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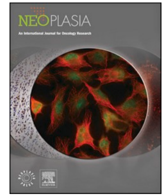

Review Article

Advanced progress of spatial metabolomics in head and neck cancer research

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ABSTRACT

Head and neck cancer ranks as the sixth most prevalent malignancy, constituting 5 % of all cancer cases. Its inconspicuous onset often leads to advanced stage diagnoses, prompting the need for early detection to enhance patient prognosis. Currently, research into early diagnostic markers relies predominantly on genomics, proteomics, transcriptomics, and other methods, which, unfortunately, necessitate tumor tissue homogenization, resulting in the loss of temporal and spatial information. Emerging as a recent addition to the omics toolkit, spatial metabolomics stands out. This method conducts *in situ* mass spectrometry analyses on fresh tissue specimens while effectively preserving their spatiotemporal information. The utilization of spatial metabolomics in life science research offers distinct advantages. This article comprehensively reviews the progress of spatial metabolomics in head and neck cancer research, encompassing insights into cancer cell metabolic reprogramming. Various mass spectrometry imaging techniques, such as secondary ion mass spectrometry, stroma-assisted laser desorption/ionization, and desorption electrospray ionization, enable *in situ* metabolite analysis for head and neck cancer. Finally, significant emphasis is placed on the application of presently available techniques for early diagnosis, margin assessment, and prognosis of head and neck cancer.

Introduction

Head and neck cancer comprises a heterogeneous group of diseases [1–3], encompassing malignancies in the oral cavity, nasal cavity, tonsil, paranasal sinuses, pharynx, larynx, thyroid, and salivary glands [4–6]. Predominantly, squamous cell carcinoma (head and neck squamous cell carcinoma, HNSCC), arising from oral mucosal epithelium (oral cavity squamous cell carcinoma, OCSCC), oropharynx (oropharyngeal squamous cell carcinoma, OPSCC), hypopharynx, and larynx (laryngeal squamous carcinoma, LSCC), constitutes 90 % of cases. The remaining 10 % include lymphomas, adenocarcinomas, and sarcomas originating from lymphocytes, connective tissue cells, or salivary glands [7]. Globally, HNSCC stands as the sixth most prevalent cancer, with over 887,000 new cases annually and an estimated 450,000 deaths [8]. Its complex risk factors contributing to high morbidity and mortality involve long-term betel nut or tobacco chewing, local irritation, smoking, alcoholism, human tumor virus infections, etc. [9,10]. Current HNSCC treatments encompass surgery, radiotherapy, chemotherapy, biological therapy, and others, though despite significant technological advances, patient prognosis remains relatively stagnant. For instance, OCSCC exhibits a mere 50 %–60 % 5-year survival rate [11]. Seeking treatment in advanced clinical stages is a common scenario among OCSCC patients, detrimentally impacting prognosis [12]. Consequently, cancer prevention strategies focus on "three early" measures: early detection, diagnosis, and treatment, with early diagnosis proving pivotal in prognosis improvement.

Currently, clinical diagnosis of head and neck cancer utilizes blood tests, ultrasound, computed tomography, magnetic resonance imaging, and biopsy. Biopsy, a standard diagnostic method, remains invasive, time-consuming, and potentially metastasis-promoting [13]. Hence,

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uncovering molecular mechanisms underpinning head and neck cancer onset holds paramount importance for early diagnosis and prognosis enhancement. Presently, cancer biomarker studies primarily employ genomics, proteomics, transcriptomics, and related methods, which necessitate tissue homogenization and compromise spatiotemporal data [14–16].

The extensive heterogeneity of head and neck cancer presents challenges in molecular-level analysis. Comprehending molecular changes mandates considering chemical information and molecule spatial distribution within the tumor microenvironment. Spatial metabolomics, an emerging metabolomics realm, adeptly retains tissue specimen spatial information [17]. This methodology yields insights into tumor microenvironment pathobiology, revealing spatial metabolite expression patterns.

Advancements in spatial metabolomics have elucidated cellular and molecular mechanisms driving head and neck cancer occurrence and progression [18]. Differentially expressed biomolecules, such as proteins, lipids, and metabolites, between tumor and normal tissues may evolve into diagnostic, prognostic, and therapeutic markers for head and neck cancer [19–21]. Spatial metabolomics, rooted in *in-situ* mass spectrometry, primarily contributes to analytical chemistry research. In recent years, scholars have extended its application to life science research [22]. Hence, this paper comprehensively reviews spatial metabolomics' progress via *in-situ* mass spectrometry in head and neck cancer investigation.

Metabolic reprogramming of cancer cells

Cancer cells necessitate nutrients for metabolic reprogramming to sustain their growth and proliferation, which is a hallmark of cancer [23]. This metabolic shift leads to altered pathways, ensuring adequate ATP production for survival and rapid proliferation in the nutrient-deprived tumor microenvironment, thus facilitating tumor occurrence and progression [24]. Notably, cancer cell metabolism significantly diverges from normal cells. Thus, spatial metabolomics, facilitating qualitative, quantitative, and spatial analyses of metabolites, along with differential metabolite identification and screening, holds potential for early cancer diagnosis. Metabolic abnormalities primarily encompass changes in glucose, amino acid, and lipid metabolism (Fig. 1), which help cancer cells manage energy deficits, heightened oxidative stress, apoptosis, metastasis, and immune evasion [25].

An early and well-established metabolic alteration in cancer cells is increased glucose consumption [26]. Otto Warburg et al. observed elevated glucose uptake and lactic acid production, even in oxygen's presence, in cancer cells compared to normal cells [27]. Many cancer cells adopt glycolysis to generate ATP despite ample oxygen, known as the "Warburg" effect [28]. This underscored that cancer cells experience metabolic changes. Consequently, they rely on the "Warburg" effect's ATP and metabolic intermediates to fuel biological macromolecule synthesis, promoting their survival, growth, and reproduction [29]. Signaling pathways altered in cancer often impact glucose metabolism through various mechanisms. For instance, insulin or growth factor-induced receptor tyrosine kinases (RTKs) activate the PI3K-AKT pathway, boosting glycolysis [30]. The Myc pathway similarly enhances aerobic glycolysis and regulates glutamine biosynthesis [31]. Pyruvate, a glycolysis end product, tends to accumulate in cancer cells and can be converted to lactic acid by lactate dehydrogenase [32]. Excessive lactic acid creates an acidic microenvironment, fostering cancer cell proliferation and invasion [33]. High lactic acid levels in HNSCC correlate negatively with overall patient survival [34]. Thus, pyruvate and lactic acid, glycolytic metabolites, offer diagnostic potential.

Current recognition extends beyond dysregulated glucose metabolism; cancer cells also increase uptake of other nutrients to support their survival, growth, and invasion, such as glutamine [35]. Cancer cells enhance amino acid synthesis, decomposition, and transport to

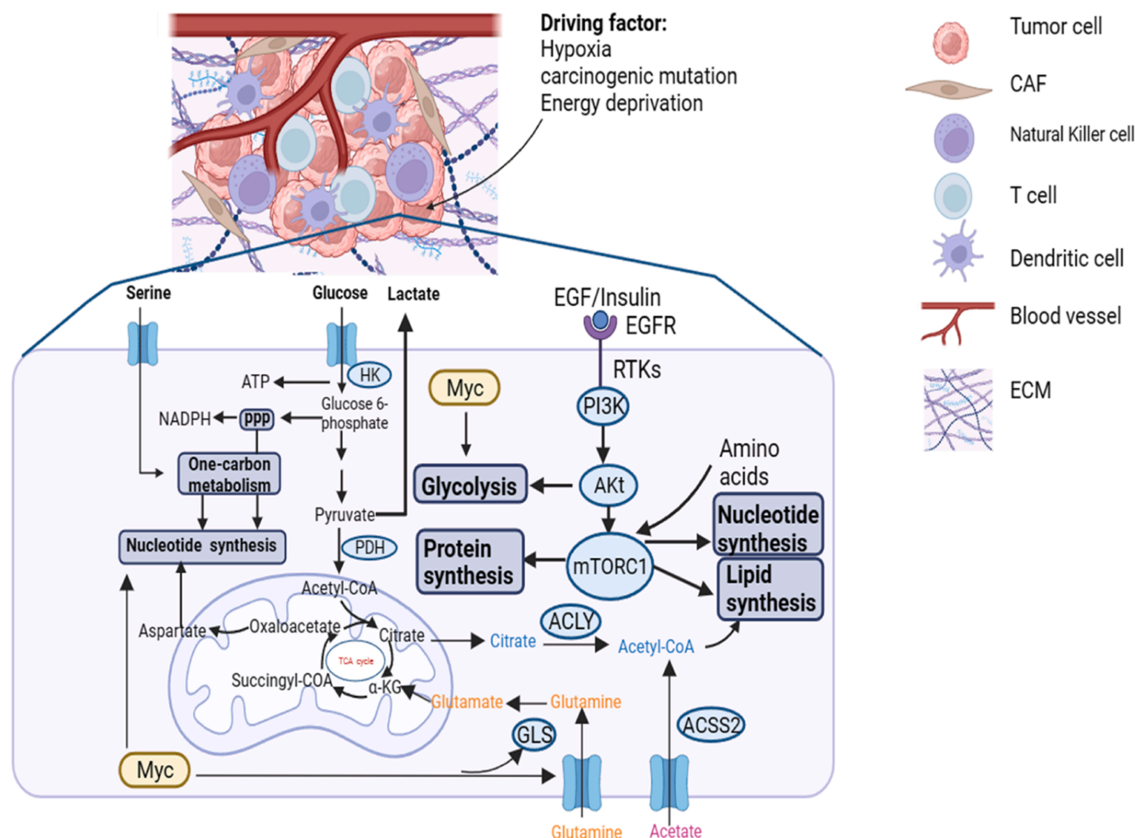

Fig. 1. Tumor cells undergo metabolic reprogramming of glucose, lipids, and amino acids in response to complex pressures. Figure created with biorender.com.

provide energy, maintain REDOX balance, homeostasis, and enable protein and lipid synthesis [36]. Carcinogenic MYC is tied to heightened glutamine breakdown, inducing glutamine addiction, whereas the PI3K/AKT pathway does not affect glutamine uptake and metabolism [31,37]. Rapid cancer cell growth demands more glutamine than they can produce internally, necessitating uptake via transporters from the extracellular environment. In OSCC, numerous studies confirmed that cancer cells' distinctive amino acid uptake and utilization. For example, the up regulation of sodium-dependent neutral amino acid transporter type 2 (ASCT2 also termed as SLC1A5) has been observed by the researchers, and reducing glutamine consumption by inhibiting ASCT2 inhibited OSCC proliferation and tumor growth, highlighting glutamine's significance [38,39]. While recent research revealed that even high glucose and glutamine consumption may fall short of sustaining cancer cell survival. Additional amino acids, like serine, vital for cancer cell survival, provide carbon and nitrogen units [40,41].

Lipids serve energy storage, metabolism, signal transduction, and biofilm constituents [42–44]. Altered lipid metabolism in cancer is increasingly acknowledged [45–47]. Elevated lipid levels, including fatty acids and cholesterol, are evident in cancer tissues, with specific cancers exhibiting lipidomic alterations [48]. Reprogrammed lipid metabolism's relevance to tumor progression underscores lipid metabolites' and lipidomics have great diagnostic potential [49,50]. While metabolomics has been extensively used in cancer research, traditional methods homogenize tissue samples, leading to spatial metabolite distribution loss. In this context, a technique for *in situ* tissue sample analysis, spatial metabolomics, is urgently needed.

Current spatial metabolomics methods in cancer research

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI), secondary ion mass spectrometry (SIMS-MSI), and desorption electrospray ionization mass spectrometry (DESI-MSI) are prominent techniques for visualizing the *in-situ* spatial distribution of biomolecules. By concurrently capturing diverse detectable chemical substances and furnishing distinct spatial distribution insights, mass spectrometry imaging (MSI) proves a robust approach. These three methods have optimally utilized their unique attributes in biological and clinical head and neck cancer research, and Table 1 outlines their distinctive characteristics [51–55].

Secondary ion mass spectrometry (SIMS-MSI)

SIMS involves bombarding the sample surface with high-energy primary ions, triggering energy absorption by surface molecules and subsequent ejection of secondary ions. Mass analyzers then collect and analyze these secondary ions, generating surface information maps for the sample [56]. In contrast to MALDI or DESI techniques, SIMS mandates ultra-high vacuum conditions for sample analysis [57]. Renowned for its exceptional spatial resolution, SIMS empowers the imaging of tissues, single cells, and microorganisms at micron and submicron levels [58–60]. Offering spatial resolutions as fine as tens of nanometers, SIMS-MSI surpasses MALDI and DESI, rendering it particularly apt for single-cell imaging [61]. SIMS can be coupled with diverse mass analyzers, including Time-of-Flight (ToF) and Fourier Transform Ion Cyclotron Resonance (FTICR), thereby augmenting its ability to scrutinize intricate biological systems [62]. Just as in the case of MALDI and DESI, SIMS-MSI holds widespread utility in cancer research, encompassing studies on colon cancer, breast cancer, medulloblastoma, and others [63–65].

Table 1
The characteristics of SIMS-MSI, MALDI-MSI and DESI-MSI.

MSI methods	SIMS-MSI	MALDI-MSI	DESI-MSI
Ionization conditions	Ultrahigh vacuum	Vacuum	Ambient
Spatial resolution	Down to 50-100 nm	Down to 1μm	Down to 10μm
Destructive nature	Maximally destructive	Minimally destructive	Minimally destructive
Sample preparation	Freeze fracture and drying	Matrix deposition	Minimum

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI)

Among the various spectral ionization techniques for direct tissue analysis, MALDI stands as a prevalent choice due to its well-balanced attributes encompassing sample preparation, chemical sensitivity, and spatial resolution [66]. Spatial resolution represents a leading edge in MALDI-MSI advancement, now achieving 10-micron resolution, often adjustable to around 1 micron [67]. Addressing MALDI's sensitivity, the development of MALDI-2 emerged, boosting sensitivity for specific molecular species by up to 100 times through the incorporation of a secondary laser [68,69]. Operating within a vacuum, MALDI-MSI employs a substrate for analyte desorption/ionization. The substrate promotes analyte-substrate co-crystallization on the sample surface, with laser irradiation triggering analyte eutectic energy absorption, ultimately leading to evaporation and ionization [70]. The substrate constitutes a key challenge in MALDI-MSI, influencing spatial resolution and analytical sensitivity. Substrate application is thus a focal point in sample preparation. Notably, ion inhibition due to uneven or inadequate substrate deposition poses a significant drawback [71]. Despite ongoing efforts to address ion inhibition, this issue remains complex and unresolved in MSI.

Given its nondestructive nature, MALDI-MSI preserves tissue integrity, allowing subsequent HE staining and ion images to be correlated with histopathology, enhancing molecular distribution evaluation [72]. This property enables MALDI-MSI to be used independently and in conjunction with patient clinical data, thereby enhancing clinical diagnosis accuracy by combining metabolic information with clinical context.

Desorption electrospray ionization mass spectrometry (DESI-MSI)

DESI-MSI generates charged spray droplets through nitrogen assistance, impacting the sample surface to simultaneously ionize the charged solvent and analyte [73]. Unlike traditional mass spectrometry (MS) imaging methods like MALDI, DESI operates under atmospheric pressure, sidestepping labeling, matrix assistance, and intricate sample preparation [74]. Regarded as a low-tissue-damage MSI technique, DESI-MSI allows repeated imaging of the same tissue section and subsequent histopathological analysis [75,76]. DESI excels in analyzing lipids and small molecule metabolites within biological tissues under environmental conditions. However, enhancing DESI-MSI's sensitivity has posed a substantial challenge. Wang et al. introduced trifluoroacetic acid to the DESI spray solvent, resulting in approximately 21-fold and 62-fold signal intensity increases for cholesterol and other metabolites in mouse brain tissue, respectively [77]. Recent research indicated that nano DESI-MSI sensitivity and specificity could be enhanced by introducing silver ions to the solvent [78].

Moreover, DESI's spatial resolution, typically around 50-200 μm, presents a notable challenge [79]. Efforts to address this limitation include utilizing nano DESI-MSI to achieve finer spatial resolution, even as low as 10 μm [80–82]. These studies underscore the potential future advancement of DESI-MSI.

Application of spatial metabolomics in head and neck cancer research

As the metabolic reprogramming of head and neck cancer becomes increasingly recognized, its identification can be achieved through the detection of altered metabolites. Spatial metabolomics technology bears

significant value in clinical contexts, encompassing the identification of histopathological characteristics, exploration of metabolic markers, early disease diagnosis, assessment of tumor metastasis potential, and facilitation of drug discovery [51,83]. Through the provision of intricate molecular insights, this approach enables the discovery of novel biomarkers and potential therapeutic targets, ultimately paving the way for advancements in cancer diagnosis and treatment [84] (Fig. 2, Table 2).

Application of spatial metabolomics in head and neck cancer diagnosis

The subtle onset of head and neck cancer often leads to advanced stages upon treatment initiation, underscoring the imperative of early detection and precise diagnosis to enhance the 5-year survival rate. Spatial metabolomics proves instrumental in achieving early diagnosis by scrutinizing distinct metabolite signatures within tissue specimens, allowing for timely intervention to impede cancer cell growth. This technology's value extends to various clinical applications, including identification of histopathological characteristics, pursuit of metabolic markers, early disease diagnosis, assessment of metastatic potential, and acceleration of drug discovery [51,83]. By furnishing intricate molecular insights, spatial metabolomics facilitates the discovery of novel biomarkers and potential therapeutic targets, thus propelling innovations in cancer diagnosis and treatment [84].

Spatial metabolomics' effectiveness in early diagnosis has been demonstrated across other cancers as well, such as prostate cancer, glioma, and breast cancer. Notably, Prostate cancer is the second most common type of cancer worldwide and a common cause of cancer-related death in men [85], Livia S. Eberlin et al. utilizing DESI-MSI technology to identify highly expressed cholesterol sulfate in cancerous and precancerous tissues, signifying a prostate cancer biomarker [86]. Shibdas Banerjee et al. utilized DESI-MSI to assess prostate cancer tissue specimens, detecting significant reductions in tricarboxylic acid cycle intermediates and revealing distinctions in glucose/citrate ratio distribution between cancerous and normal areas [87].

In glioma biopsies, assessing the percentage of tumor cells is achieved through the measurement of a single metabolite, N-acetylaspartate (NAA). Recent research by Cooks' group introduced additional biomarkers to enhance DESI-MS's accuracy in distinguishing glioma from brain parenchyma [88]. This comprehensive metabolic analysis identified diagnostic metabolites distinguishing normal brain tissue from glioma tissue, including GABA, creatine, glutamate, carnitine, and hexanol. GABA, carnitine, and creatine demonstrated sensitivities exceeding 90 % and specificities surpassing 80 %, enabling the distinction of healthy tissue from glioma tissue. The inclusion of hexanol exhibited promising discriminating abilities as well [88].

Moreover, DESI-MSI application extended to invasive breast cancer (IBC) and ductal carcinoma *in situ* (DCIS) differentiation, with Adriana Leandra Santoro et al. leveraging DESI-MSI to identify lipid composition differences between these breast cancer subtypes and adjacent benign tissue [89]. In the specific context of head and neck cancer, spatial metabolomics' potential for early diagnosis is still nascent. Eduardo Sommella et al. employed MALDI-MSI to distinguish healthy tissue areas from tumor tissue areas in patients with salivary gland tumors, revealing

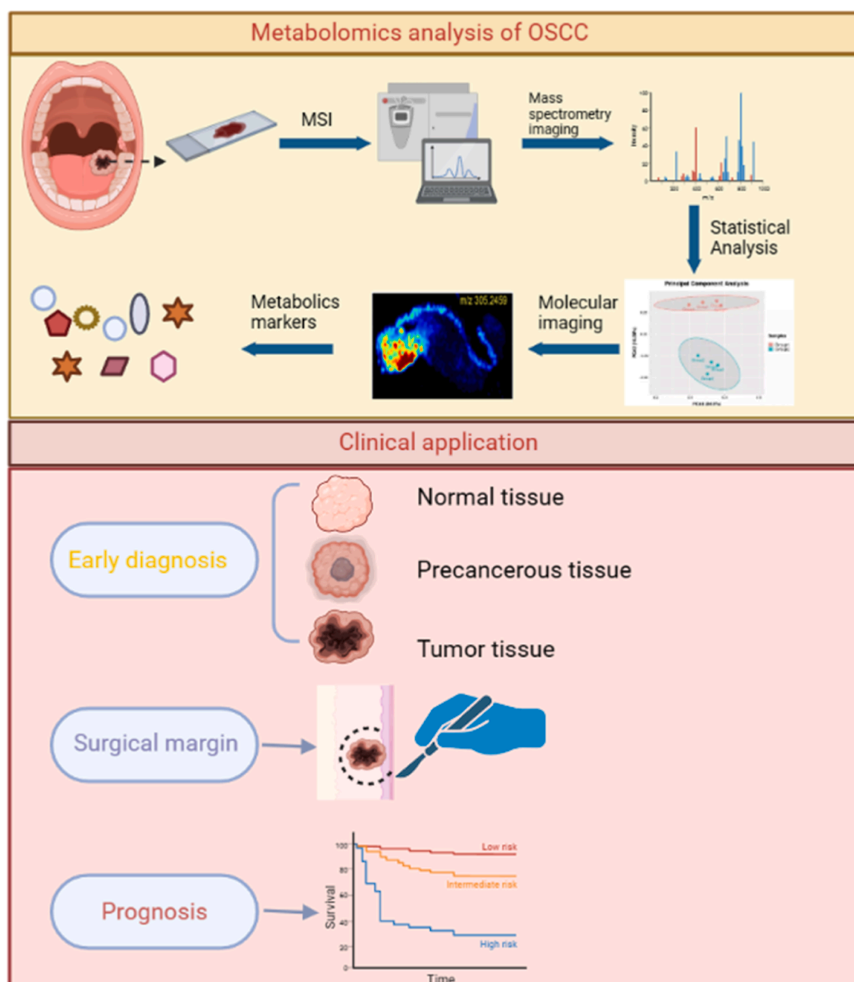

Fig. 2. Spatial metabolomics flow chart and clinical application of head and neck cancer. Figure created with biorender.com.

Table 2
Detection of metabolites by situ mass spectrometry for the study of human cancer.

Cancer type	Technique	Differential Metabolites	Refs.
Prostate cancer	DESI-MSI	Cholesterol sulfate.	[86]
	DESI-MSI	erythrose, glutamate, Glucose, sorbitol, succinate, malate, salicylate, aconitate, citrate.	[87]
Gliomas	DESI-MSI	GABA, creatine, glutamate, carnitine, hexanol.	[88]
breast cancer	DESI-MSI	glutamine, glutamate, taurine, uric acid, ascorbic acid, glutathione.	[89]
	DESI-MSI	Phosphatidylinositols, oleic acid.	[99]
Gliomas/ meningiomas	DESI-MSI	plasmenylethanolamines, Phosphatidylinositols, Phosphatidylserines.	[95]
	DESI-MSI	2-Hydroxyglutaric acid, Phosphatidylinositols.	[96]
Stomach cancer	DESI-MSI	cardiolipin, unsaturated fattyacid, Glycerophosphoethanolamine, glycerophosphoserine,glutamate, Palmitoyl glycine, glutamine.	[97]
pancreatic cancer	DESI-MSI	oleic acid, palmitic acid, arachidonic acid.	[98]
salivary gland tumor	MALDI-MSI	lysophosphatidylcholine, sphingolipid, fatty acid, phosphatidylcholine, phosphatidyl ethanolamine, glutamine, glutamate.	[90]
oral cancer	MALDI-MSI	phosphatidylcholine, sphingolipid, acylglycerol.	[92]
	DESI-MSI	Cholesterol sulfate, oleic acid.	[93]
	DESI-MSI	fatty acid esters of hydroxy fatty acids , fatty acid.	[101]

altered metabolites related to the tumor's energetic demands [90].

Addressing squamous cell carcinoma's tissue heterogeneity, Franziska Hoffmann et al. employed MALDI-MSI to uncover characteristic m/z values differentiating tumor and non-tumor regions [91]. Comparably, Katarzyna et al. contrasted lipid and protein biomarkers through MALDI mass spectrometry analysis to effectively differentiate oral cancer from normal mucous membranes, highlighting the suitability of both domains as biomarkers [92]. Cedric D'Hue et al. applied DESI-MSI alongside spectral principal component analysis and linear discriminant analysis to accurately distinguish oral tongue squamous cell carcinoma from adjacent normal epithelium [93].

Application of spatial metabolomics for intraoperative imaging of head and neck cancer margins

Acquiring a well-defined "clear" surgical margin presents a significant challenge, being a primary contributor to local recurrence occurrences. Consequently, the establishment of a safe surgical margin distance is imperative to curbing postoperative recurrence rates and enhancing patient survival rates. Conventional frozen section histology remains a prevalent technique for intraoperative tissue diagnosis. However, it necessitates the expertise of a skilled pathologist for microscopic evaluation, introducing subjectivity into the results. In contrast, spatial metabolomics technology demonstrates the ability to effectively differentiate between cancer tissue and normal tissue, thereby determining diverse incisal margin states, through comprehensive metabolite profile analysis. This innovative approach has found gradual application in the evaluation of cancer surgical margins [94].

Notably, the research conducted by Cooks' group achieved successful differentiation of distinct brain tumor types using DESI-MSI, consequently assessing brain glioma resection margins during surgery [95, 96]. Livia S. Eberlin et al.'s pioneering work utilized DESI-MSI to rapidly identify differential metabolites between tumor tissue, normal tissue, and tumor-adjacent tissue (incisal margin tissue) during gastric cancer surgery, showcasing the promising clinical application potential of this technology [97]. Further explorations by Eberlin et al. successfully employed DESI-MSI/Lasso to distinguish pancreatic cancer from normal tissue, thereby aiding in pancreatic cancer surgical margin assessment [98]. In a parallel endeavor, David et al. utilized DESI-MSI to discriminate between normal and breast cancer tissue in mastectomy samples from 14 patients, evidencing the technique's capability for surgical margin determination [99].

Initially, our research group utilized GC-MS and UHPLC-MS/MS metabolomics technology to pinpoint amino acid metabolite combinations as markers for negative and abnormal hyperplasia incisal margins in oral squamous cell carcinoma [100]. Despite their utility, these techniques exhibited limitations in capturing tissue spatial characteristics and precisely determining safe margin distances. Subsequently, through DESI-MSI, we identified 14 lipid molecular composition diagnostic models capable of discerning tumor from normal tissue, as well as distinguishing various incisal margin states [101]. Among these, 9 lipid molecules emerged as negative margin markers for OSCC, while 1 lipid molecule stood as a positive margin marker. This advancement implies that clinicians can establish DESI-MSI molecular diagnostic models to guide surgical decision-making, thereby reducing patient postoperative recurrence rates in OSCC treatment. Consequently, the application of spatial metabolomics holds great promise in elevating the precision of diagnosis and treatment for oral and maxillofacial head and neck tumors, ultimately improving patient prognoses.

Application of spatial metabolomics in head and neck cancer prognosis

In the realm of cancer research, the application of classical metabolomics techniques has proven instrumental in discovering predictive and prognostic markers. Florian N Loch et al. [102] successfully employed MALDI-MSI to discern specific peptides significantly associated with adverse prognostic parameters, including lymphatic vascular invasion (pL), lymph node metastasis (pN), and vascular invasion (pV, $p < 0.001$), in pancreatic cancer patients. This study exemplifies the feasibility of MALDI-MSI in identifying peptide signatures corresponding to prognostic indicators of pancreatic cancer. While limited studies explore the implementation of spatial metabolomics in the prognosis of head and neck cancer, with most confined to oral cancer prognosis, noteworthy advances have emerged. Agata Kurczyk et al. [103] evaluated the prognostic potential of intratumoral molecular heterogeneity in primary oral cancer and lymph node metastasis through mass spectrometry imaging. They revealed that higher phenotypic heterogeneity detected in excised tumor tissues of locally advanced oral cancer patients was associated with better prognosis. Similarly, Yao et al. [104] utilized MALDI-based proteomics to assess differential expression of peptides/proteins between oral squamous cell carcinoma tissues and adjacent normal tissues, identifying 5 protein expression alterations. Notably, they identified an up-regulation of LRP6 expression in oral squamous cell carcinoma, correlating it with various clinicopathological parameters such as smoking, alcohol consumption, tumor differentiation status, lymph node metastasis, and survival time. This study proposes LRP6 as a potential biomarker for oral squamous cell carcinoma, holding promise for guiding treatment approaches.

Challenges and perspective

In recent years, the exploration of early cancer diagnosis and treatment via spatial metabolomics has emerged as a prominent research focus, yielding notable achievements in both early diagnostic efficacy and clinical applications. On one hand, the identification of specific cancer-associated metabolites facilitates the timely diagnosis of tumors,

enabling prompt therapeutic interventions and tumor progression control. On the other hand, pinpointing distinctive metabolites within tumor cells allows for effective differentiation between tumor and normal tissues. This differentiation, in turn, facilitates precise surgical resection margins, curbing postoperative recurrence, and ultimately enhancing patient survival rates. Furthermore, the potential biomarkers found in cancer tissue offer the prospect of accurately prognosticating patient outcomes. However, while DESI spatial metabolomics holds immense promise, it faces challenges stemming from high costs, intricate procedures, and stringent technical demands. As an emerging field, DESI spatial metabolomics must navigate these obstacles to ensure its widespread utilization in the realm of early cancer diagnosis and treatment.

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CRediT authorship contribution statement

Huiting Zhao: Writing – original draft, Investigation. **Chaowen Shi:** Writing – review & editing. **Wei Han:** Supervision. **Guanfa Luo:** Software. **Yumeng Huang:** Supervision. **Yujuan Fu:** Visualization. **Wen Lu:** Visualization. **Qingang Hu:** Validation, Writing – review & editing. **Zhengjun Shang:** Funding acquisition. **Xihu Yang:** Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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