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Review Article

Spatial Omics in Decoding Oral Squamous Cell Carcinoma Heterogeneity: Microenvironment Crosstalk and Multi-Omics Integration

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Highlights

- *Tumor heterogeneity and TME interactions in OSCC decoded by spatial omics.*
- *Advances in transcriptomics, proteomics, and metabolomics for OSCC insights.*
- *Multi-omics integration reveals biomarkers and precision therapy targets.*

Abstract

Oral squamous cell carcinoma (OSCC) is a major issue in the sphere of head and neck cancer since it is very heterogeneous, which also leads to the poor treatment results and low survival rates in the advanced stages. Here, the review will discuss how spatial omics methods explore tumor heterogeneity in OSCC, which includes cellular, molecular, and immune microenvironment alterations that occur due to cancer stem cells, stromal interactions, genetic instability, epigenetic reorganization, and metabolic reprogramming. The focus of heterogeneity is given on the contribution of the tumor microenvironment such as immune cells, cancer-associated fibroblasts and extracellular matrix remodeling to the stimulation of progression, metastasis, as well as therapeutic resistance. The introduction of spatial omics technologies, including spatial transcriptomics, proteomics, and metabolomics, has revolutionized the field by preserving tissue architecture, enabling high-resolution mapping of gene expression, protein distribution, and metabolite profiles. Significant developments around the spatial omics technologies are discussed, while how they are used in the identification of ligand-receptor network, signaling pathways, and spatial patterns of heterogeneity in OSCC are described. Integration of multi-omics approaches bridges gaps between transcriptomic, proteomic and metabolic, facilitating the discovery of biomarkers for prognosis, immune evasion mechanisms, and precision therapies targeting epithelial-to-mesenchymal transition and immunosuppressive networks. Despite challenges in data integration, cost, and clinical translation, spatial omics holds promise for personalized oncology, with future directions involving artificial intelligence-driven modeling to enhance diagnostic accuracy and therapeutic efficacy in OSCC management.

Keywords

oral squamous cell carcinoma; tumor heterogeneity; spatial omics; spatial transcriptomics; spatial proteomics; tumor microenvironment; precision oncology

1 Introduction

Oral squamous cell carcinoma (OSCC) is a head and neck malignancy that commonly develops in the oral mucosa[1]. It is the most frequent type of oral cancer, accounting for more than 90% cases[2]. Unfortunately, the 5-year survival rate is up to 80 % when detected at the early stage (T1). If diagnosed at the later stage (T3 or T4), the chances would decrease dramatically to 20–40 %[3]. OSCC treatments include surgical intervention followed by radiotherapy, chemotherapy, and immunotherapy, among which chemotherapy is the main adjuvant therapy for advanced OSCC[4]. However, although chemotherapy can improve survival rates, it often fails to achieve satisfactory results due to intrinsic and extrinsic drug resistance, low target specificity, and severe adverse drug reactions[5–8].

Tumor heterogeneity was noticed and proposed over 40 years ago[9,10]. Cancer may consist of multiple clonal subpopulations of cancer cells, which differ from one another in many characteristics, and all tumors are heterogeneous. While tumor heterogeneity encompasses both inter-tumor (the heterogeneity between different tumors) and intra-tumor variations, the tumor heterogeneity referred to in this text is intra tumor heterogeneity if there is no specific clarification, due to its critical implications for personalized therapeutic strategies. The pivotal role of intra tumor heterogeneity in prognostic indicators and for methods to individualize therapy has rendered it a longstanding focal point of scholarly attention and discourse[9,11]. The existence of tumor heterogeneity necessitates a shift from our current, static treatment strategies, tailoring therapies instead to target the specific characterizations of different cancer cell clones.

Spatial omics technologies characterized by high resolution and precision are increasingly garnering favor from academia. While conventional bulk and single-cell sequencing have identified key mutations and cell types in tumors like OSCC, they lose the critical spatial information of where these elements exist within the tumor ecosystem, as life processes unfold within three-dimensional space, even in simple organisms or single tissues.

This review will prove that spatial omics provides this missing dimension, visualizing the direct interactions between cancer cells and their microenvironment for us to understand OSCC progression and therapeutic resistance that was previously less accessible. We will start from discussing the foundational techniques to the state of the art, achievements attained and potential clinical applications of currently popular multi spatial omics technologies within the field of OSCC heterogeneity research. Regarding tumor heterogeneity in OSCC, we will then focus more on how do spatial omics discover tumor behavior, alongside the pathways and molecular mechanisms and provide solutions to clinical problems. Whereas for spatial multi-omics we highlight both Next-Generation Sequencing (NGS)-based and imaging-based methods. We end with obstacles now and an outlook to the future.

2 Tumor Heterogeneity in OSCC

Tumor heterogeneity is a major factor contributing to suboptimal treatment outcomes, which is equally applicable to OSCC[12]. To decrease or even eliminate the impact of this challenge on treatment strategies, we must develop a clear understanding of it.

2.1 Overview of Tumor Heterogeneity

Tumor heterogeneity exists concurrently at both temporal and spatial dimensions (Figure **1A**).

Temporally, the distribution of cells within tumors dynamically shifts throughout progression stages, generating heterogeneity. Spatially, cells exhibit heterogeneity at different tumor locations, exemplified by contrasting distributions in anaerobic central regions versus well-perfused peripheral areas. Tumor heterogeneity exhibits distinctive characterizations that encompass epigenetic, transcriptional, phenotypic, secretory, and metabolic components, among the others[13–15]. Two major forces drive heterogeneity: genomic instability and natural selection. It is also mediated by other factors which include epigenetic modifications and tumor microenvironment (TME)[15,16]. However, not all clones will be able to gain genetic changes related to the phenotypes that are favorable to survive by inheritance. Under the pressure of natural selection, only a portion of these cells ends up being the main constituents of the tumor under the pressure of natural selection (Figure **1B**).

Figure 1.

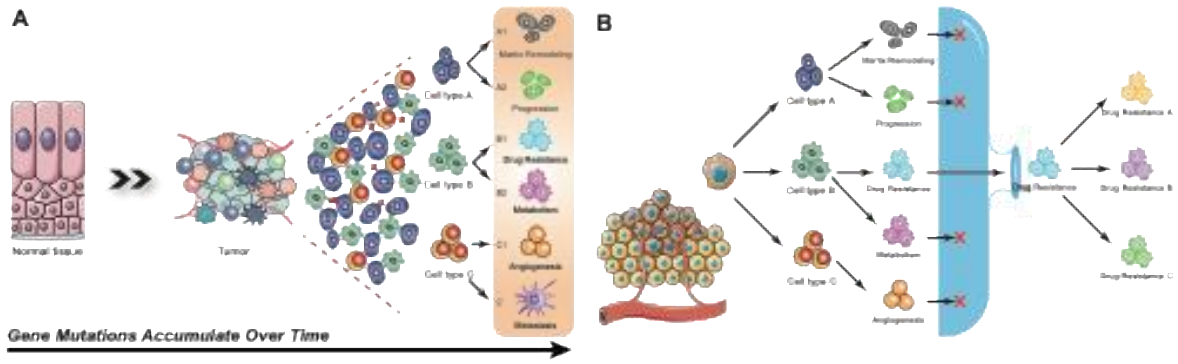

Figure 1. The Formation and Evolution of Tumor Heterogeneity

(A) Tumor Heterogeneity in Time and Space. Spatial heterogeneity: diverse cell populations in the orange box vs. earlier stages. Temporal heterogeneity: positional, morphological, and functional variations among cells in the box. Horizontal axis: tumor progression over time.

(B) Bottleneck Effects in Tumor Progression. External selection pressures, such as radiotherapy or drug therapy, create a “bottleneck effect,” allowing only specific cell subpopulations with advantageous phenotypes to survive. For example, drug-resistant cells gain a selective advantage, gradually increasing their proportion within the tumor cell population.

2.2 Cellular Heterogeneity

Cellular heterogeneity is a fundamental characteristic of many cancers, including OSCC. To understand it, we need to start with understanding its origins. Although there is still no clear consensus on this point, cancer stem cells (CSCs) have been identified as a potential contributor. When gene abnormalities accumulate in somatic stem cells, due to carcinogenic exposures like tobacco, alcohol, or Human Papillomavirus (HPV), transformation into CSCs starts by undergoing procedures like epithelial-to-mesenchymal transition (EMT)[17]. They are believed to initiate the carcinogenic process, drive the progression, and promote the acquisition of heterogeneity, regardless of the manner in which CSCs proliferate[10,18–20].

Yet, these transformed stem cells cannot do it alone. Certain seemingly “normal” cell or tissue populations that also exist within healthy tissues are recruited and activated to become part of the TME, thereby aiding in the tumor's further progression[21]. The role of endothelial cells in TME in promoting tumor angiogenesis has long been acknowledged. However, the contribution of stromal cells to cancer hallmarks, and thus to the very essence of the disease, cannot be underestimated. We can generically classify the stromal components of TME into

three major categories: angiogenic vascular cells (AVCs), infiltrating immune cells (IICs), and cancer-associated fibroblasts (CAFs).

The importance of cells of these types to TME development, tumor progression, and therapeutic outcome has been recognized for their complex interactions with cancer cells and other stromal components[22]. In the example of CAFs, there is evidence that CAFs can interact with CSCs in OSCC, thus promoting tumor progression[23]. Other studies suggest that CAFs secretion also plays a crucial role in tumor drug resistance acquisition[24]. Moreover, in OSCC, CAFs awake tumor and star recurrence by "interrupting" OSCC cell dormancy, and can also lead to metastasis through EMT mechanisms[25,26]. When it turns into tumor-associated macrophages (TAMs) from IICs, studies have also demonstrated their role in interacting tumor cells, like CSCs, and promoting tumorigenesis within the OSCC TME[27,28]. Tumorigenesis is associated with TAMs polarization, and the underlying key factors and mechanisms have been identified. Gene *Thbs1* in oxidative stress mediates M1-like TAMs polarization while enzyme SOAT1 in lipid metabolism induces M2-like TAMs Polarization[29,30]. These findings reveal potential therapeutic and contribute to the development of new therapies for OSCC.

2.3 Molecular Heterogeneity

Tumor molecular heterogeneity refers to the variability in molecular profiles, including genetic mutations, epigenetic modifications, transcriptomic patterns, proteomic alterations, and metabolic differences, observed among cancer cells[31]. Knowledge of molecular heterogeneity helps to demonstrate intrinsic subpopulations of OSCC, each exhibit distinct functional behaviors, and as a result, prognoses and response to therapy can vary. The understanding

of the specialized mechanisms and processes within the various tumor subpopulations may provide hope towards personalized treatment approaches also in the case of patients with OSCC.[32,33].

With the leading-edge techniques of Spatial Transcriptomics (ST) and Spatial Metabolomics (SM), we can finally examine transcriptomic, proteomics and metabolomic landscapes. The homogeneous and heterogeneous mechanisms of the origin of OSCC have been clarified by researching in the field of transcriptional and metabolomic changes in the progression from oral precancerous lesions to OSCC[34,35]. Specific expression patterns can be observed in key enzyme molecules related to lipid, amino acid and glucose metabolism, indicating metabolic reprogramming in OSCC[36]. Therapies targeting metabolic pathways may also prove effective against tumors[37].

Epigenetic modifications are likewise a key manifestation of molecular heterogeneity. Modifications to histones and DNA methylation/demethylation can upregulate or suppress the expression of proto-oncogenes and tumor suppressor genes, thereby influencing perineural invasion (PNI) and contributing to adverse tumor prognoses[38]. Research has demonstrated through multi-omics approaches that oral microbiota can mediate the occurrence of OSCC by epigenetically activating DNA promoter methylation[39]. It has also been demonstrate that CAFs can regulate DNA methylation level, promote angiogenesis in OSCC[40]. MicroRNAs (miRNAs or miRs), a prevalent class of short non-coding RNAs, serve as crucial regulatory factors in epigenetics. As epigenetic modulators, miRNAs can bind to complementary target sequences in mRNA, induce mRNA degradation and interfering with the translational process, thereby

preventing or altering the translation of the resultant protein product, without modifying the underlying gene sequences[41]. A novel circRNA-miRNA-mRNA regulatory axis which significantly contributes to oral cancer progression and malignancy has been identified through omics profiling[42].

2.4 Immune Microenvironment Heterogeneity

Tumor immune microenvironment (TIME) of OSCC is characterized by changes in immune factors (tumor and matrix), checkpoints and cell populations that benefits immunosuppression, allowing tumor immune evasion[43,44]. The principal regulatory molecules within the immune microenvironment are secreted by both tumor cells and heterogeneous immune cells, modulating tumor behavior through their interactions while concurrently remodeling the extracellular matrix (ECM) and influencing the morphology and function of the immune microenvironment. These critical immune cells and signaling pathways along with their associated receptors and signaling pathways display heterogeneous expression patterns within tumors, thereby posing both challenges and opportunities for cancer therapeutics.

Among these signaling pathways, certain key molecules have garnered particular attention, such as Interleukin-6 (IL-6) and IL-10, Programmed Death-Ligand 1 (PD-L1) and Transforming Growth Factor-β (TGF-β)[45]. The impact of TGF-β on TIME in OSCC has been extensively studied. It is a multifunctional cytokine secreted by CAFs, TAMs and regulatory T cells (Tregs) within the OSCC TME[46]. By regulating behavior of downstream cells in TME, such as M2 polarization of macrophages, It is reported to exert immunosuppressive effects and promote tumor immune evasion and progression in OSCC at different stages[47–49]. Meanwhile, it is

demonstrated that formation and remodeling of ECM is also modulated by TGF-β[50]. This process, intended for tissue repair, can become uncontrolled with the excessive accumulation of collagen, fibronectin, hyaluronic acid and proteoglycans during chronic and persistent inflammation, thereby creating conditions for tumorigenesis[50,51]. Concurrently, TGF-β exhibits heterogeneous secretion across different tumor subclones and stromal compartments, thereby mediating pathways such as EMT and tumor-ECM communication. These pathways are widely recognized as key potential therapeutic targets for tumor growth, therapeutic resistance and invasive capacity[52–55]. Targeted inhibition of TGF-β signaling holds promise for restoring anti-tumor immunity[56].

3 Spatial Omics and Application in OSCC

OSCC is a heterogeneous malignant neoplasm characterized by complex molecular pathways and diverse genetic features, and this diversity poses a great challenge in researching and managing this cancer[57]. Traditional omics approaches suffer from the loss of spatial information[58]. Therefore, spatial omics has emerged, enabling the in situ visualization of cellular interactions and heterogeneity while preserving spatial context of tissues, thereby elucidating how tissues function or malfunction within tumors[59].

From spatially resolved transcriptomics (Method of the Year 2020 by *Nature methods*[60]) to spatial proteomics (Method of the Year 2024 by *Nature methods*[61]), spatial omics is emerging as a pivotal driving force in life sciences research. Technological revolutions in Immunohistochemistry (IHC), In Situ Hybridization (ISH) and NGS-based methods are

advancing spatial omics to the subcellar level, offering greater flexibility across the detection of spatial information regarding gene and protein expression, genetic mutations, epigenetic marks, chromatin structure, and genome organization[59,62–65].

The general workflow involves applying specialized probes to a prepared tissue section to bind target molecules. These molecules are identified through methods like next-generation sequencing (NGS) or mass spectrometry, and their original coordinates are used to reconstruct a detailed spatial map (Figure **2**).

Figure **2**.

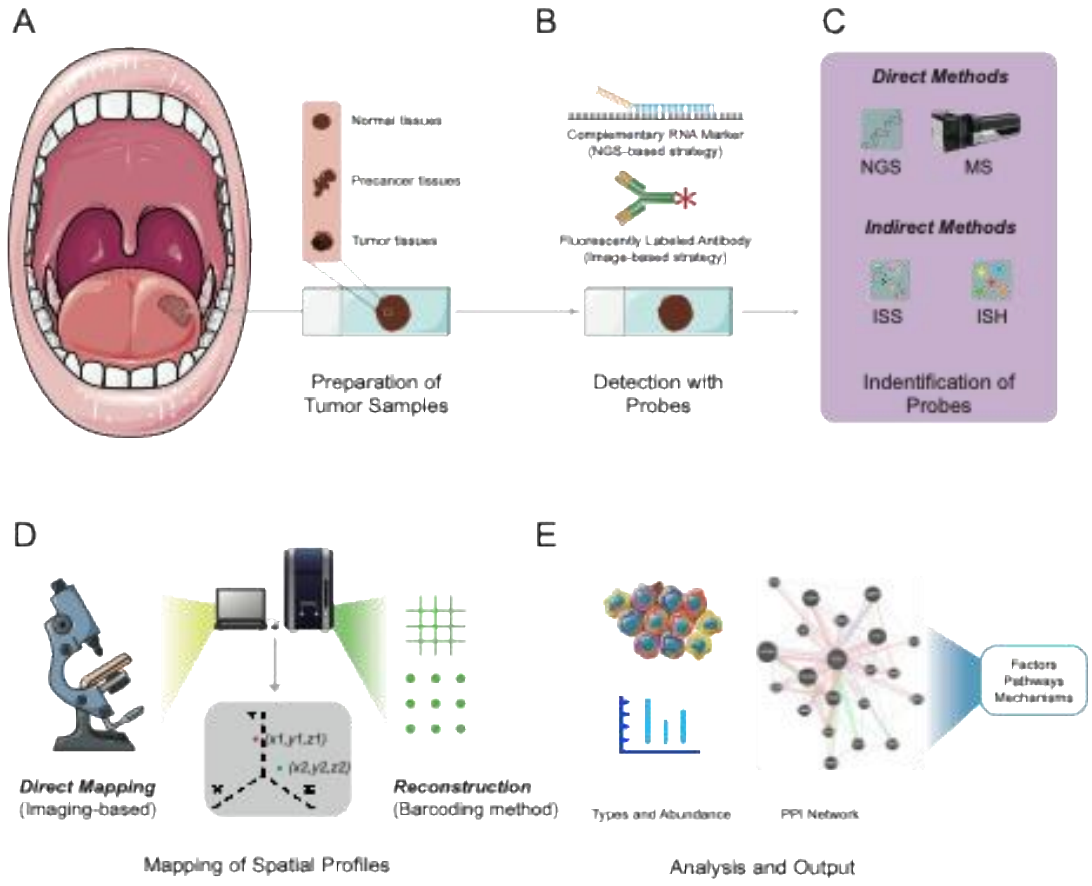

Figure 2. Workflow for Studying Tumors by Spatial Omics

(A) Tumor sample preparation. For studying heterogeneity, including normal/precancerous and tumor tissues is needed. **(B)** Probe-based detection of expression profiles.

Oligonucleotide probes (barcode-encoded at one end) or fluorescently labeled antibodies bind specific nucleic acids, proteins, or small molecules. **(C)** Identification of bound molecules. Use next-generation sequencing (NGS) or mass spectrometry (MS) for direct detection, or indirectly via individual probe markers. **(D)** Spatial mapping. Imaging methods yield direct images; sequencing methods reconstruct patterns from pre-acquired coordinates. **(E)** Analysis and output. Quantify/validate metrics like biomolecular abundance, regulatory functions, and protein-protein interaction (PPI) networks to uncover key factors, pathways, and mechanisms in tumor behavior.

Note. PPI network generated using the GeneMANIA platform[66]. No additional permission required for academic use, as per GeneMANIA website.

This spatially resolved data allows for comprehensive analysis of biomolecular abundance, regulatory pathways, and interaction networks, thereby elucidating how tissues function or malfunction within tumors. Thus, spatial omics has now turned into the most effective tool for scientists[60,61].

From bulk omics to high-resolution spatial mapping, driven by advancements in imaging and sequencing, spatial omics has developed branches such as transcriptomics, proteomics, and metabolomics.

3.1 Spatial Transcriptomics

Spatial transcriptomics (ST) is born with the goal of mapping every gene and gene isoform, at subcellular resolution, in a whole tissue sample with spatial dimensions. Though none of the techniques can reach satisfactory detection levels across all metrics in terms of sensitivity, resolution, throughput or ease of use, we can still obtain our desired data by balancing between the choice of techniques[67].

3.1.1 Spatial Transcriptomics Strategies

(1) Next-generation sequencing (NGS)-based strategies are designed to capture transcriptomic information within tissue by tagging reads originating from different

Platform	Visium	Visium HD	Slide-seqV2	Stereo-seq
Developer/ Company	10x Genomics	10x Genomics	Broad Institute	BGI
Principle/ Mechanism	Array-based spatial barcoding with poly-T capture for mRNA; NGS readout.	Enhanced array-based barcoding; probe-based chemistry with CytAssist for probe transfer; NGS readout.	Bead-based puck array; high-density beads with barcodes; optimized library prep for sensitivity; NGS readout.	Chip-based nanometer-scale barcoding; high-density array; NGS readout.
Spatial Resolution	55 μm feature size (spots).	2 μm square pixels (higher fidelity).	~10 μm feature size (near cellular).	0.22–1 μm feature size (nano-scale).
Throughput/ Gene Detection	Whole transcriptome (>20000 genes); medium throughput (millions of reads per slide).	Whole transcriptome (>20000 genes); high throughput (millions of reads improved capture).	Whole transcriptome; high sensitivity (~50% RNA capture efficiency ~45000 UMIs per array).	Whole transcriptome (>20000 genes); ultra-high throughput (billions of reads per cm^2).
Sample Compatibility	Fresh frozen and FFPE (with CytAssist).	FFPE; adaptations for fresh.	Fresh frozen	Fresh frozen; adaptations for FFPE.
Key Advantages	Versatile for fresh/FFPE; simple workflow; integrates with histology; no special instruments beyond NGS.	Superior spatial fidelity vs. standard Visium; minimizes diffusion; complements imaging tools; unbiased discovery.	High detection efficiency (10x over original Slide-seq); excels in rare transcript detection and trajectory analysis.	Highest resolution among sequencing-based; large-area coverage; cost-effective for scale; ideal for heterogeneous tissues.
Key Limitations	Lower resolution (multi-cellular per spot); diffusion artifacts; requires downstream deconvolution for single-cell insights.	Higher cost; requires CytAssist instrument; data processing intensive for large datasets.	Limited to fresh tissues; bead synthesis complex; lower scalability for very large areas; computational demands for indexing.	Massive data volumes require advanced computing; sample prep complexity; potential overkill for low-res needs.

locations with short sequences carrying spatial information (spatial barcodes/IDs, similar

Table 1. Comparison of sequencing-based spatial transcriptomics methods.

These four technologies primarily rely on NGS for whole-transcriptome profiling while capturing spatial information through array or bead-based barcoding. They differ in

resolution, throughput, sample compatibility, and sensitivity, with advancements focusing on higher spatial fidelity and RNA capture efficiency.

to the labels on goods in shops). Transcriptomic information is captured within the tissue through complementary base pairing of barcodes and RNAs in tissue, and transcriptomic information is stored as the base sequence information reverse-transcribed from barcodes into cDNA. Following sequencing of cDNA, the information is computationally re-attributed to its original spatial location based on these barcodes.

Technologies including Visium[58], Visium HD[68], Slide-seqV2[69], and Stereo-seq (Table **1**) are currently the most widely applied NGS-based strategies. These techniques each offer advantages in areas such as user-friendliness and formalin-fixed paraffin-embedded (FFPE) compatibility, or sensitivity and ultra-high resolution. This diversity requires choice for research needs, whether in clinical applications or precision studies.

(2) Image-based strategies rely on the combination of transcriptomic data with fluorescent labelling signals in situ without reconstruction. The primary approach involves acquiring spatial information using fluorescent labels paired with transcripts. These methods are chiefly categorized as in situ sequencing (ISS) and in situ hybridization (ISH). ISS employs in situ amplification to enhance signals, though it suffers from low sensitivity and difficulties in reading long-chain signals. ISH relies on pre-set complementary sequences to capture specific transcriptomic content. While its analyzed items are clearly defined, it is typically restricted to predefined gene panels.

The imaging-based technologies Xenium, CosMx, STARmap PLUS[70], and MERFISH[71] (Table 2.) provide true single-cell/subcellular resolution, but their respective pros and cons also require researchers to make their own choices.

Platform	Xenium	CosMx	STARmap PLUS	MERFISH
Developer/ Company	10x Genomics	NanoString	Broad Institute (Wang et al.)	Harvard/Vizgen (MERSCOPE)
Principle/ Mechanism	ISS-based with padlock probes rolling circle amplification (RCA) and cyclic fluorescent imaging.	ISH-based with gene-specific probes and optical signatures; cyclic hybridization.	ISS-based with in situ sequencing and amplification; enhanced for larger panels.	ISH-based with multiplexed error-robust FISH; binary encoding for probes.
Spatial Resolution	Subcellular (<1 μm; transcript-level).	Subcellular (<1 μm; xyz coordinates).	Subcellular (~1 μm; 3D capable).	Subcellular (<1 μm; high-plex imaging).
Throughput/ Gene Detection	Targeted (up to 5K genes); high cell throughput (~100000–millions per run).	Targeted (up to 6K genes including non-coding); medium-high throughput (~100000 cells)	Targeted (~1K genes); very high cell throughput (up to millions).	Targeted (hundreds to 1K genes; customizable up to 1K).
Sample Compatibility	Fresh frozen and FFPE.	Fresh frozen and FFPE.	Fresh frozen; tissue sections.	Fresh frozen and FFPE.
Key Advantages	Large sample area (full slide); shorter runtimes (~half of CosMx); reliable high-quality data; integrates with Visium.	Larger panels; good for multi-tissue; automatic data to cloud (AtoMx); identifies major cell types well.	High plex for ISS; excellent for 3D brain mapping; integrates with spatial imaging; cost-effective for large tissues.	Error-robust (binary coding); fully customizable panels (no design fees); open platform for any species; good for subtypes despite lower counts.
Key Limitations	Limited to pre-designed panels (human/mouse focus); longer prep; data access via instrument.	Longer runtimes (2x Xenium); preselect ROIs (limited area); higher noise potential; custom design fees; data export required via subscription.	Limited public data/customization; lower plex vs. ISH peers; requires specialized analysis for massive datasets.	Lower sensitivity/specificity in some cases; limited to predefined panels; longer imaging times; challenging annotation without markers.

Table 2. Comparison of imaging-based spatial transcriptomics technologies.

These technologies focus on in situ hybridization (ISH) or sequencing (ISS) for subcellular resolution but with targeted gene panels (hundreds to thousands). They emphasize direct visualization of transcripts in tissue context, differing in plexity, runtime, and customization.

Below is a structured comparison.

Due to the significant differences between NGS-based and imaging-based strategies, researchers must make intentional choices for different study subjects. Generally speaking, NGS-based methods hold the ability to provide unbiased genome-wide coverage, but its relatively low resolution is its limitation, making it relatively suitable for exploring gene expression profiles at scale and capturing the overall level of cellular expression within a region. In contrast, imaging-based techniques such as MERFISH, allowing for subcellular resolution, are better suited for hypothesis validation, for example, to precisely locate a specific subpopulation of immune cells in comparison to cancerous stem cells. However, the reliance on a panel of predefined genes restricts its region of interest (ROI). This situation, of course, is also changing with advances in technology. For example, the resolution of Visium HD is now at the subcellular level as well, which provides more choices with the disadvantage of a higher cost. An approach that combines the strengths of the diverse approaches might be applying them at different stages of the research.

3.1.2 Application of Spatial Transcriptomics in OSCC

Spatial transcriptomics technology captures cellular transcriptomic profiles while preserving the integrity of the tissue architecture, thereby revealing the cellular behaviors and heterogeneity of OSCC, which can be further translated into guidance for clinical applications.

Spatial distribution of gene expression patterns, such as differences between upregulated genes in tumor core and leading edge architectures revealed the spatial underpinnings of heterogeneity in tumor and TME, allowing us to predict drug targets, evaluate therapeutic responses and to further predict patient survival rates[72].

Identification of cellular subpopulations and their composition, including cancer cells, immune infiltrating cells, and stromal components such as tumor-associated fibroblasts (CAFs)[35]. CAF subsets contribute to tumorigenesis, metastasis, and therapy resistance by remodeling ECM, promoting angiogenesis, secreting growth factors and cytokines, and suppressing anti-tumor immunity[73]. Researches have also demonstrated the tumor-suppressing role of other CAF subsets, suggests that cellular heterogeneity provides diverse and complex patterns for tumor function[74]. In OSCC, spatial transcriptomic has found that CAFs can interact with epithelial cells which located at the tumor's stromal front, causing oxidative stress overload[75]. It is also reported that CAFs promoting cancer cell metastasis through EMT[25]. The in-depth exploration of CAFs is not just a glimpse of tumor biological basis. Furthermore, by uncovering and comparing various signaling pathways, the promising targets for improving patient prognosis will be identified. By understanding the mechanisms of CAF action, characteristic diagnostic clues will be recognized, thereby contributing to the development of personalized treatments in precision medicine.

Ligand-receptor interaction networks, highlighting crosstalk between tumor and microenvironmental cells, form the basis for tumor communication with the external environment[76]. A protein kinase RNA-like ER kinase (PERK)-signaling cascade is highlighted, as a key sensor in the unfolded protein response, in driving metabolic reprogramming and epigenetic changes in TAMs via the *PERK-ATF4-PSAT1* axis[77]. By targeting the unfolded protein response relevant *PERK/activating transcription factor 4 (ATF4)* signaling pathway, tumor control in both primary tumors and lymph node metastases may be achieved, and the direct identification of these potential targets provides guidance for the development of

targeted therapeutics. [78].

3.2 Spatial Proteomics

Spatial proteomics enables the measurement of protein abundance, distribution, and interactions within tissue sections, with overall characteristics similar to spatial transcriptomics. In OSCC, it revealed tumor heterogeneity, tumor-stroma interactions, and signaling pathway activations, offering insights into mechanisms of progression, metastasis, and therapeutic resistance[79].

3.2.1 Spatial Proteomics Strategies

(1) The concept of imaging-based strategies, previously introduced in transcriptomics, is similarly applied to proteomics. Compared to the short RNA sequences mentioned in transcriptomics, referred to as Barcodes, the approach here relies more upon antibody (as the binding part) and fluorescent protein molecules (as the indicator). This involves utilizing ISS/ISH techniques with antibody-based or aptamer-based probes conjugated with fluorescent, oligonucleotide, or metal tags to detect proteins.

Iterative fluorescence methods enable multiplexed protein imaging through cyclic antibody staining, imaging, and signal quenching/bleaching to reuse channels. The binding parts (antibodies) are repeatedly replaced, but the biological information is retained for measurement. Owing to distinct marker characteristics (such as fluorescent color) and powerful computational processing capabilities, a single experimental round can detect a substantial number of antibodies. IBEX employs bleaching for >65 parameters with quick 2-5 day runs and open-source flexibility, though bleaching may

compromise tissue integrity. CycIF combine bleaching with chemical quenching for cost-effective, non-destructive workflows on FFPE tissues but risks cumulative signal loss. Oligonucleotide-tagged methods, such as the upgraded CODEX platform PhenoCycler-Fusion (PCF), barcode antibodies for hybridization and detection, supporting >100 biomarkers while preserving samples for multi-omics integration. PCF is an automated high-plex phenotyping technology that requires lengthy multi-day protocols and increased costs. Mass spectrometry (MS)-based spatial proteomics maps protein distributions by labeling antibodies with some form of stable isotopes or metals, ablating tissue regions, and analyzing ionized peptides/proteins using MS for quantitative, interference-free detection at single-cell resolution (~1 μm).

Imaging Mass Cytometry (IMC) functions as scanners (such as the one in the supermarket) that uses UV laser to carefully burn off tiny spots on tissue samples labeled with special metal-tagged antibodies. The measurements of these are then made by a process known as time-of-flight mass spectrometry (up to ~40 markers). Its benefits incorporate strong capability to analyze TME, great reproducibility and easy interpretation of data. Disadvantages include a fixed resolution that is approximately of ~1 μm, inability to rescan samples, and high cost of equipment.

In Multiplexed Ion Beam Imaging (MIBI), particles on metal-tagged tissue samples are gently knocked off by means of a focused beam of ions, and the resulting secondary ions can be analyzed, supporting tunable resolution (30-50 nm in HD mode) and multiple scans (~100 targets). The main advantages include high subcellular resolution, the ability

to see the whole-tissue image, and reduction of the artifacts in the fluorescent images. Disadvantages include long acquisition times, high cost of tags and instruments and increased complexity of data.

3.2.2 Application of Spatial Proteomics in OSCC

Spatial proteomics has evolved into one of the most powerful tools in precision oncology for OSCC. By preserving spatial context at near-single-cell resolution, it allows clinicians and researchers to answer critical real-world questions that traditional bulk proteomics or IHC cannot address: Which proteins are driving lymph node metastasis in this particular patient's tumor? Why does one region of the tumor respond to anti-PD-1 while the adjacent region is completely resistant? How is the tumor stroma (especially CAFs) being reprogrammed to promote invasion and treatment resistance? Can we identify patients who will truly benefit from immune checkpoint inhibitors or EGFR-targeted therapies based on spatially resolved biomarker patterns?

In the field of spatial mapping of protein markers and signaling pathway activation profiles, new spatial proteomics technologies are continually being developed and applied, studies on OSCC and head & neck squamous cell carcinoma (HNSCC) have provided direct clinical insights.

For instance, one study applied digital spatial profiling with an immune pathway panel to mucosal HNSCC samples. It demonstrated regional differences in immune checkpoint molecules like PD-1/PD-L1, LAG-3, and TIM-3, along with activation markers such as Granzyme B and Ki-67, as well as immunosuppressive cytokines across the tumor core,

invasive margin, and stroma[80]. This helped explain varying responses to PD-1 blockade by revealing hot, cold, and excluded immune phenotypes within the same tumor.

Another investigation conducted proteogenomic and spatial analysis on primary OSCC and matched metastatic lymph nodes. It found that extracellular matrix remodeling proteins including COL11A1, THBS2, and Tenascin-C secreted by activated CAFs create pre-metastatic niches particularly in cases with lymph node involvement. These matrix signatures were linked to cetuximab resistance and poor prognosis[25].

Furthermore, a research effort integrated mass spectrometry-based spatial proteomics with single-cell transcriptomics in a large OSCC group. It successfully divided patients into three practical subgroups: an immunogenic type with high PD-L1, CD8, and IFN-γ signaling that responds well to anti-PD-1; a stromal-rich or desmoplastic type with elevated TGF-β and CAF markers that resists immunotherapy but may benefit from TGF-β inhibitors; and a metabolic or Epidermal Growth Factor Receptor (EGFR)-driven type suitable for EGFR monoclonal antibodies or combined approaches[81].

In addition, emerging panoramic ultra-high-resolution techniques have been employed in glioma samples and are now adapting to OSCC. These techniques map gradients of EGFR, phospho-EGFR, and related signaling from the tumor center to the invasive front, potentially aiding in predicting responses to anti-EGFR therapy prior to treatment[82].

Moreover, spatial proteomics is revealing that TP53 gain-of-function mutations in non-HPV OSCC actively shape an immunosuppressive TIME through downregulation of interferon-stimulated genes and T-cell chemokines. This explains the disappointing response of TP53-

mutated OSCC to checkpoint inhibitors and supports ongoing trials combining PD-1 blockade with STING or type I IFN pathway agonists[83].

3.3 Spatial Metabolomics

Spatial metabolomics is a research method designed to meet the need for assessing metabolites and metabolic conversion rates, given the fact that metabolic rewiring is a critical component of tumor progression[36].

3.3.1 Spatial Metabolomics Strategy

Mass spectrometry imaging (MSI) is a label-free technique that can spatially map hundreds of metabolites and drugs directly from a tissue section[84]. Nowadays, MS techniques are commonplace in cancer research. Matrix-assisted laser desorption ionization (MALDI) has been applied for imaging peptides and proteins in biological samples for many years[85,86]. Subsequently, the development of desorption electrospray ionization (DESI) technology has established MSI as a powerful technique, capable of spatial visualization of molecular like amino acids, proteins, lipids, polysaccharides and oligonucleotides[87,88]. Beyond the fundamental identification and quantification of metabolites, MS can also utilize stable non-radioactive isotope labeling (such as ^{13}C, ^{2}H, or ^{15}N) to assess the impact of nutrients and metabolic pathways on specific observed metabolic changes[86,89]. Moreover, ideas for improving MS continue to be proposed and applied. Previously, the integration of 3D MALDI MSI data with H&E and IHC images was reconstructed from 162 consecutive human OSCC tissues, leading to a better insight into functional heterogeneity of tumors[90].

3.3.2 Application of Spatial Metabolomics in OSCC

For OSCC, metabolomics-based assessment of tumor heterogeneity can assist clinical treatment across multiple critical stages, including prevention, diagnosis, therapy, and prognosis. The spatial metabolomics characteristics of oral submucous fibrosis (OSF)-derived OSCC reveals metabolic reprogramming procedure, confirming remarkable metabolic differences between amino acid metabolism (in malignant epithelial region, associated with poorer prognosis in patients) , galactose metabolism, lipid metabolism and ABC transporters (in OSF and stroma regions)[35]. During diagnosis, spatial metabolomics can also distinguish tumor margins from normal mucosa by detecting variations in characteristic amino acids and fatty acids and fatty acid esters of hydroxy fatty acids, thereby aiding pathological diagnosis and surgical planning[91,92]. Meanwhile, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), a key enzyme in the generation of collagen cross-links, has been demonstrated as a poor prognostic biomarker for OSCC and may affect the metastasis through EMT pathway, examined by MS and IHC[93]. Additionally, distinct metabolic profiles were observed across different regions of OSCC tumors, revealing characteristics such as elevated metabolic rates between the tumor center and invasive margin. This suggests that activated purinergic signaling may serve as a potential target for therapeutic strategies targeting TME[94].

3.4 More Spatial Omics Technologies and Application

The above three spatial omics technologies discussed in this review are not all inclusive; more highly valuable spatial omics approaches exist for uncovering additional insights into OSCC. Spatial epigenomics and microbiomics offer profound value in OSCC research by unveiling

spatially resolved molecular and microbial landscapes that drive tumor heterogeneity, progression, and therapeutic resistance.

Spatial epigenomics, leveraging technologies such as spatial Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) and Cleavage Under Targets and Tagmentation (CUT&Tag), bring chromatin accessibility to the field and spatially maps epigenetic modifications like DNA methylation across tumor genes, offering new opportunities to investigate epigenetic regulation, cell function, and fate decision in normal physiological processes and pathological mechanisms[95,96].

Spatial microbiomics has experienced an evolution from traditonal methods and 16S rRNA sequencing to metagenomics and spatial genomics. We can combine spatial transcriptomics like 10x Visium and GeoMx digital spatial profiling to reveal spatial, cellular and molecular host–microbe interactions[97]. It described the patterns of microbial community distribution in OSCC TME, highlighting dysbiotic interactions that exacerbate inflammation, immune evasion, and metastasis. These interactions can inform microbiome-modulating interventions to enhance immunotherapy efficacy and prognosis.

A combination of these technologies enables precise investigation of tumor ecosystems, particularly in heterogeneous malignancies like OSCC.

4 Multi-Omics and Application

4.1 Integration of Multiple Tumor Analysis Methods

The technologies we employ to detect tumor heterogeneity are continually evolving. On a cellular population basis, we can examine the mean gene expression phenotype of whole

tissues using bulk analysis[98]. On the other hand, analysis of single cells enables us to distinguish between cell subtypes[99]. Today, spatial omics permits us to understand the spatial patterns of gene expression within cells (or the patterns of cell-ECM interactions) at the subcellular level. Although these levels of omics exhibit a progression in terms of resolution, each of them has its own benefits and compliments functionally. The integration of these multi-level approaches often yields synergistic effects where the whole is greater than the sum of its parts.

This principle remains valid for spatial omics technologies. Spatial transcriptomics reveals potential functional instructions, which could be seen as the cell's “action plan”; Spatial proteomics maps cellular effectors, analyzing the “actual execution” of cellular functions. What sparks might their collision create?

As discussed above, the methods by which spatial proteomics and transcriptomics are technically performed with similarities, making it possible for the parallel analysis. In practice, platforms like Nanostring GeoMx DSP can already profiles expression of RNA and protein from distinct tissue compartments and cell populations, creating more convenience for the application of multi-omics methods[100].

Moreover, the combination with epigenomics help focus on principles of gene regulation; the integration of spatial metabolomics would produce a more detailed account of biological transformation; and the incorporation of microbiomics enables an understanding of how external factors influence TME. Spatial heterogeneity of those fields illustrates that there are bottlenecks and mismatch regulation areas in the process of transitioning genetic instructions

to functional protein expression and on to metabolism, a process of genotype to phenotype.

To summarize, single cell level methods are limited to provide information of just one element of the biological processes, compared to multi-omics that can provide multidimensional view of different facets of the biological processes. Multi-omics help us gain a systems level perspective of biological phenomena and processes

4.2 Application of Multi-Omics in OSCC

Integration of multi-omics not only bridges the information gap between transcription, translation and effection but also plays a crucial role in analyzing TME, discovering biomarkers, and designing precision treatment strategies.

4.2.1 Exploration of TME

In the present day, innovative multi-omics technologies can give deep insights into TME, focusing on intercellular and cell-matrix interactions, as well as matrix remodeling and metabolic status. By bulk and single-cell omics screening the region of interest and uncover tumor behaviors, we can identify distinct cellular subtypes in OSCC, which may exhibit distinct levels of genetic variation, malignancy, and poor prognosis.

Subsequently, the Caveolin-1 (CAV-1+) epithelial subtype can be shown by spatial omics to engage in an interaction with T cells through the Nectin Cell Adhesion Molecule 1- Cluster of Differentiation 96 (NECTIN1-CD96) signaling network, which could cause immune evasion[101]. Furthermore, spatial omics methods were employed to define the EMT process, a critical mechanism in oral cancer progression, invasion and metastasis[25]. Key cells, pathways, and molecules that influence EMT activation through oxidative stress signaling were revealed[75].

4.2.2 Identification of Biomarkers and Signaling Pathways

Overall, the focus of tumor research remains on developing, evaluating, and refining treatment strategies, which inherently relies on the identification of specific signaling molecules or pathways.

Clinicians can tackle real-world challenges in precision therapy, such as how can we overcome resistance to ferroptosis inducers or metal-dependent cell death pathways that drive cancer stem cell survival and metastasis. One validated CD44-targeted therapy using mP6/Rg3 micelles promote ferroptosis in cancer stem cells by inhibiting ATP Binding Cassette Subfamily B Member 1 (ABCB1), improving OSCC pathology[102]. The other confirmed that this mechanism correlates with lymph node metastasis and resistance to both immunotherapy and anti-*EGFR* targeted therapies. This research further identified Doramapimod as a potential therapeutic candidate[103].

Other questions on the way: why do some OSCC patients develop rapid resistance to immunotherapy, anti-EGFR agents, or cisplatin, and how can we predict this pre-treatment, as revealed in research on cisplatin-resistant cells that showed associations with EMT, inflammatory signaling, and metabolic adaptation, suggesting adjunctive strategies like allyl isothiocyanate to inhibit proliferation and induce apoptosis, while also linking metal-dependent resistance to these therapy failures[104].

Which immune or metabolic regulators in TME could serve as actionable targets to enhance invasion control and overall survival as demonstrated in a study on the C-C Motif Chemokine Ligand 26 (CCL26) protein within TME, indicating its role in tumor cell invasion while also

investigating its interactions with other immune cells[105]. Additionally, the epigenetic factor miR-181a-5p and its regulatory pathways in lipid metabolism were also identified through multi-omics analysis, suggesting its role in maintaining oral mucosal homeostasis[106].

To improve the results of combination therapy, can we attempt to map spatial-based prognostic models and refine them individually to achieve the desired outcomes? A recent systematic analysis examined six major mechanisms of OSCC: inflammation, proliferative signaling, immune regulation, oxidative stress, angiogenesis, and epigenetic regulation. The study focused on relevant signaling pathways and targets, discussing the efficacy of various therapeutic approaches[107], along with discovering that Transcription Factor 7+ (TCF7+) T cells were significantly associated with increased survival rates in OSCC, contributing to the adjustment and evaluation of tumor therapy efficacy.[108]

In general, multi-omics is transforming the general level into personal care, decoding the TME dynamics and speeding up the process of biomarker-based interventions, overcome resistance, and improve patient prognosis.

5 Conclusions and perspectives

In conclusion, spatial omics is an important tool for analyzing OSCC and other cancers and gives a clear picture of the heterogeneity of the tumors. Regarding TME-tumor cell interactions, OSCC research has developed a mature workflow model for research on similar diseases such as head and neck cancer, lung cancer, and breast cancer. In the protein/gene expression field, it identifies varied patterns in the expression and assists in the identification of biomarkers for prognosis and immunotherapy in OSCC. On pathways, it reveals important

pathways such as EMT-oxidative stress and PERK-ATF4 in OSCC growth, suggesting therapies that can be applied to metabolism alterations in other solid cancer types. These approaches have been widely used in oncology as well as non-oncology spheres and will certainly be applied in areas of research that have not yet been examined. In the oral cavity, metabolomics and spatial proteomics may be used to study the pattern of secretion in salivary glands, which can guide the treatment of diseases like Sjögren's syndrome. The complexity of the oral microbiome environment can be compared to TME, implying that spatial multi-omics can also be potentially useful in studying its heterogeneity.

Despite continuing progress, spatial omics has critical challenges regarding clinical translation. Spatial omics is characterized by flaws such as a lack of standardization, such that results are difficult to compare across research or implement in clinical settings. In the case of oncology, validation of spatial biomarkers for OSCC immunotherapy or prognosis requires further trials, yet because of the high cost and complexity of data, this cannot be produced.

For standardization, challenge is to integrating data across scales and modalities. Vertically, combining bulk omics (which averages signals across tissues), single-cell omics (which captures individual cell details but loses location), and spatial omics (which adds positional context) is tough. The disparities between resolutions and data types result in difficulties matching the multi-omics approaches, leading to the inability to align them without losing accuracy or adding bias. Horizontally, the combination of transcriptomics (gene expression), proteomics (protein levels), metabolomics (metabolite profiles), and epigenomics (DNA modifications) complicates integration because different forms of data are used, and they have

different dimensions and biological layers. This scenario requires complex computational models to put them in a mutually consistent perspective of OSCC heterogeneity.

In the case of spatial transcriptomics in particular, low throughput and high costs are major problems. Experiments such as 10x Visium or MERFISH can cost thousands of dollars per sample, and this prohibits routine experiments or large cohorts. Also, throughput is limited, and many techniques process hundreds to thousands of genes or cells at once and therefore are slower than bulk sequencing for performing extensive studies. These issues complicate scaling up for OSCC research because labs cannot cope with the cost and time.

Spatial omics needs to bridge the gap between innovative science and everyday clinical use by overcoming significant challenges of standardization and cost-effectiveness. Implementing robust standardization is paramount. This includes enacting elaborate Standard Operating Procedures (SOPs) for sample processing, especially for clinically relevant FFPE tissues, to achieve data consistency. In addition, cross-study comparisons as well as meta-analyses require the adoption of a common data format, which incorporates expression matrices, spatial coordinates, and histology images.

At the same time, it is critical to make it more affordable and scalable for more extensive use. Feasible measures include developing lower-cost, non-proprietary technologies for probe synthesis to reduce reagent costs. By taking advantage of sample multiplexing methods, including the deployment of Tissue Microarrays (TMAs), one can analyze a large number of different samples in a single run, which can reduce the costs per sample by a large margin. Moreover, automating laborious processes through robotics may reduce reagent waste and

maximize throughput, which makes large-scale clinical studies more achievable and also brings the transition of the technology to the field of diagnostics a step closer, as seen in how the Assist version of the Visium platform has been refined.

Moving forward, spatial omics, being an exciting new technology, will allow us to venture into more frontiers that have not been well charted. Combining multi-omics and AI analysis will allow real-time spatial mapping and dynamic monitoring of individual cells to reveal the heterogeneity of OSCC tumors and mechanisms of TME evolution.

List of abbreviations

ABCB1, ATP Binding Cassette Subfamily B Member 1
ABC transporters, ATP-Binding Cassette Transporters
ATAC-seq, Assay for Transposase-Accessible Chromatin with Sequencing
ATF4, Activating Transcription Factor 4
AVCs, Angiogenic Vascular Cells
CAFs, Cancer-Associated Fibroblasts
CAV1+, Caveolin-1 Positive
CCL26, C-C Motif Chemokine Ligand 26
CD44/96, Cluster of Differentiation 44/96
CODEX, Co-Detection by Indexing
CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR-Associated Protein 9
CSCs, Cancer Stem Cells
CUT&Tag, Cleavage Under Targets and Tagmentation
DESI, Desorption Electrospray Ionization
ECM, Extracellular Matrix
EGFR, Epidermal Growth Factor Receptor
EMT, Epithelial-to-Mesenchymal Transition
ER, Endoplasmic Reticulum
FFPE, Formalin-Fixed Paraffin-Embedded
FISH, Fluorescence In Situ Hybridization
GLUT-1, Glucose Transporter 1
H&E, Hematoxylin and Eosin
HIF-1α, Hypoxia-Inducible Factor 1-Alpha
HNSCC, Head and Neck Squamous Cell Carcinoma
HPV, Human Papillomavirus
IHC, Immunohistochemistry
IICs, Infiltrating Immune Cells
IL-6/10, Interleukin-6/10
IMC, Imaging Mass Cytometry
IPA, Immuno-Oncology Proteome Assay
ISH, In Situ Hybridization
ISS, In Situ Sequencing
MALDI, Matrix-Assisted Laser Desorption Ionization
MERFISH, Multiplexed Error-Robust Fluorescence In Situ Hybridization

MIBI, Multiplexed Ion Beam Imaging
miRNAs/miRs, MicroRNAs
MS, Mass Spectrometry
MSI, Mass Spectrometry Imaging
NECTIN1, Nectin Cell Adhesion Molecule 1
NGS, Next-Generation Sequencing
OSCC, Oral Squamous Cell Carcinoma
OSF, Oral Submucous Fibrosis
PCF, PhenoCycler-Fusion
PD-1, Programmed Cell Death Protein 1
PD-L1, Programmed Death-Ligand 1
PERK, Protein Kinase RNA-like Endoplasmic Reticulum Kinase
PLOD2, Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 2
PNI, Perineural Invasion
PPAR, Peroxisome Proliferator-Activated Receptor
PSAT1, Phosphoserine Aminotransferase 1
PSERP, Panoramic Spatial Enhanced Resolution Proteomics
scATAC-seq, Single-Cell Assay for Transposase-Accessible Chromatin with Sequencing
scRNA-seq, Single-Cell RNA Sequencing
SM, Spatial Metabolomics
ST, Spatial Transcriptomics
STAT3, Signal Transducer and Activator of Transcription 3
TAMs, Tumor-Associated Macrophages
TCF7+, Transcription Factor 7 Positive
TGF-β, Transforming Growth Factor-Beta
TIME, Tumor Immune Microenvironment
TME, Tumor Microenvironment
TP53, Tumor Protein 53
Tregs, Regulatory T Cells
UMI, Unique Molecular Identifier

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