fresh-frozen tissue)	Poly-adenylated RNA only	Approx. 10 cells/feature; a center-to-center distance of 100 µm; spot size 55 µm	Whole coding transcriptome	9,10
NanoString GeoMx DSP	Maximum of 14.6 × 36.2 mm (whole tissue sections, fresh-frozen tissue and FFPE)	Any RNA to which a probe can be designed	Approx. 20–200 cells/ROI	Panel-based detection; User-defined ROIs	9
Stereo-seq	An effective area of 13.2 cm × 13.2 cm (frozen tissue)	Poly-adenylated RNA	Subcellular resolution; a center-to-center distance of 500 or 715 nm; spot size 220 nm	400 spots for tissue RNA capture per 100 mm^2	32

ROI, Region of interest.

NanoString GeoMx DSP

NanoString GeoMx DSP technology is designed to analyze the spatial expression of RNA within user-defined regions of interest (ROIs) in a tissue section. Tissue sections are prepared to expose the *in situ* hybridizing RNA to specific gene probes. Specifically, DSP relies on fluorescent markers to visualize the selected ROI. Each ROI is exposed to UV light, facilitating the release of photocleavable barcodes from gene-specific probes. Finally, the cleaved barcodes are identified using next-generation sequencing or the NanoString nCounter system. Curated panels of probes enable high-confidence analysis of tissue sections from formalin-fixed paraffin-embedded (FFPE) samples, allowing high-throughput detection of 18,000 genes.[9]

Stereo-seq

Chen *et al*. combined DNA nanoball (DNB)-patterned arrays with *in situ* RNA capture to create high-resolution spatial omics sequencing, named Stereo-seq. Currently, Stereo-seq can be used to depict spatial cell heterogeneity, cell fate, and cell-cell interactions in developing tissues with single-cell resolution. As reported, the use of random barcode-labeled DNB achieves a large barcode pool with 425 distinct spots. Similar to 10× Genomics Visium described above, UMI and polyT sequences are ligated onto each spot through hybridization with an oligonucleotide sequence. Frozen tissue sections loaded onto the chip surface are fixed and permeabilized to gain the tissue polyA-tailed RNA, followed by reverse transcription and amplification. Notably, the Stereo-seq chip has an effective area of up to 13.2 cm × 13.2 cm and characterizes the single cell-type composition of complex tissues with high sensitivity.[32–34]

The detailed comparison of the two transcriptomic platforms is presented in Table 1.[9,10,20,26,28,29,32,35]

Application of scRNA-seq and spatial transcriptomics in metastatic GC

Finding new breakthroughs is urgent as existing traditional treatments of GC have reached the therapeutic plateau. Therefore, a more precise understanding of metastatic GC is needed to identify new targets and enhance the clinical management of the disease. Utilizing scRNA-seq and spatial transcriptomics could clearly identify the signatures of both primary and metastatic tumors, as well as cell-cell interactions within the TME .[36]

Malignant cells in GC exhibiting heterogeneity

Understanding intratumoral heterogeneity, *i.e.* the molecular variation among cells within a tumor, promises to improve the diagnosis and treatment of malignant cancer.[37] Analysis of scRNA-seq data often reveals that each normal cluster comprises cells from multiple patients, whereas each tumor cluster consists of cells from a single patient, indicating a high level of intertumoral heterogeneity. Furthermore, Sun *et al*. identified differentially expressed genes (DEGs) between the tumor cell clusters and normal cell clusters, finding that tumor cells from one patient formed two distinct tumor clusters. Interestingly, one cluster appeared less advanced than the other, highlighting intratumoral heterogeneity.[38] GC with peritoneal carcinoma (PC) underpins tumor cell survival, leading to treatment resistance, a major obstacle to improving patient outcomes.[39,40] Wang *et al*. performed scRNA-seq on PC cells collected from 20 advanced-stage GC patients.[21] Further studies divided the PC samples into two main subtypes: gastric phenotype (mainly gastric cell lines) and GI-mixed type (mixed gastric and colorectal cells), revealing high inter-patient heterogeneity in PC tumor cells. In addition, gastric-dominant had shorter survival compared to GI-mixed. Moreover, a 12-gene fundamental signature derived from PC cells showed prognostic significance when applied to independent cells.[21,41,42] In summary, scRNA-seq is a robust and unbiased tool to assess intratumoral heterogeneity.[43] Single-cell analysis of heterogeneity shows a way to overcome drug resistance and develop new drugs.

Exploring cell populations and interactions in GC

The unique ecosystem resulting from the interaction between

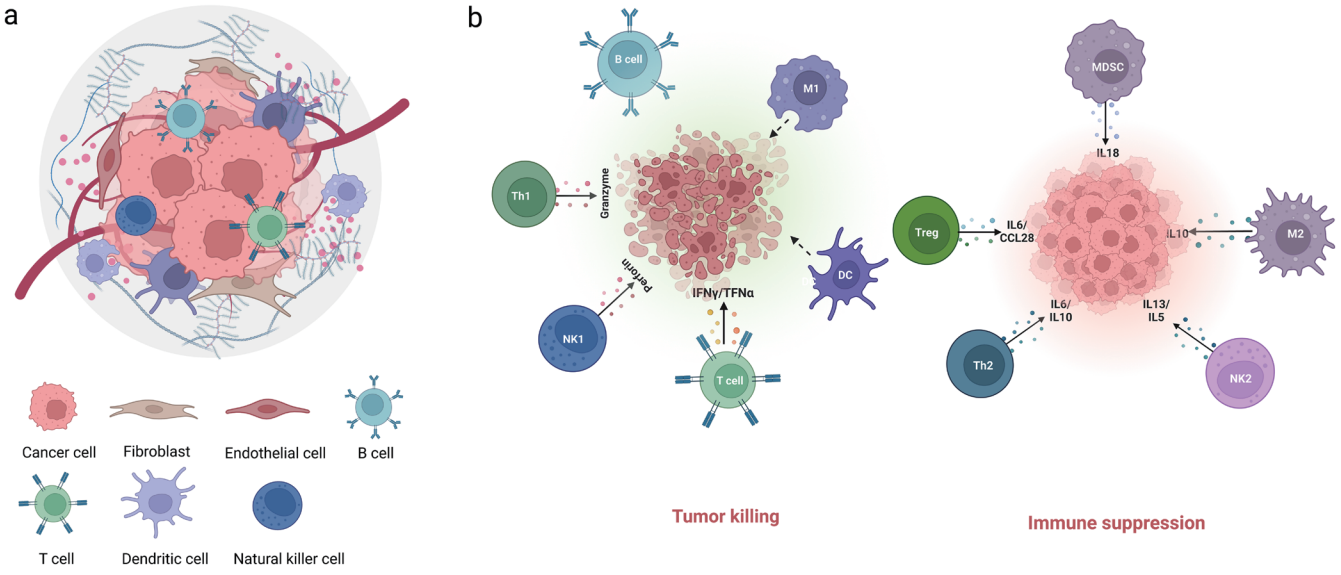

Fig. 3. The architecture of the tumor microenvironment. (a) The tumor microenvironment comprises cancer cells, fibroblasts, endothelial cells, and various immune cells. The complicated and dynamic interactions result in great heterogeneity of the tumor microenvironment. (b) Immune cell actions include tumor killing and immunosuppressive effects; therefore, a full understanding of various immune cell effects is beneficial to the development of immune therapies. Figure adapted from BioRender.com.

the tumor and the associated microenvironment promotes tumor growth and invasion.[44] The TME of GC consists of stromal cells, macrophages, dendritic cells (DCs), Tregs, etc. As reported, myeloid populations or the stromal cells can regulate the status of lymphocytes through complex ligand-receptor interactions.[38,45] Tumor-recruited immune cells, such as M2 macrophages, are known to promote immune escape, subsequently contributing to metastasis, as shown in Figures 3 and 4.[46–48] Therefore, the analysis of cell populations and cell-cell interactions is critical for early diagnosis and the development of cancer immunotherapies.

Epithelium

GC is a malignant tumor originating from the gastric epithelium.[49] Epithelial cells consist of a complex cell lineage comprising mucinous, secretory, and endocrine cells, working together to maintain tissue homeostasis.[50,51] Malignant epithelial cells exhibit enrichment in various protein-related processes, including negative regulation of protein modification and positive regulation of protein

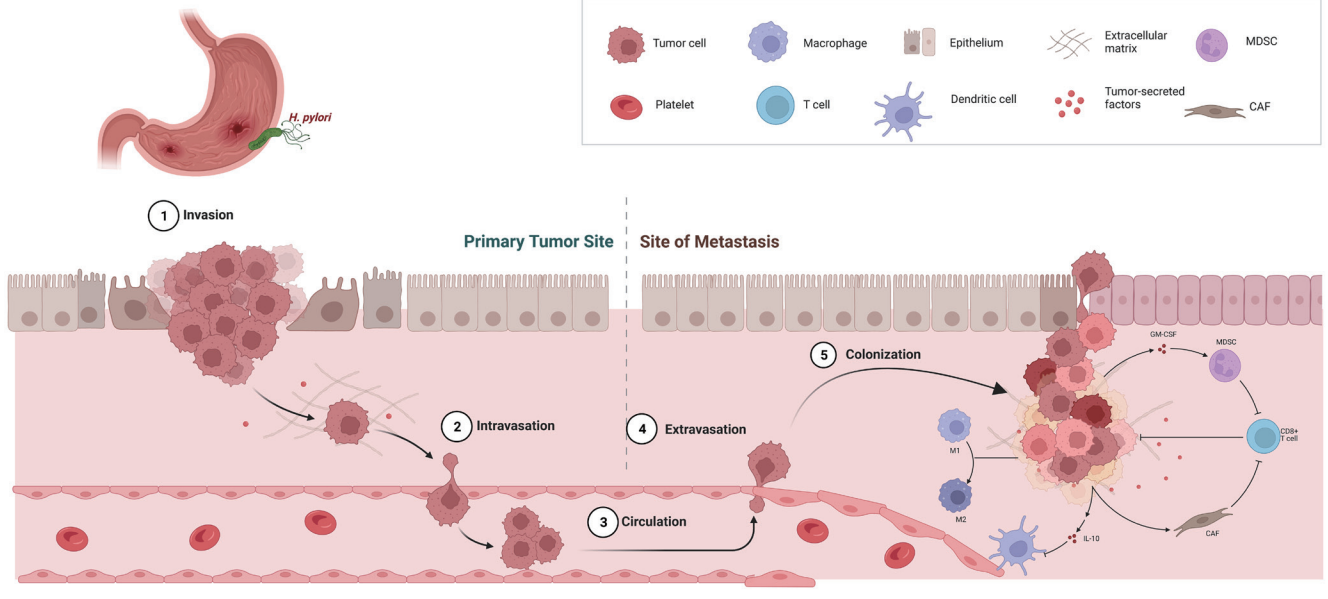

Fig. 4. Overview of metastasis and TME. The interaction between cancer cells and the immune microenvironment leads to immune reprogramming and metastasis. Cancer immunoediting facilitates tumor escape from immune destruction, consequently contributing to a poor prognosis. Figure adapted from BioRender.com.

localization. These cells also demonstrate copy number variants (CNVs) in patients with distal gastric adenocarcinoma and liver metastases.[48] Wang *et al*. discovered that ERBB2, CLDN11, and CDK12 were related to GC lymph node metastasis, while FOS and JUN were considered potential driving genes in GC.[36] High-resolution scRNA-seq revealed high-level expression of NOTCH2, NOTCH2NL, KIF5B, and ERBB4 in primary cancers, while metastatic cancer displayed overexpression of CDK12, ERBB2, and CLDN11, playing an associated role in metastasis.[24,52–54] CLDN11, a member of the tight junction protein family, is reported to be shared within lymph node metastasis-prone subclones.[36,55] Fan *et a*l performed scRNA-seq analysis of PC cells from 20 gastric adenocarcinoma patients and found that SOX9 was expressed in epithelial cells of both primary and metastatic gastric adenocarcinoma and was associated with poor prognosis. Further study revealed that SOX9 is highly associated with cancer stem cell traits, tumorigenicity, and metastases in GC.[21,56]

Using spatial DSP, Vikrant *et al.* confirmed the loss of LIPF in tumor epithelial cells compared to normal samples and identified LIPF as a lineage-specific target in GC. In addition, there are higher transcript levels of KLF2 in diffuse-type epithelial cells compared to intestinal-type epithelial cells.[5,57] Furthermore, Bianca *et al.* defined stroma AReactive invasion front areas (SARIFAs) as a spatial structure of tumor glands comprising at least five tumor cells directly contacting adipocytes in the invasion front. The most upregulated genes in SARIFA-positive cases included COL15A1, FABP2, FABP4, and FGB. Because SARIFA has a high prognostic relevance, it has the potential to be a biomarker of malignant GC, thus providing a basis for GC treatment.[58]

Stromal cells

Cancer-associated fibroblasts (CAFs), pericytes, and endothelial cells express core components of the extracellular matrix (ECM). Gene expression profiling in patients has revealed that genes related to inflammation, cytokines, and growth factor-associated proteins are highly enriched in the surrounding stroma but not in the cancer cells themselves. Thus, stromal cells play an important role in promoting GC cell migration and metastasis.[59] As reported, transcriptional reprogramming of stromal cells in the TME promotes tumor growth.[60] Tumor-associated endothelial cells exhibit greater activation of SOX18 and SOX7, which regulate endothelial cell growth,[61] and they secrete large numbers of cytokines that interact with SDC1, SDC4, and ITGB1 in cancer cells.[62] In addition, Li *et al.* detected 1,873 endothelial cells using scRNA-seq and found that multiple vascular endothelial growth factor receptors play an essential role in angiogenesis and ACKR1 specifically expressed in tumor endothelial cells associated with poor prognosis.[20]

CAFs represent one of the most important components in the TME and play a critical role in tumor development and progression. CAF subsets express genes at high levels involved in ECM remodeling, including genes encoding collagen and collagen metabolism enzymes.[59] Intrinsic fibroblasts and mesenchymal stem cells (MSCs) originating from bone marrow constitute the primary precursor cells of CAFs. Interactions between MSCs and neutrophils result in MSC differentiation into CAFs via an interleukin (IL)-6–STAT3 axis, providing a proinflammatory environment and consequently increasing metastasis.[63,64] The CAF subpopulation is characterized by high expression of periostin, which promotes the adhesion and migration of epithelial cells and drives the maintenance and metastasis of cancer stem cells. CAFs enhance IL-17B expression in GC tissues, leading to the activation of MSCs and further accelerating the migration of GC cells.[65] Using spatial DSP, Vikrant *et al.* analyzed FAP and INHBA in fibroblast regions identified by α-smooth muscle actin, revealing increased expression of these proteins in tumor fibroblasts compared to normal fibroblasts. Consequently, there is a strong correlation between FAP and INHBA coexpression levels.[5]

Moreover, CAFs do not exist as individual cells around tumors but interact with tumor cells to promote tumor growth and survival, thus maintaining their tendency toward malignancy.[60] Tumor cells can affect the recruitment of CAF precursors and induce normal fibroblasts into CAFs. Conversely, CAFs can secrete multiple cytokines, growth factors, and extracellular matrix proteins, promoting cell proliferation, drug resistance, invasion, and metastasis. The connection between GC cells and fibroblasts through the cadherin 11-mediated juxtacrine signal activates the YAP/Tenascin-C axis, facilitating gastric cancer metastasis.[66] In addition, fibroblasts serve as the major source of Wnt ligands, and the corresponding receptors, such as LGR4–RSPO3, are expressed on tumor epithelial cells, endothelial cells, and pericytes.

The association between CAFs and epithelial cells is mediated through various ligand-integrin receptor interactions, including collagen and fibronectin. COL1A1, COL1A2, and COL3A1 are highly expressed in fibroblasts and interact with cancer cells through ITGA2, DDR1, and ITGB1, which are strongly correlated with GC genesis, development, and metastasis.[67–70] Furthermore, collagen alterations within the TME are associated with PM in GC through serosal invasion.[71] By using scRNA-seq, Li *et al.* demonstrated that inflammatory CAFs (iCAFs) and extracellular matrix CAFs (eCAFs) exhibit strong pro-invasive activity. They also recruit surrounding immune cells to build a favorable tumor microenvironment. iCAFs interact with T cells through C-X-C motif chemokine ligand 12 (CXCL12) and interleukin (IL)-6, while eCAFs promote M2 macrophage polarization by expressing periostin. eCAFs, as a pre-invasive CAF subset, reduce the overall survival time of GC patients.[3,72] In conclusion, CAFs are involved in angiogenesis, extracellular matrix remodeling, immune suppression, and EMT, providing a favorable microenvironment for tumor cells.[73] A TME-specific intercellular communication network has the potential to influence cellular behavior.

Immune cells

The infiltrating state of immune cells in GC metastases may provide specific diagnostic and therapeutic strategies for organ-specific metastases. ScRNA-seq and spatial transcription are used to analyze different immune cell subtypes and their heterogeneous transcription factors in GC patients at single-cell resolution.[48,74] The migratory properties of immune cells can play an important role in elucidating the biology of tumor-infiltrating immune cells. In addition, when analyzing tumor-infiltrating immune cells, it is essential to consider sample collection and selection of relevant control tissues within each study, as shown in Table 2.[5,20,23,24,36,58,61,67,68,72,75–82]

T cell

ScRNA-seq assays were conducted on immune cells isolated from peripheral blood, GC tissues, and corresponding adjacent nontumor tissues to analyze transcription factor levels. The expression of IRF8 transcription factor was observed to be downregulated in both $CD8^+$ tumor-infiltrating lymphocytes (TILs) from GC tissues and blood, indicating a more advanced stage in these GC patients.[61] In addition, the exhaustion levels of cytotoxic $CD8^+$ T lymphocytes (CTLs) were found to be relatively low in primary gastric tumors. Further studies have found that exhausted T cells

Table 2. Exploring cell populations and interactions by scRNA-seq and spatial transcriptomics

Cell subsets	Signature genes	Enriched tissues	Platforms	Ref.
Epithelium				
Mucous and secretory lineages	NOTCH2, NOTCH2NL, KIF5B, ERBB4, CDK12, ERBB2, CLDN11, KLF2, COL15A1, FABP2, FABP4, FGB	Tumor tissue	10× Chromium system, SMART-seq2, DSP	24,36,58
Stromal cells				
Cancer-associated fibroblasts	COL1A1, COL1A2, COL3A1, FAP, INHBA	Tumor tissue	10× Genomics, SMART-seq2, DSP	5,67,68
Endothelial cells	PECAM, ENG, VWF, SELE	Tumor tissue	10× Genomics	20,23
Immune cells				
CD8[+] T cells	Naïve markers: CCR7, SELL	Normal gastric tissue, peripheral blood	10× Genomics, SMART-seq2	23,61
	Tissue effector memory markers: CD69, ITGAE, ITGA1	Tumor, peripheral blood		
	Cytotoxic genes: GZMB, GZMA, PRF1, IFNG, NKG7	Tumor, peripheral blood		
	Exhausted T cells: LAG3, CTLA4, VSIR	Tumor, peripheral blood		
$CD4^+$ T cells	Naïve markers: CCR7, SELL	Peripheral blood, normal gastric tissue	10× Genomics	20,23
	Effector CD4 T cells: GZMA, GZMB, CXCL13, BATF, HLA	Normal and tumor tissue		
B-cell	ETS1, FHIT	Tumor, peripheral blood	10× Genomics	75
NK cells	GZMA, XCL2, CCL5, PRF1, CCL3, CCL4, GITR, CD96, KIR2DL4	Tumor, peripheral blood	10× Genomics	23,61
DCs	IL3RA, CLEC4C	Peripheral blood	10× Genomics	20,23,76
Macrophages	CD68, CD163, MRC1, INHBA, PTGS2, C1QC, CX3CR1, MARCO, CCL5, IL2RG, CD14, FCGR3A, CD68	Tumor, peripheral blood	SMART-seq2, 10× Genomics	77–79, 81,82
MDSC	ARG1, CD66b, VISTA, IDO1	Tumor tissue	DSP	72,80

in the TME exhibit high expression of inhibitory receptors, such as PD-1, CTLA-4, TIM-3, TIGIT, and LAG3, and elevated expression levels of MKi67, a marker of active proliferation.[8] Sun *et al.* uncovered that Tc17 ($CD8^+IL17^+$ T) cells expressed the highest level of ITGAE (CD103), a member of the integrin family, among all detected T cell subtypes in the single-cell analysis. This suggests that Tc17 cells may be involved in cell-to-cell contacts through ITGAE-CEACAM5 interactions. Further studies indicated that tissue-resident $CD8^+$ T cells could differentiate into Tc17 cells in the TME, which further shift to exhausted phenotype. In addition, the CD40LG-CD40 and CCL20-CCR6 interactions from CD4_C4/C6 and DC_LAMP3/cDC1_XCR1 suggest that $CD4^+$ T cells promoted the recruitment and activation of DCs.[38] The proportion of Tregs in tumor samples was significantly higher than that in adjacent normal samples, suggesting the expansion or recruitment of Tregs in gastric tumors ScRNA-seq also revealed increased expression of multiple genes associated with immune suppression in Tregs, including DUSP4, IL2RA, TNFRSF4, LAYN, and LGALS1.[20]

B-cell subsets

ScRNA-seq analysis of B-cell subsets between cancerous and paracancerous tissues revealed DEGs, including EIF1AY, KRT19, LCN2, and RPS4Y1.[61] Pathway analysis revealed that the upregulated genes in the B-cell cluster were enriched in the TNF, nod-like, and CXCR chemokine receptor-binding pathways. In addition, a special group of B cells, named T-cell-like B cells, express both the marker CD3D of T lymphocytes and the typical surface markers CD79A, MS4A1 (CD20), CD40, and CD19 of B lymphocytes.[75] In particular, T-cell-like B cells with the marker genes CD19 and CD3D were further validated in GC with lymph node metastasis and ovarian metastasis.[48]

Natural killer cells

As reported, natural killer (NK) cells actively participate in immunosurveillance to prevent GC. Differential analysis of gene expression in cancerous and adjacent tissues at the single-cell level showed that IL8, G0S2, HSPA6, and CXCL1 are upregulated, while IGJ, TFF1, and NCR2 are downregulated.[61] These genes might be involved in cytokine-cytokine receptor interactions, MAPK signaling, T-cell receptor signaling, and chemokine signaling.[61]

Dendritic cell subtypes

DCs present cancer antigens and secrete inflammatory cytokines and chemokines, initiating and regulating both innate and adaptive immune responses against tumors. In GC patient blood, DCs were found to express multiple inhibitory receptors, such as FTL

and IL8, and secrete cytokines, including CCL4 and CCL5.[23] Li *et al.* characterized clusters as DCs based on low expression of CD14 and high expression of the DRα gene. Multiple studies have demonstrated that LAMP3$^+$ DCs tend to express more types of chemokines, cytokines, and inhibitory ligands. LAMP3$^+$ DCs can regulate the functions of tumor-infiltrating T cells, such as driving naïve T cells toward Tregs and activated CD8$^+$ states.[8,20,76,83] Sun *et al.* found that LAMP3+DCs, characterized by the specific expression of LAMP3 and CCR7, are involved in mediating T cell activity and forming intercellular interaction hubs with tumor-associated stromal cells. Conversely, these DCs inhibit the activity of anti-tumor T cells by expressing CD274 at high levels.[38,84]

Macrophages

Macrophages, a major component of the tumor microenvironment, either promote or inhibit tumorigenesis and metastasis according to their status.[77] Roca *et al.* found that macrophage-secreted CHI3L1 promoted GC metastasis *in vitro* and *in vivo*.[78] Leukemia inhibitory factor (LIF) as the top secreted molecule is regulated by SOX9 in GC with PC and mediates SOX9-induced M2 macrophage repolarization. Fan *et al.* uncovered that targeting SOX9/LIF axis in GC increased infiltration and cytotoxicity of CD8$^+$ T cells and decreased M2 macrophage infiltration.[56] GSVA analysis of hallmark pathways revealed increased activities of WNT signaling, hedgehog signaling, angiogenesis, EMT, and IL10 signaling in tumor-associated macrophages (TAMs), while C1QC$^+$ macrophages were upregulated in MHC class II antigen presentation.[20]

Macrophages secrete cytokines interacting with SDC1, SDC4, and ITGB1 in cancer cells, leading to EMT activation and GC metastasis. Inhibiting these interactions could suppress GC metastasis.[62] Studies have demonstrated that the CXCL 5-CXCR 2 interaction between cancer cells and macrophages can promote GC metastasis.[79,85] Further understanding of the molecular mechanisms underlying macrophage plasticity holds promising prospects for immunopathology for GC.

In general, the above statements suggest that the observed plasticity at the transcriptional level in GC may result from oncogenic and exogenous mediators, such as ligand-receptor interactions in the TME. Alterations in the immune environment can be observed in the early stages of multistep progression, providing an opportunity for immunotherapy.

Drug response

For patients with advanced GC, effective treatment is particularly crucial due to the poor survival rate. By examining cellular changes before and after drug administration, it is possible to discover potential mechanisms affecting drug response by scRNA-seq.

Studies of advanced GC indicate the presence of immune remodeling during chemotherapy. Research indicates a decrease in both proinflammatory genes and MHC class I antigen-presenting genes after chemotherapy. The expression of M2 type macrophage-related genes decreases, suggesting that macrophages transform from M1 cells to M2 cells after chemotherapy.[86] One study demonstrated that treated samples exhibited damaged immune cells but increased endothelial cells and fibroblasts. T cells exhibited lower cytotoxic and proliferative properties, along with the downregulation of immune pathways. Using paired pretreatment and on-treatment samples during 5-FU treatment, Ryul *et al.* identified chemotherapy-induced NK-cell infiltration, macrophage repolarization, and increased antigen presentation among responders. Nonresponders exhibited increased LAG3 expression and reduced numbers of DCs, highlighting the remodeling of the TME during chemotherapy response and resistance.[87] Single-cell transcriptome was used to detect differentially expressed proteins among normal gastric mucosa, primary GC and PM tissues. Ye *et al.* found that MYH9-induced expression of CTNNB1 was found to promote GC metastasis, which may be inhibited by staurosporine, indicating a novel approach for the treatment of GC peritoneal metastasis.[88]

In conclusion, scRNA-seq may offer an opportunity to expand the portion of patients benefiting from chemotherapy alone or in combination with immunotherapy.

Conclusion and perspective

Advances in understanding the molecular alterations in GC have provided valuable knowledge to reveal the complex biological phenomenon underlying metastasis. However, due to the complexity and systematization of GC metastasis, many questions about the mechanisms of GC metastasis remain unanswered. Within a relatively short period, scRNA-seq and spatial transcriptomics have illuminated and reinforced many complex facets of cancer cells and associated TME. The precise roles and plasticity of cancer cells and the TME continue to be investigated, with single-cell and spatial studies identifying even greater levels of subtype diversity within this already complex cellular compartment.

In recent years, the targeted therapy of CAF has garnered significant interest, with numerous related clinical trials under way. FAP is a major cell surface marker of immunosuppressive CAFs. Elimination of FAP + CAFs are associated with increased CD8 + T cell infiltration. Depleting FAP+ CAFs via genetic deletion or chimeric antigen receptor T cells has shown promising anti-tumor activities in preclinical animal models.[89] Targeting activation signaling and downstream effectors of CAFs, such as IL-6, IL-6 receptor or JAKs, kinase inhibitor imatinib have been well verified.[90] In a word, it is urgent to find specific markers and categorize them into different subpopulations using scRNA-seq.

A growing number of studies have confirmed that the composition and functional status of different cell types influences different responses to cancer immunotherapy. By utilizing scRNA-seq and spatial transcriptomics, further understanding of the TME has great potential not only in identifying reliable biomarkers but also in discovering novel therapeutic strategies to complement existing therapeutic drugs.[8] For example, scRNA-seq can analyze the contribution of the rejuvenation vitality in pre-existing T cells and the recruitment of new T cells in response to anti-PD-1 treatment in GC patients. The findings revealed that the recruitment of new T cells may be more significant for anti-PD-1 therapies in basal and squamous cell carcinomas. T cells derived from peripheral tissues are essential for generating effective immunotherapy.[91] Moreover, TCF1$^+$CD8$^+$ T cells, known for their stem-like phenotype, express low levels of PD-1 and TIM-3 inhibitory receptors, thereby enhancing the immune response to tumor suppressors. In addition to effector memory T cells, tissue-resident memory and peripheral T cells can also be important sources supporting the rejuvenation and recruitment of tumor-infiltrating T cells.[92,93] In addition, a scRNA-seq study demonstrated the critical role of cytotoxic CD4$^+$ T cells in mediating the anti-tumor effects of anti-PD-L1 therapy in an MHC class II-dependent manner.[94] The development of immune checkpoint inhibitors has enhanced cancer therapy by providing clinical treatment for some previously incurable patients. A comprehensive understanding of the complex immune cell composition and molecular pathways could help identify the mechanisms underlying immunotherapy response and indicate the potential of new targets to overcome resistance.[95–97]

By consolidating the reported studies, we can gain deep insights into the mechanisms of GC metastasis. Considering the persistent increase in the number of patients with metastatic GC, we urgently hope that the understanding and findings regarding the mechanism of cancer metastasis can be continuously applied to clinical practice. The road ahead will involve the integration of single-cell and spatial analytics in the comprehensive monitoring of patients during clinical trials, providing effective solutions to address the various mechanisms behind resistance and ineffective treatment, and opportunities for progress in the treatment of devastating diseases.

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Conflict of interest

The manuscript was submitted during Dr. Jiang-Jiang Qin's term as an editorial board member of *Oncology Advances*. The authors have no other conflict of interests to declare.

Author contributions

Study design, manuscript writing (CXZ); Critical revision, critical funding, administration (JJQ). All authors have made a significant contribution to this study and have approved the final manuscript.

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