

# Research Progress in Multimodal Guidance for Brain Tumor Surgery and Preservation of Functional Brain Networks

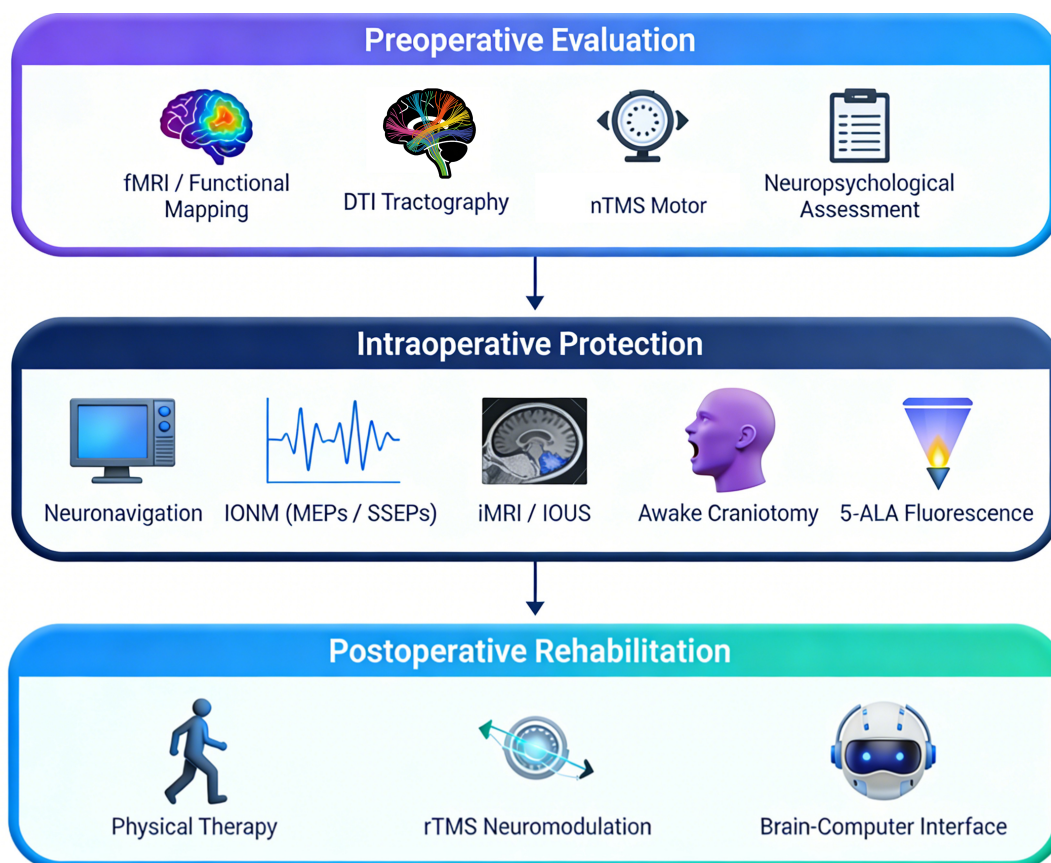
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## Graphical Abstract



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# Research Progress in Multimodal Guidance for Brain Tumor Surgery and Preservation of Functional Brain Networks

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## Abstract

The evolution of brain tumor surgery has progressed from "microsurgery" to "precision neurosurgery," currently entering a new stage characterized by the deep integration of multimodal technologies and the concept of preserving functional brain networks. This article systematically reviews the current state of research on preserving brain functional networks during brain tumor surgery. It summarizes the composition of brain functional networks, the impact of brain tumors on these networks, the classification of multimodal technologies, and the application progress of protecting key functional networks during brain tumor surgery. It emphasizes that precision surgical strategies based on preserving brain functional networks can significantly improve patient quality of life and provide a new paradigm for translational brain science research. Despite progress, key challenges remain, including interindividual functional variability, intraoperative brain shift, and the limited reproducibility of non-traditional network mapping. Future research should focus on dynamic monitoring of functional networks, long-term functional prognosis correlation analysis, and cross-modal data fusion integrated with artificial intelligence to establish new precision surgery standards guided by multimodal functional network preservation.

**Keywords:** Brain tumor; Multimodal techniques; Functional brain networks; Neuroprotection

## The Need for Functional Brain Network Preservation in the Era of Precision Neurosurgery

The central challenge in brain tumor treatment lies in achieving a balance between maximal tumor resection and the preservation of neurological function. Although traditional anatomy-guided surgery has benefitted from microsurgical innovations, approximately 36%–56% of patients still experience postoperative neurological deficits, including language, motor, or cognitive impairments [1]. With advancements in neuroimaging, neurosurgery has transitioned from the era of "microsurgery" to that of "precision neurosurgery", placing brain function preservation at its core. Research indicates that brain functions rely on the synergistic operation of dynamic networks involving multiple brain regions, driving an evolution in surgical strategy from purely "anatomical resection" towards "network preservation" (Figure 1). This shift is particularly critical in surgeries for tumors located in eloquent areas such as the frontal lobe and basal ganglia [2].

Multimodal technology is not merely a simple image overlay, but rather achieves deep integration of "structural-functional-molecular" information through cross-modal data fusion

algorithms. It constructs personalized brain connectome maps, enabling visualization and quantification of the topological relationships between tumors and functional networks, thereby overcoming the limitations of single-data approaches [3-5]. Such advances have shifted the focus of surgical planning from lesion-centered localization to network-oriented functional preservation [6-7]. In this regard, connectome-based neurosurgery has emerged as an integrative paradigm that explicitly incorporates large-scale brain network organization into surgical decision-making, extending beyond traditional modular brain mapping. In this context, functional brain networks provide the theoretical framework through which multimodal information can be interpreted and applied in precision neurosurgery.

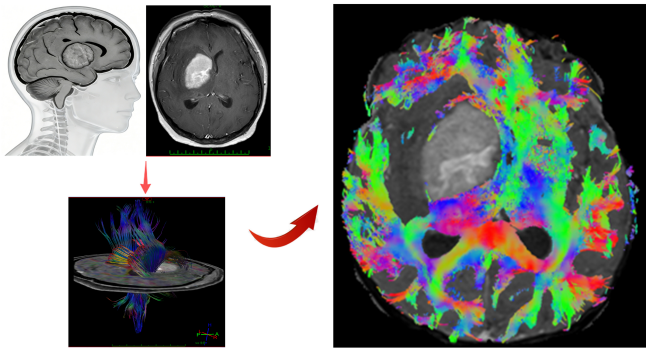
Functional brain networks refer to functionally synergistic systems formed by spatially separated brain regions through the synchronization of neural activity [8-9]. The strength of their connections can be quantified using neuroimaging techniques and supports multi-level functions ranging from basic perception to high-level cognition [10]. However, it should be acknowledged that the definition and reproducibility of functional brain networks—particularly those derived from resting-state fMRI—

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**Figure 1. From anatomical excision to network preservation: an evolving paradigm in brain tumor surgery.**

This figure illustrates the paradigm shift in brain tumor surgery from traditional anatomical resection to modern network-based preservation.

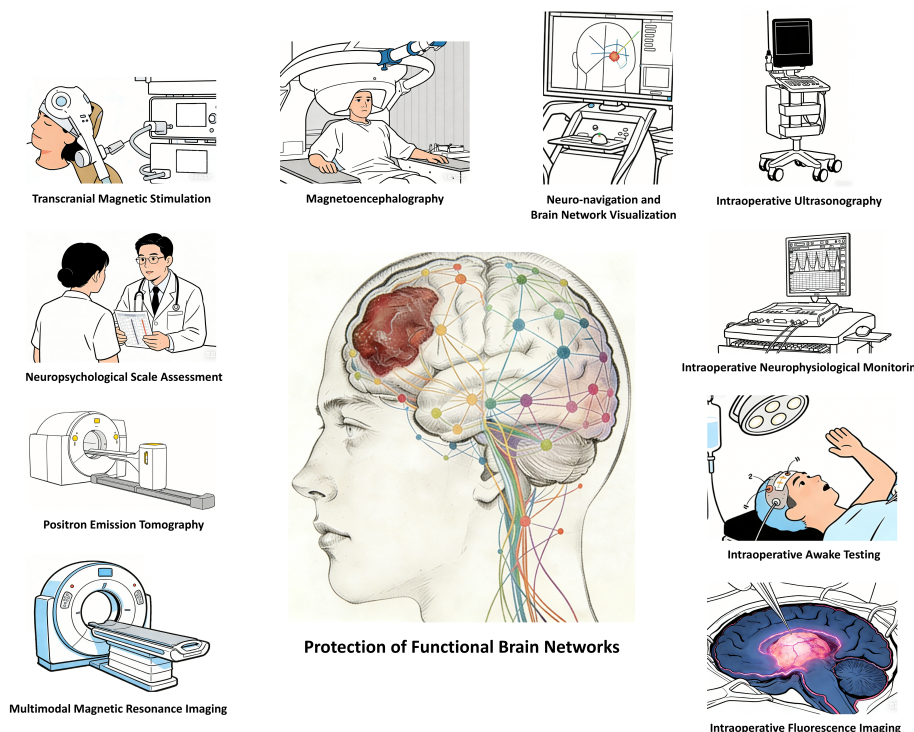
remain subject to ongoing methodological debate. Recent reviews highlight that resting-state fMRI is sensitive to acquisition parameters, preprocessing pipelines, and substantial inter-individual variability, which poses challenges for clinical translation [13-14]. These networks exhibit specific topological properties (e.g., modularity, small-world properties), whose strength and dynamic changes are constrained but not fully determined by underlying structural connectivity, with structure-function coupling itself varying systematically across the cortical hierarchy [2, 11-12]. They are further organized through rich-club architecture—a topological property in which highly connected hub regions (such as the precuneus and superior frontal gyrus) are preferentially interconnected with one another beyond what would be expected by chance, thereby enabling efficient global information integration. Accordingly, the clinical significance of multimodal guidance lies in its

ability to simultaneously characterize these structural and functional dimensions, thereby allowing surgeons to identify critical cortical regions and subcortical pathways more precisely [15]. During brain tumor surgery, combining two types of critical information, static structural connectivity and dynamic task-based activation networks, enables precise localization of critical functional areas and neural fiber tracts. This approach optimizes surgical strategies to preserve brain function and maximize patients' postoperative quality of life [15].

This article systematically reviews the composition of functional brain networks, multimodal protective technologies, and future research directions, aiming to provide a theoretical basis for establishing new surgical protocols grounded in the preservation of functional brain networks, with the ultimate goal of maximizing tumor resection rates and improving patient quality of life.

### Composition of Functional Brain Networks

Functional brain networks are conceptualized as organized assemblies of neural regions defined by the temporal correlation or functional coherence of spontaneous neural activity. Their functional organization is not random, but follows a hierarchically coordinated architecture, with primary sensorimotor systems supporting basic perception and action and higher-order association systems subserving socio-affective, cognitive, and executive functions [16-18]. This hierarchical organization is established early in development and refined through experience, providing the scaffolding upon which complex cognition emerges. Based on their functional attributes, functional brain networks can be broadly categorized into traditional and non-traditional systems. The complex and adaptive operations of the brain arise from the integrative and cooperative interactions among these distributed networks (Figure 2, Table 1).



**Figure 2. Composition and function of brain functional networks.**

This figure depicts the major human brain functional networks and their core functions.

Traditional functional brain networks are directly associated with fundamental sensory and motor functions, primarily including the Sensorimotor Network (SMN), Visual Network (VN), Auditory Network (AN), and Language Network (LangN). In contrast, non-traditional functional brain networks participate in higher-order cognitive, affective, and introspective processes. These include the Default Mode Network (DMN, including the posterior cingulate cortex, precuneus, medial prefrontal cortex, angular gyrus, and parahippocampal gyrus), Salience Network (SN), Central Executive Network (CEN), Limbic Network (LimbN), Dorsal Attention Network (DAN), and Ventral Attention Network (VAN) [19-34]. Although these networks lack clearly defined physical structural boundaries, they are crucial for maintaining cognitive integrity and emotional stability. It should be emphasized, however, that the level of evidence

varies across networks: the DMN has been most robustly identified across individuals and clinical populations, whereas the reproducible identification of SN, CEN, DAN, and VAN in tumor patients remains methodologically challenging. Consensus has not yet been reached regarding which non-traditional networks can be reliably preserved through surgical intervention—an area requiring further empirical investigation.

### Impact of Brain Tumor Invasion on Functional Brain Networks

Brain tumors disrupt network topology and interfere with neural signal transmission, thereby causing deficits in cognition, motor function, and other domains [35-37]. Glioma growth and infiltration exhibit marked network dependency: high-grade gliomas (HGG) have been reported to preferentially invade rich-club hub regions such as the insula and temporal lobe,

Table 1. Composition and functions of functional brain networks.

| Category                                  | Name                            | Constituent Structures                                                                                       | Anatomical Location                                   | Core Functions                                   | Symptoms After Damage                                       |
|-------------------------------------------|---------------------------------|--------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|--------------------------------------------------|-------------------------------------------------------------|
| Traditional Functional Brain Networks     | Sensorimotor Network (SMN)      | Precentral gyrus, Postcentral gyrus, Supplementary motor area                                                | Brain convexity (Frontoparietal lobe)                 | Somatosensory integration, Motor control         | Hemiplegia, Sensory loss, Motor ataxia                      |
|                                           | Visual Network (VN)             | Primary visual cortex (V1), Extrastriate cortex (V2-V5)                                                      | Occipital lobe and temporo-occipital junction         | Visual information processing                    | Hemianopia, Visual agnosia, Metamorphopsia                  |
|                                           | Auditory Network (AN)           | Transverse temporal gyrus (Heschl's gyrus), Superior temporal gyrus                                          | Superior part of temporal lobe                        | Auditory signal processing                       | Auditory agnosia, Tinnitus, Speech comprehension impairment |
|                                           | Language Network (LangN)        | Broca's area (Left inferior frontal gyrus), Wernicke's area (Posterior part of left superior temporal gyrus) | Left hemisphere dominant (Frontotemporal lobe)        | Language expression and comprehension            | Aphasia (Expressive /Receptive)                             |
|                                           | Default Mode Network (DMN)      | Posterior cingulate cortex, Medial prefrontal cortex, Angular gyrus, Parahippocampal gyrus                   | Midline structures (Medial frontal-parietal-temporal) | Introspection, Episodic memory, Social cognition | Autism, Cognitive decline in Alzheimer's disease            |
| Non-traditional Functional Brain Networks | Salience Network (SN)           | Anterior insula, Anterior cingulate cortex, Dorsolateral prefrontal cortex                                   | Fronto-insulo-cingulate circuit                       | Attentional switching, Conflict detection        | Schizophrenia, Executive dysfunction                        |
|                                           | Central Executive Network (CEN) | Dorsolateral prefrontal cortex, Posterior parietal cortex                                                    | Bilateral fronto parietal lobe                        | Working memory, Decision making                  | Attention deficit, Impaired planning                        |
|                                           | Limbic Network (LimbN)          | Amygdala, Hypothalamus, Anterior cingulate cortex, Orbitofrontal cortex                                      | Medial anterior brain                                 | Emotional processing, Stress response            | Anxiety, Depression, Emotional dysregulation                |
|                                           | Dorsal Attention Network (DAN)  | Frontal eye field, Intraparietal sulcus, Superior parietal lobule                                            | Dorsal posterior parietal-frontal lobe                | Goal-directed attention                          | Neglect syndrome, Attention distraction                     |
|                                           | Ventral Attention Network (VAN) | Temporoparietal junction, Ventral prefrontal cortex, Anterior insula                                         | Right ventral temporo-parieto-frontal lobe            | Stimulus-driven attention capture                | Spatial neglect (Right hemisphere damage)                   |

Note: Functional brain networks are divided into traditional and non-traditional systems according to their major functional domains. The constituent structures listed are representative core regions rather than a complete set of anatomical components. The symptoms after damage refer to common manifestations associated with dysfunction of each network, including neurological deficits, cognitive-behavioral disturbances, or disorders in which network abnormalities are prominently implicated

whereas low-grade gliomas (LGG) tend to favor locally densely connected regions in the frontal cortex [38-40]. However, the available evidence is heterogeneous, and not all studies confirm a strict HGG-hub versus LGG-periphery dichotomy; therefore, these patterns should be regarded as probabilistic tendencies rather than absolute rules. This selective infiltration pattern might reflect the biological and connectomic properties of hub-enriched regions, including their high metabolic activity and dense integration within large-scale brain networks [41-42].

At the clinical level, such network-dependent invasion is reflected not only in global connectome disruption but also in focal damage to eloquent cortical regions and subcortical pathways [43]. A multimodal imaging analysis of 115 patients with low-grade glioma (LGG) showed that injury involving the precentral gyrus, middle frontal gyrus, and insula, as well as major white matter tracts such as the arcuate fasciculus and corticospinal tract, was directly associated with reduced language fluency [44]. In addition, among 45 patients with insular glioma, 98% showed tumor proximity to or invasion of higher-order cognitive functional networks, further emphasizing the widespread impact of glioma infiltration on distributed brain systems and the limitations of conventional anatomy-based surgery in achieving true functional preservation [45].

#### **Effects of brain tumor mass effect on functional brain networks**

Brain tumor mass effect alters functional brain networks through both structural distortion and secondary functional reorganization [46-48]. Compression of white matter pathways, edema-related displacement, and disruption of large-scale connectivity can affect key systems such as the CEN and DMN [42]. Benign tumors more often cause displacement of adjacent fiber tracts, whereas aggressive gliomas are more likely to induce substantial tract disruption and broader connectome-level impairment, although these effects should be interpreted as a spectrum rather than a strict binary distinction based on FA alone [49]. In parallel, the brain may undergo compensatory remodeling through perilesional recruitment, inter-hemispheric redistribution, and reconfiguration of hub-related interactions [50].

Importantly, glioma-induced network disruption is not a passive process but involves active glioma-neuron interactions; recent work demonstrates that glioblastoma can disrupt cortical network activity at multiple spatial and temporal scales and remodel neuronal circuits in ways that promote regional immunosuppression [51-52]. Clinically, this means that adaptive remodeling may preserve function in the short term but still leave patients vulnerable to postoperative cognitive decline, especially when higher-order association systems are involved [50].

#### **Significance of Functional Brain Network Preservation**

In brain tumor treatment, the delicate balance between tumor resection and preservation of brain function presents a critical clinical decision-making challenge [53-54]. Preservation of traditional functional brain networks is crucial for postoperative quality of life. Intraoperative neuronavigation and mapping enable maximal safe resection, which has been associated with improved motor and language preservation as well as extended survival in selected glioma cohorts [55-56]. Gliomas

infiltrate functional brain networks; focal resection risks damaging critical white matter tracts (e.g., arcuate fasciculus, corticospinal tract), causing permanent neurological deficits [57]. Traditional functional brain network preservation provides the structural substrate for neuroplasticity, facilitating long-term recovery.

Preservation of non-traditional networks (e.g., default mode network (DMN), salience network (SN)) represents a critical advance toward whole-brain network protection [58-59]. Up to approximately 36%–56% of patients exhibit cognitive deficits attributable, at least in part, to non-traditional network damage, although the precise contribution of individual networks to specific deficits varies across studies [1]. Safeguarding these networks is increasingly considered important to reduce postoperative memory decline and emotional disturbance. Intraoperative preservation of the DMN helps prevent episodic memory impairment, thereby significantly improving postoperative memory retention and the patient's ability for self-care [60].

## **Methods and Techniques for Functional Brain Network Preservation**

### **Preoperative Multimodal Evaluation**

#### **Multimodal Imaging Evaluation**

In preoperative planning for brain tumor surgery, high-resolution structural imaging, functional activation mapping, and diffusion-based white matter tractography should be integrated to define the individualized spatial and network relationships between the tumor and functional brain networks [61-63]. Contemporary network-based frameworks emphasize that multimodal assessment is necessary not only to localize the lesion but also to identify functionally preserved tissue, estimate network vulnerability, and guide maximal safe resection [64-65]. Advanced diffusion imaging and connectome-based analyses can further characterize tumor-related displacement, compression, or infiltration of major white matter pathways, thereby improving patient-specific surgical planning [66]. In addition, individualized functional imaging may reveal preserved sensorimotor and cognitive network organization within or adjacent to tumor tissue, highlighting the value of personalized, network-informed preoperative evaluation [6, 67].

### **Neuronavigation-guided preoperative planning**

Neuronavigation-guided preoperative planning integrates structural MRI, diffusion-based tractography, functional mapping, and, when appropriate, navigated transcranial magnetic stimulation to generate patient-specific anatomy-function maps for surgical planning [68]. In preoperative assessment, these multimodal data help define the spatial relationship between tumors and eloquent cortical and subcortical systems, thereby supporting trajectory design and functional preservation [69-70]. In selected glioma patients, amino-acid PET such as <sup>11</sup>C-MET-PET can further complement MRI by improving delineation of tumor extent and infiltration margins [71]. Together, these approaches support more individualized surgical decision-making and may help reduce the risk of postoperative neurological deficits.

### Preoperative Transcranial Magnetic Stimulation (TMS)

TMS technology, through precise neurofunctional localization and multimodal imaging integration, provides critical preoperative assessment and planning for functional brain network preservation [72]. Navigated TMS (nTMS), fused with electromagnetic navigation and 3D MRI, can preoperatively map primary motor cortex somatotopy and delineate tumor–motor cortex spatial relationships, helping to anticipate functional risk before surgery [73]. Combined with DTI corticospinal tract reconstruction, it quantifies tumor-tract distances, providing "function-structure" coupling models for trajectory planning that have been associated with reductions in postoperative motor deficits compared with anatomical-only localization [74–75].

Repetitive TMS inhibits naming errors to localize language areas, compensating for fMRI limitations from tumor edema. This electrophysiological approach enables preoperative risk stratification, multimodal navigation, and personalized surgical strategies, maximizing resection while optimizing functional brain network preservation [76]. Additionally, TMS assesses preoperative functional network plasticity [77]. Tumor mass effect induces network remodeling; TMS detects excitability changes and connectivity alterations, informing compensatory capacity for individualized planning. In patients intolerant of awake craniotomy (e.g., obesity, OSA), TMS serves as an alternative to intraoperative direct cortical stimulation for functional localization [78].

### Preoperative Neuropsychological Assessment

Neuropsychological scales provide critical clinical value in preoperative and postoperative brain tumor evaluation by quantifying cognitive changes to inform surgical planning, risk prediction, and rehabilitation [79–81]. Rather than presenting isolated scales as universal recommendations, a domain-oriented assessment strategy is preferred, focusing on language, attention, executive function, and verbal memory according to tumor location and planned surgical strategy. Selection of specific scales is guided by their validation in neuro-oncological cohorts, sensitivity to focal cognitive deficits, and feasibility of repeat administration. For patients with aphasia, the "Aphasia Battery of Chinese (ABC)" is recommended due to its comprehensive evaluation of expressive and receptive language domains in Chinese-speaking populations [44]; for those with cognitive dysfunction, the "Montreal Cognitive Assessment (MoCA)" is advised given its established sensitivity for mild cognitive impairment [82–83]; for motor impairments, the "Motor Assessment Scale (MAS)" is suggested for its standardized evaluation of functional motor performance [84]; for verbal memory deficits, the "Hopkins Verbal Learning Test (HVLT)" is utilized owing to its validation in glioma populations [85]; and for spatial memory disorders, the "Blessed Dementia Rating Scale (BDRS)" is applied to establish preoperative functional baselines [86].

The integrating neuropsychological assessment with diffusion-based neuroimaging (e.g., DTI tractography/connectomics) helps identify tumor-related network disruption and interpret its functional relevance for cognitive risk stratification [42]. Scales serve as "functional landmarks" across tumor management: preoperative risk boundary definition, intraoperative functional preservation guidance, postoperative recovery trajectory monitoring [87–88]. Future integration of advanced

neuroimaging and molecular biomarkers may facilitate a shift from group-level cognitive profiling toward individualized prediction, with the goal of improving cognitive preservation and oncological decision-making in glioma care [89–90].

### Intraoperative Methods and Techniques for Functional Brain Network Preservation

To achieve maximal safe resection while preserving neurological function, multimodal strategies have been implemented across the preoperative, intraoperative, and postoperative stages (Figure 3).

### Functional Brain Network Reconstruction and Intraoperative Navigation

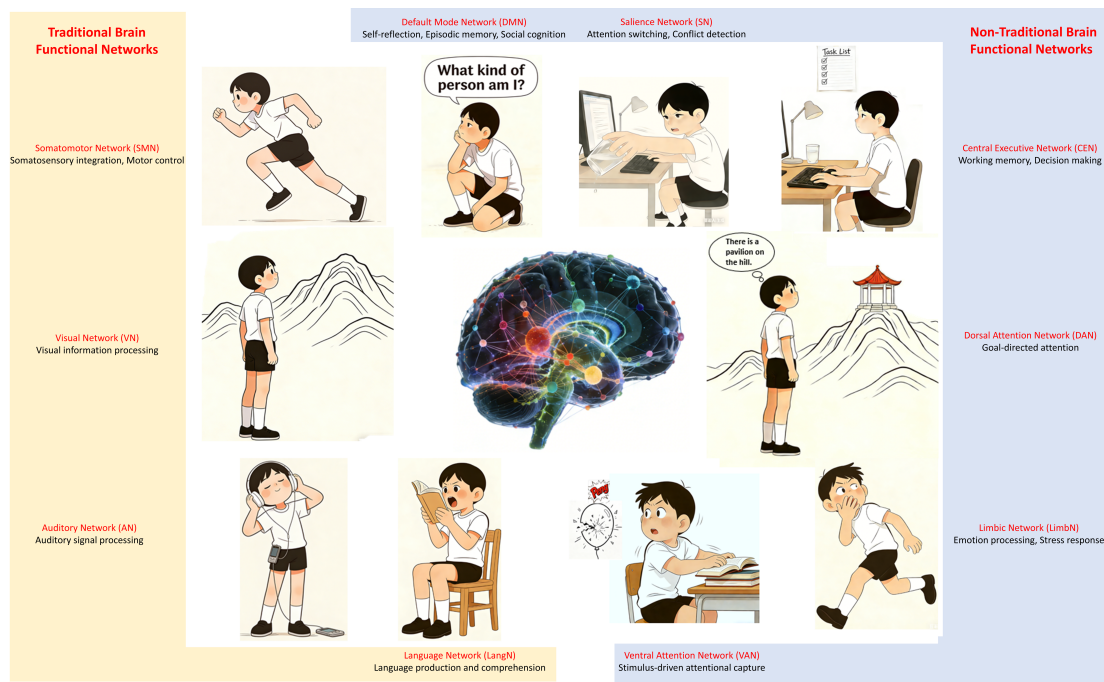
During brain tumor resection, multimodal neuronavigation establishes a framework for functional brain network preservation by integrating preoperative imaging with real-time anatomical scenes. By superimposing preoperative fMRI-based functional hotspots and DTI-based tractography models onto the surgical microscope view—often utilizing augmented reality—surgeons can visually identify the spatial relationship between the tumor and critical networks, such as language and motor pathways. When surgical instruments approach predefined safety boundaries, these systems provide distance-based proximity alerts to prevent inadvertent damage to subcortical tracts. However, because preoperative models are subject to intraoperative brain shift following dural opening and tumor resection, navigation data frequently require real-time updating with intraoperative MRI (iMRI) or ultrasound to maintain spatial accuracy [91–92].

Intraoperative navigation also facilitates the real-time integration of preoperatively acquired TMS mapping data with intraoperative direct electrical stimulation (DES) and continuous neuromonitoring [93]. While preoperative TMS provides a reliable initial cortical map showing high spatial concordance with DES, intraoperative DES remains the gold standard for functional localization, allowing dynamic adjustment of resection margins. Concurrently, continuous monitoring of motor evoked potentials (MEPs) and somatosensory evoked potentials (SEPs) provides real-time feedback on pathway integrity; notably, a significant amplitude drop in MEPs serves as a critical predictor of postoperative motor deterioration. This integrated mapping and monitoring approach has been shown to significantly reduce the incidence of permanent postoperative neurological deficits, thereby achieving an optimal balance between maximal safe resection and functional preservation [94–96].

### Intraoperative Neurophysiological Monitoring (IONM)

IONM is a key adjunct in brain tumor surgery involving eloquent cortex or critical white-matter pathways. Its primary objective is to maximize tumor resection while preserving neurological function through real-time monitoring of neural pathways, proving particularly crucial during operations within eloquent brain areas. The IONM technical framework has become increasingly sophisticated, primarily encompassing: (1) Evoked potential monitoring, such as Motor Evoked Potentials (MEPs), Somatosensory Evoked Potentials (SSEPs), Brainstem Auditory Evoked Potentials (BAEPs), and Visual Evoked Potentials (VEPs) [97]; (2) Cortical and subcortical mapping techniques, where direct electrical cortical stimulation and





**Figure 3. Strategies for brain functional network preservation.**

This figure summarizes the multimodal strategies used pre-, intra-, and post-operatively to protect brain functional networks during tumor surgery.

electrocorticography (ECoG) can precisely delineate functional boundaries, significantly reducing the risk of permanent postoperative neurological deficits [98]; and (3) Cranial nerve monitoring, exemplified by facial nerve monitoring during vestibular schwannoma resection, which, combined with EMG recordings of muscle activity, substantially lowers the incidence of postoperative facial palsy [99]. Several studies indicate that patients undergoing brain tumor surgery with IONM have a significantly lower probability of functional sequelae compared to those without it [95, 100-101]. Furthermore, IONM enhances surgical safety. A German multicenter study demonstrated an IONM application rate of 46.4%, and its use was associated with a significant reduction in in-hospital mortality compared to cases where IONM was not employed [102].

### iMRI and Intraoperative Ultrasonography (IOUS)

iMRI and IOUS enable dynamic correction of imaging discrepancies during surgery, addressing brain shift occurs after dural opening and tumor debulking [103-104]. iMRI acquires detailed, high-resolution images of intracranial structures, clearly displaying the tumor's location, morphology, and its relationship to surrounding eloquent tissues. This assists surgeons in precisely determining tumor boundaries, avoiding localization errors due to brain displacement, thereby enhancing resection accuracy and extent, monitoring surgical complications, guiding intraoperative strategy adjustments, and achieving maximal safe resection. Similarly, IOUS provides real-time guidance for surgical maneuvers, clearly visualizing the tumor's feeding arteries and draining veins, helping surgeons avoid critical vasculature and reduce intraoperative bleeding risk. A meta-analysis indicates that for pediatric brain tumors, IOUS is comparable to iMRI in estimating the extent of resection [105]. Combined with its lower cost and reduced requirements for radiation and contrast agents, IOUS is more readily applicable

and promotable in primary hospitals or resource-limited settings, providing intraoperative imaging assistance for a broader patient population [106-107].

### Awake Craniotomy (AC) and Intraoperative Functional Assessment

Guided by multimodal technology, awake craniotomy (AC) combined with intraoperative neurophysiological monitoring has become a cornerstone strategy for preserving functional brain networks. AC utilizes real-time cortical and subcortical electrical stimulation to dynamically map neural circuits, enabling maximal safe tumor resection while sparing critically functioning peritumoral tissue. High-level evidence from recent meta-analyses demonstrates that, compared with surgery under general anesthesia, AC significantly reduces the incidence of late severe neurological deficits and improves the extent of resection across various glioma subgroups [108-110].

The anesthetic management for AC must be carefully tailored to the individual patient, primarily involving either an Asleep-Awake-Asleep (SAS) technique or a continuous Conscious Sedation (Awake-Awake-Awake, AAA) approach [111]. SAS provides a robust sedation buffer during cranial opening and closure, making it highly suitable for patients with anxiety sensitivity or those anticipating particularly lengthy and complex resections. Conversely, AAA favors uninterrupted baseline monitoring and patient cooperation throughout the procedure [112]. The optimal choice is continuously refined based on tumor location, psychological assessment, and the multidisciplinary team's expertise, rather than rigid operative time constraints [108-109, 113-114].

Beyond traditional basic language and motor mapping, the integration of AC with advanced neurocognitive assessment has significantly evolved. Modern intraoperative functional assessment now incorporates tailored neuropsychological paradigms

to monitor higher-order cognitive domains—such as executive function, visuospatial processing, and social cognition—in real time. By continuously correlating these intraoperative cognitive task performances with preoperative network models, surgeons can precisely adjust the resection trajectory to avoid disrupting distributed functional network hubs and connecting white matter tracts. This individualized approach ensures the integrity of large-scale cognitive networks, fundamentally optimizing postoperative quality of life [115-116].

### Intraoperative Tumor Fluorescence Imaging

5-Aminolevulinic acid (5-ALA) fluorescence-guided surgery is an established adjunct in the resection of contrast-enhancing malignant gliomas [117]. After preoperative oral administration, 5-ALA is metabolized into fluorescent protoporphyrin IX within tumor cells, allowing intraoperative visualization of tumor tissue under blue-violet light. This improves identification of residual enhancing tumor and supports more complete resection when integrated with standard microsurgical and functional-guidance techniques [118]. In the landmark multicenter phase III randomized controlled trial by Stummer et al., 5-ALA-guided surgery achieved a higher rate of complete resection of contrast-enhancing tumor than white-light microsurgery alone (65% vs 36%,  $p < 0.0001$ ) and improved 6-month progression-free survival (41.0% vs 21.1%,  $p = 0.0003$ ), without increasing severe adverse events [119].

### Rehabilitation Strategies for Postoperative Functional Brain Networks

#### Assessment Methods

Multimodal imaging may support postoperative rehabilitation planning by enabling comparison of tract integrity across preoperative and postoperative examinations. Preoperative DTI can delineate tumor-related compression or infiltration of critical white-matter pathways, such as the corticospinal tract and arcuate fasciculus, whereas postoperative DTI can help assess tract preservation, edema-related change, and structural injury [120]. When interpreted together with neurological or neuropsychological assessments, these imaging findings may help stratify functional risk, distinguish transient from persistent deficits, and estimate recovery potential during rehabilitation [121-122].

#### Rehabilitation Methods

Early postoperative inpatient rehabilitation focuses on improving patient consciousness levels, emphasizing early physical therapy intervention and multidisciplinary collaboration [123-124]. Progressive training from bed rest to walking reduces delirium risk by 43% and shortens hospital stay by 1 day [125]. For moderate-to-severe motor paralysis patients, repetitive facilitation training promotes early postoperative mobilization. Marciniak et al. analysis of 132 brain tumor patients demonstrates that multimodal-guided physical therapy significantly improves motor and cognitive efficiency, independent of tumor type [126-128].

In addition to conventional physical therapy, noninvasive neuromodulation techniques such as repetitive transcranial magnetic stimulation (rTMS) are increasingly utilized to facilitate neural plasticity [129-130]. Recent randomized, sham-controlled trials indicate that navigated low-frequency rTMS, when applied to the unaffected hemisphere or motor cortex along-

side standard physical therapy, can effectively accelerate the recovery of specific motor deficits following the resection of motor-eloquent supratentorial tumors [131].

Furthermore, brain-computer interface (BCI) technology is emerging as a highly promising tool in neurorehabilitation. Although predominantly validated in broader neurological injuries such as stroke, BCI systems utilize visual or proprioceptive feedback during motor imagery to activate residual cortical areas [132-133]. Advanced neuroimaging demonstrates that such BCI-guided training can stimulate targeted neuroplasticity and strengthen functional connectivity within compromised sensorimotor networks, offering a novel synergistic approach to optimize long-term upper limb functional scores in post-surgical neuro-oncology patients [134-136].

### Significance of Functional Brain Network Assessment for Rehabilitation Training

Functional brain network assessment provides critical guidance for postoperative brain tumor rehabilitation, offering a comprehensive scientific framework from preoperative planning to postoperative intervention [137]. Preoperative fMRI and DTI localize tumor-infiltrated cortical nodes and subcortical tracts; combined with intraoperative mapping and clinical scales, these modalities help optimize surgical trajectories to preserve residual functioning networks. Postoperatively, network-based evaluations enable the design of personalized rehabilitation protocols. For instance, rather than adopting a uniform rehabilitation strategy, identifying specific disruptions in the sensorimotor network or the default mode network (DMN) allows clinicians to implement concurrent, targeted physical and cognitive therapies tailored to the patient's unique deficit profile [137-140].

This "network-based subtyping" overcomes the limitations of traditional, one-size-fits-all rehabilitation approaches. Recent advanced neuroimaging studies demonstrate that brain tumors not only cause localized structural damage but also induce global alterations in functional network topology—such as changes in network segregation and hub centrality—that are closely correlated with specific cognitive outcomes; notably, increased network segregation following therapy may reflect either compensatory adaptation or maladaptive reorganization, depending on its impact on cognitive performance [138, 141]. Monitoring these functional connectivity patterns longitudinally helps estimate a patient's neuroplastic potential, thereby facilitating dynamic adjustments of training intensity and supporting a more precise, individualized neurorehabilitation strategy [142-143].

### Challenges and Future Directions of Functional Brain Networks

Despite the clear clinical value of functional brain network preservation, significant challenges persist in daily neuro-oncological practice [144-145]. First, interindividual functional variability and intraoperative brain shift resulting from cerebrospinal fluid loss frequently degrade the spatial accuracy of preoperative navigation data. Second, resting-state fMRI-based network identification remains susceptible to acquisition and preprocessing variability, limiting reproducibility, particularly for non-traditional networks such as SN, CEN, and attention

systems. Furthermore, acquiring and interpreting multimodal network imaging is time-consuming, resource-intensive, and challenging for widespread adoption in resource-limited settings [146]. A critical unresolved issue is the accurate characterization of network resilience during tumor infiltration and the ability to confidently predict postoperative compensatory plasticity using individualized network models [111, 147]. Future directions must address these limitations by shifting the surgical paradigm from an "experience-driven" approach to a precision "data-driven" framework. A highly promising avenue involves integrating large-scale connectomics data with artificial intelligence (AI) and machine learning algorithms [3, 122, 148]. Such integration aims to develop robust "network risk assessment tools" capable of generating personalized surgical trajectories and real-time intraoperative updates [149]. Along with multidimensional technological innovations and improved clinical translation, these data-driven models are expected to ultimately optimize the delicate balance between maximal oncological control and the preservation of global neurocognitive function [150].

## Summary

Preserving neural functional networks during brain tumor resection embodies a "functional prognosis priority" decision-making logic, seeking the optimal balance between maximal tumor removal and neural function preservation. Brain functional networks critically regulate cognition, motor function, language, and other essential abilities. Protecting these networks during extensive tumor resection effectively reduces postoperative neurological deficits, helping patients maintain self-care capabilities and social functions while significantly improving quality of life. This paradigm drives neurosurgery's transformation from "anatomy-oriented" to "functional network-oriented" approaches, ultimately achieving dual optimization of "tumor control" and "quality of life." Multimodal technology-driven network preservation marks brain tumor surgery's transition from "anatomical resection" to a new era of "brain functional network protection." Integrating multi-source data precisely delineates tumor-network relationships and constructs topology-based surgical strategies, substantially enhancing patient survival quality. Future breakthroughs in dynamic monitoring and data fusion, alongside standardized surgical protocols, will synergistically optimize neural function preservation with tumor eradication.

## Abbreviations

5-ALA: 5-aminolevulinic acid; AC: Awake Craniotomy; BCI: Brain-Computer Interface; CEN: Central Executive Network; DMN: Default Mode Network; DTI: Diffusion Tensor Imaging; fMRI: functional Magnetic Resonance Imaging; IONM: Intraoperative Neurophysiological Monitoring; TMS: Transcranial Magnetic Stimulation.

## Author Contributions

Mu Fengchun and Li Huaixu: Conceptualization, Investigation,

Writing - original draft. Wan Jinghai and Yang Ming: Writing - review & editing.

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## Ethics Approval and Consent to Participate

Not Applicable.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

All data discussed in this review are derived from previously published studies and are available in the cited references. No new data was generated or analysed in this study.

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# The Application of 3D Printing Technology in Otological Diagnosis and Treatment from Morphological Reconstruction to Functional Restoration

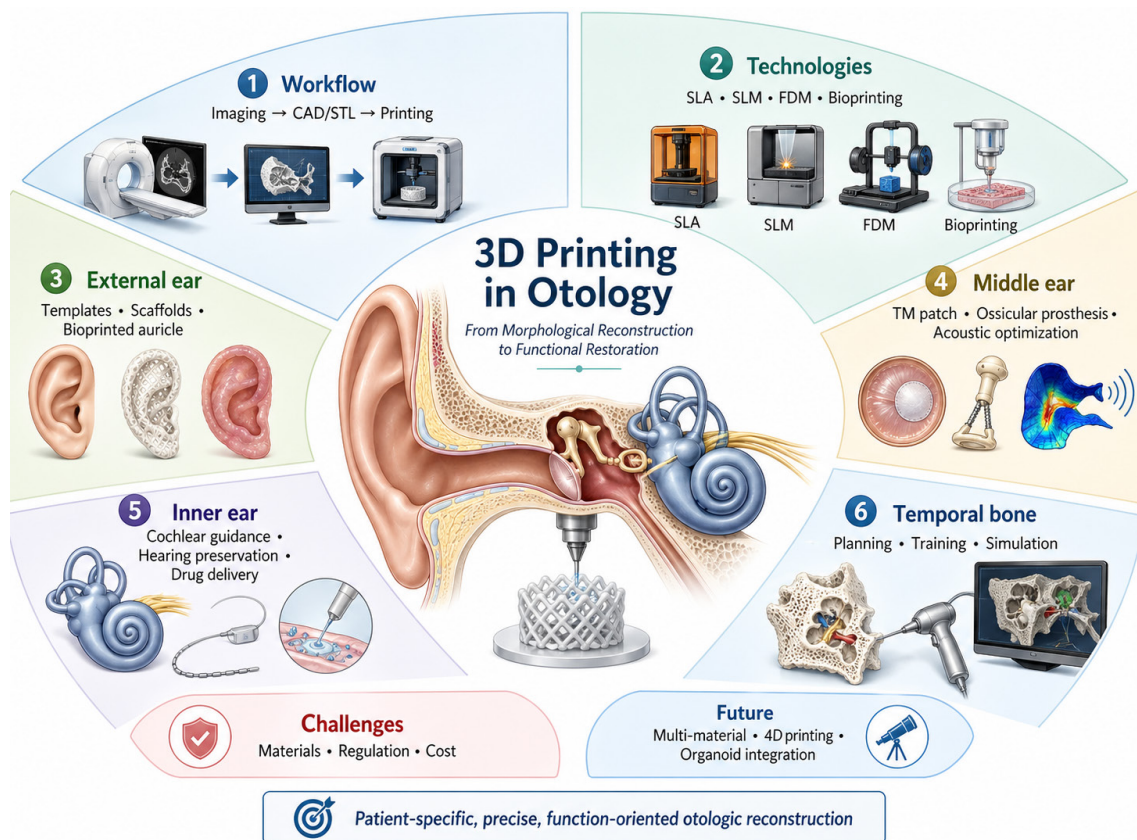
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## Graphical Abstract



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# The Application of 3D Printing Technology in Otological Diagnosis and Treatment from Morphological Reconstruction to Functional Restoration

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## Abstract

Through patient-specific design and precise anatomical fabrication, 3D printing is reshaping otology from experience-based surgery toward digital precision medicine. This review summarizes recent advances in the application of 3D printing in otology, focusing on technical platforms, morphological reconstruction, and functional restoration. At the technical level, stereolithography, selective laser melting, fused deposition modeling, and 3D bioprinting are suited to preoperative simulation, titanium-alloy implant fabrication, educational models, and tissue-engineered scaffolds, respectively. For morphological reconstruction, 3D printing enables precise replication of structures ranging from auricular frameworks and middle-ear ossicular chains to temporal-bone anatomical models. For functional restoration, patient-specific ossicular prostheses can optimize sound conduction, surgical guides can assist cochlear implantation and help preserve residual hearing, sustained-release devices may help overcome barriers to inner-ear drug delivery, and tissue-engineered scaffolds have opened new possibilities for inner-ear neural repair and regeneration. Although challenges related to material compatibility, regulatory approval, and cost-effectiveness remain, advances in multi-material printing and 4D printing may further expand the clinical potential of 3D printing in personalized otologic reconstruction that integrates anatomical form with functional recovery.

**Keywords:** 3D printing; otology; morphological reconstruction; functional restoration; personalized medicine

## Introduction

Because of the anatomical complexity of the ear, the precision required for auditory and vestibular function, and substantial interindividual anatomical variation, otology remains one of the most technically demanding subspecialties within otolaryngology and head and neck surgery. Conditions ranging from congenital microtia and ossicular chain disruption caused by chronic otitis media to otosclerosis and sensorineural hearing loss requiring cochlear implantation all impose stringent requirements on therapeutic strategies [1]. Although conventional treatments are widely used in clinical practice, they still face substantial limitations in anatomical matching, functional restoration, operative precision, and long-term stability. Therefore, achieving accurate morphological reconstruction while maximizing auditory functional recovery has become a central challenge in contemporary otology [2].

The emergence of 3D printing offers a promising solution to this challenge. By enabling patient-specific design, precise replication of complex structures, and rapid prototyping, this

technology is well suited to the highly variable anatomy of the ear. Since Kaneko [3] first applied three-dimensional shape measurement to microtia reconstruction in 1993, 3D printing in otology has evolved from the production of anatomical models to the design and fabrication of personalized implants, and further toward tissue engineering and regenerative medicine. Its applications now extend from external auricular reconstruction and middle-ear ossicular chain repair to the protection and restoration of inner-ear neural function, as well as temporal bone models for surgical training [4-5]. In this context, 3D printing is gradually reshaping otologic diagnosis and treatment by linking anatomical reconstruction with functional recovery.

This review summarizes recent progress in the application of 3D printing technology in precision otology. First, it outlines the technical foundations of 3D printing and the main application strategies in otologic practice. It then discusses the current research status and clinical value of 3D printing in outer-ear reconstruction, middle-ear ossicular chain repair, inner-ear functional restoration, and temporal bone surgical training. Finally, it summarizes the major challenges and future directions of

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this field, including material biocompatibility, regulatory pathways, cost-effectiveness, multi-material printing, 4D printing, and tissue-engineered reconstruction. By integrating current evidence, this review aims to provide clinicians and researchers with a structured understanding of 3D printing in otology and to support its further translational development.

Technical Workflow and Application Strategies of 3D Printing in Otology

The clinical application of 3D printing in otology relies on a complete digital-to-physical workflow that transforms patient-specific imaging data into anatomical models [6], surgical guides, implants, or tissue-engineered constructs. Because otologic structures are small, complex, and highly variable among individuals, successful application requires not only accurate image reconstruction but also careful matching between printing technology, material properties, clinical purpose, and cost-effectiveness [1]. This section therefore summarizes the technical workflow of 3D printing in otology and further discusses demand-oriented application strategies for preoperative planning, surgical guidance, personalized implantation, and regenerative reconstruction.

Digital Workflow: from Medical Imaging to Printable models

The clinical application of 3D printing begins with the conversion of patient-specific anatomical information into a printable digital model. In medical practice, anatomical data are commonly obtained from computed tomography (CT), magnetic resonance imaging (MRI), or, in selected scenarios, ultrasound imaging. These imaging datasets are usually stored in the Digital Imaging and Communications in Medicine (DICOM) format [7], which provides the basis for subsequent three-dimensional reconstruction. After image acquisition, the target anatomical structures are segmented and reconstructed using computer-aided design (CAD) or medical image processing software. The reconstructed model is then exported into a printer-compatible file format, most commonly the Standard Tessellation Language

(STL) format [8]. STL files describe the surface geometry of a three-dimensional object using triangular meshes, allowing the digital model to be sliced into multiple layers for additive manufacturing [9]. Through this workflow, patient-specific anatomical structures can be transformed from two-dimensional imaging data into physical models, surgical guides, implants, or tissue-engineered scaffolds. In otology, this digital workflow is particularly valuable because the ear contains small, complex, and highly individualized anatomical structures. High-resolution imaging and accurate segmentation allow the reconstruction of delicate structures such as the ossicular chain, facial nerve canal, cochlea, external auricle, and temporal bone [10]. These models can support preoperative planning, surgical simulation, anatomical teaching, doctor-patient communication, and the design of personalized therapeutic devices.

Demand-Oriented Selection of 3D Printing Technologies in Otology

The key to applying 3D printing in otology is not the use of a single printing method, but the selection of an appropriate technology according to clinical purpose. Different otologic applications require different levels of precision, material performance, sterilizability, fabrication time, and cost. Here are several common 3D printing methods: Stereolithography (SLA) and digital light processing (DLP) offer high precision and smooth surface finish, making them suitable for preoperative models and surgical guides, although their material selection remains limited. Selective laser melting (SLM) enables the fabrication of high-strength metal implants such as custom ossicular prostheses, but is associated with high cost and the need for post-processing. Fused deposition modeling (FDM) is low-cost and easy to operate, yet its lower precision and weak interlayer bonding restrict its use to educational models and biodegradable scaffolds. Three-dimensional bioprinting incorporates cells and growth factors for tissue-engineered auricular constructs and inner ear repair, but currently lacks mechanical strength and remains at an experimental stage (Table 1). Therefore, strategies for applying 3D printing in otology can be categorized based on their roles in morphological reconstruction.

Table 1. Comparison of common 3D printing technologies in terms of materials, resolution, advantages, limitations, and representative otological applications.

| Technology     | Typical Materials                  | Resolution  | Advantages                                                    | Limitations                                          | Representative Otological Applications            |
|----------------|------------------------------------|-------------|---------------------------------------------------------------|------------------------------------------------------|---------------------------------------------------|
| SLA            | Photopolymer resins                | ~25–100 μm  | High precision, smooth surface                                | Limited material range, post-curing required         | Preoperative models, surgical guides              |
| SLM            | Titanium alloys, Co–Cr alloys      | ~50–200 μm  | High mechanical strength, suitable for permanent implantation | High cost, surface post-processing required          | Custom ossicular prostheses, auricular frameworks |
| FDM            | PLA, PCL, and other thermoplastics | ~100–300 μm | Low cost, simple operation                                    | Lower precision, weaker interlayer bonding           | Educational models, biodegradable scaffolds       |
| DLP            | Photopolymer resins                | ~50–100 μm  | Fast printing speed, high precision                           | Limited material selection                           | High-resolution anatomical models                 |
| 3D Bioprinting | Hydrogels, bioinks                 | ~20–200 μm  | Can incorporate cells and growth factors                      | Low mechanical strength, still at experimental stage | Tissue-engineered auricles, inner ear repair      |



tion and functional restoration: Preoperative and educational models, as well as customized surgical guides, play a crucial role in guiding morphological reconstruction. Functional restoration includes tympanic membrane repair and targeted drug delivery, while customized implants and tissue engineering scaffolds not only enable precise morphological reconstruction but also better assist patients in restoring ear-related functions [11].

### **Preoperative Planning and Educational Models**

Preoperative planning models and educational models represent the earliest and most mature applications of 3D printing in otology. Their main purpose is to reproduce complex anatomical structures and spatial relationships, helping surgeons understand individual anatomical variations, anticipate surgical risks, and rehearse difficult procedures before surgery. Typical applications include temporal bone models for lateral skull base surgery [12], facial nerve decompression, congenital auricular deformity assessment [13], and resident training in otologic anatomy [14].

For these models, morphological accuracy and cost-effectiveness are more important than implantability or long-term biocompatibility. Stereolithography (SLA) and fused deposition modeling (FDM) are the most commonly used techniques. SLA provides higher printing resolution and can reproduce fine structures such as the ossicles and facial nerve canal. Resin-based SLA models also offer relatively realistic tactile feedback during drilling and are therefore useful for surgical rehearsal and detailed anatomical teaching [15]. In contrast, FDM is less expensive and more suitable for large-scale production of teaching models, basic anatomical education, and use in primary-care or resource-limited settings [16].

### **Patient-specific Surgical Guides**

Surgical guides are designed to translate preoperative planning into intraoperative precision. Their core value lies in converting virtual information, including the planned entry point, direction, angle, and depth, into a physical guide that can assist surgeons during key procedural steps. In otology, such guides are mainly used in cochlear implantation, ossicular chain reconstruction, and complex temporal bone procedures [17].

Compared with ordinary anatomical models, surgical guides require higher dimensional accuracy, reliable sterilizability, close conformity to bone surfaces, and convenient intraoperative handling. SLA is currently one of the preferred techniques for fabricating surgical guides because of its high resolution and ability to generate smooth, patient-specific structures. By improving procedural accuracy and reducing intraoperative uncertainty, surgical guides may contribute to safer and more standardized otologic surgery.

### **Tympanic Membrane Repair and Targeted Drug Delivery to the Inner Ear**

Tympanic membrane perforation is a common cause of conductive hearing loss; traditional surgical repair methods suffer from issues such as tissue harvesting trauma and poor structural compatibility. 3D printing technology offers a high-precision, personalized solution for tympanic membrane regeneration [18]. Using high-resolution 3D printing processes, scaffolds with a multi-layered biomimetic structure resembling that of the natural tympanic membrane can be fabricated [19].

In terms of material selection, current research tends to favor photo-crosslinkable natural polymers and composite hydrogels, which possess excellent biocompatibility and promote cell migration, proliferation, and tissue remodeling.

Pharmacological treatment of inner ear diseases has long been limited by the blood-labyrinth barrier and the deep anatomical location of the cochlea [20]. 3D printing technology offers a new approach to addressing this challenge, centered on the fabrication of micro-drug delivery implants that precisely conform to the round window membrane or the round window niche. Utilizing patient-specific image reconstruction, these implants can be custom-shaped to match anatomical structures, ensuring that the drug delivery device remains stably positioned at the target site. Furthermore, localized micro-delivery significantly improves drug bioavailability in the inner ear while avoiding systemic side effects, providing a novel technological platform for the precise treatment of inner ear disorders such as sensorineural hearing loss.

### **Customized Implants for Structural and Functional Reconstruction**

Customized implants represent one of the most clinically valuable but technically demanding applications of 3D printing in otology. Their purpose is to overcome the mismatch between standardized implants and individual anatomical variations, thereby achieving personalized structural reconstruction and functional restoration. Major applications include ossicular chain reconstruction and auricular reconstruction.

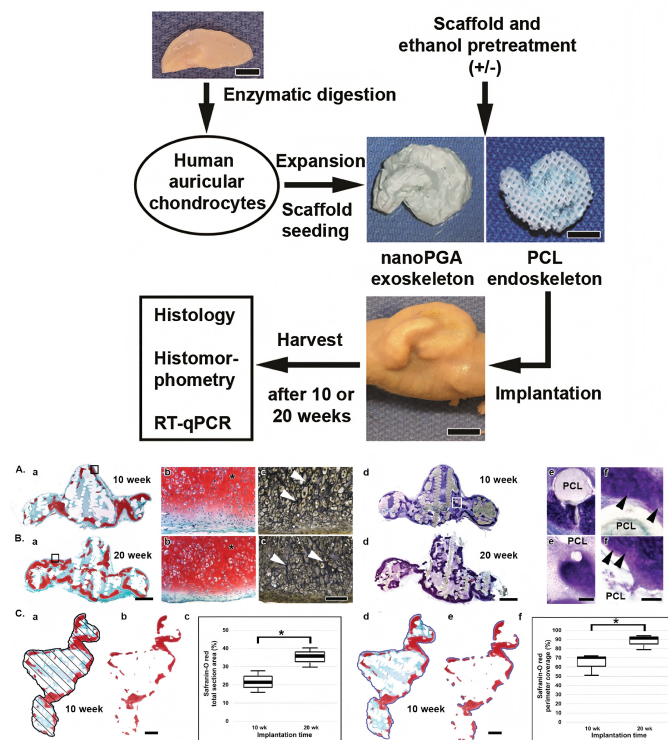
For permanent implants, the key requirements include biocompatibility, mechanical stability, anatomical fit, sterilizability, and long-term safety. Selective laser melting (SLM), especially for titanium alloy fabrication, is currently one of the most important approaches for producing customized metallic implants. Titanium alloys have favorable biocompatibility and mechanical strength, making them suitable for long-term implantation [21]. In addition, 3D printing allows the fabrication of porous, hollow, or thin-walled structures, which may help reduce implant rigidity, improve tissue integration, and better mimic the mechanical properties of native ear structures.

### **Tissue-Engineered Scaffolds and 3D Bioprinting**

Tissue-engineered scaffolds represent the frontier of 3D printing in otology. Unlike anatomical models or inert implants, these scaffolds aim to support biological regeneration through the integrated design of biomaterials, cells, and bioactive factors [22]. Potential applications include auricular cartilage engineering, inner-ear neural repair, and local drug delivery systems.

An ideal tissue-engineered scaffold should provide cell compatibility, controlled degradation, mechanical support, appropriate porosity, and the ability to release bioactive molecules in a controlled manner [19]. 3D bioprinting offers unique advantages in this context because it can spatially organize cells and biomaterials with high precision [22]. Compared with traditional cell seeding methods, bioprinting may improve cell distribution and reduce the risk of insufficient nutrient diffusion within the scaffold. Although most applications remain at the experimental or preclinical stage, tissue-engineered scaffolds and 3D bioprinting provide an important direction for future functional reconstruction in otology. For instance, 3D-printed nanoPGA/PCL composite scaffolds have been

utilized to demonstrate that optimizing scaffold surface properties through ethanol pretreatment can significantly enhance human auricular chondrocyte adhesion and promote the *in vivo* regeneration of mature, glossy cartilage [23] (Figure 1.).



**Figure 1.** Design of 3D nanoPGA/PCL scaffolds seeded with human auricular chondrocytes  $\pm$  ethanol treatment. Histological analysis of regenerated cartilage on ethanol-treated scaffolds after 10 and 20 weeks of implantation [23]. Copyright 2021 PLoS One.

## Clinical Applications of 3D Printing in Otolaryngology: From Morphological Reconstruction to Functional Restoration

The clinical value of 3D printing in otology lies in its ability to translate patient-specific anatomical information into tangible tools for reconstruction, simulation, implantation, and regenerative repair. Because the external ear, middle ear, inner ear, and temporal bone differ substantially in anatomical scale, functional requirements, and clinical accessibility, 3D printing strategies must be tailored to distinct therapeutic goals. In the external ear, the primary objective is to restore auricular shape and psychosocial function. In the middle ear, the key challenge is to reconstruct the ossicular chain while preserving efficient sound conduction. In the inner ear, 3D printing is increasingly used to assist cochlear implantation, improve local drug delivery, and support neural repair strategies [24]. In temporal bone surgery, printed models provide a practical platform for preoperative rehearsal and surgical education. Together, these applications indicate that 3D printing in otology is evolving from morphological replication toward function-oriented precision medicine [14].

## External Ear Reconstruction: From Mirror-Image Templates to Tissue-Engineered Auricles

The external ear is important for both sound collection and facial appearance. Auricular defects, most commonly caused by congenital microtia [25], trauma, burns, or tumor resection, can impair hearing-related function and psychosocial well-being. Because the auricle has a delicate three-dimensional contour and an elastic cartilage framework, auricular reconstruction remains technically demanding [26].

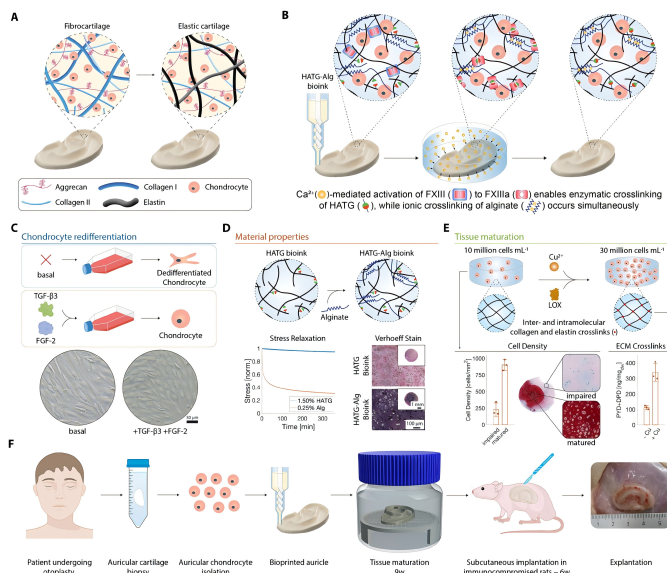
Autologous rib cartilage grafting remains a standard method for auricular reconstruction because of its good biocompatibility and low immunogenicity [27]. However, it requires cartilage harvesting and precise manual sculpting, which may lead to donor-site morbidity, cartilage exposure, infection, hematoma, and variable aesthetic outcomes [20, 26]. Porous polyethylene implants, such as Medpor, provide an alternative alloplastic option, but they are associated with risks such as infection, fracture, implant exposure, and limited adaptability in growing pediatric patients [27–28]. These limitations have driven the development of more individualized and less invasive reconstruction strategies.

3D printing has improved auricular reconstruction mainly by providing patient-specific templates and customized scaffolds. In unilateral microtia, imaging data from the healthy ear can be mirrored to generate a three-dimensional model of the affected side, offering surgeons a precise reference for framework sculpting, auricular positioning, and bilateral symmetry [29–30]. Compared with traditional two-dimensional templates, 3D-printed models can reduce intraoperative adjustment and improve anatomical accuracy [33]. More recently, directly printed auricular scaffolds and prostheses have been explored to reduce dependence on manual carving and improve structural consistency. However, their clinical use still requires careful evaluation of biocompatibility, mechanical elasticity, infection risk, skin coverage, and long-term stability.

Beyond inert scaffolds, 3D bioprinting and tissue engineering aim to generate living cartilage-like auricular constructs. In this strategy, chondrocytes or chondrogenic cells are combined with printable biomaterials to form bioinks, which are then printed into auricular-shaped structures and cultured to promote cartilage matrix deposition. Ideal bioinks should provide biocompatibility, tunable degradation, cell adhesion, nutrient diffusion, and cartilage-like mechanical strength [34]. Hydrogels, including collagen-, gelatin-, alginate-, and hyaluronic-acid-based systems, are attractive because of their extracellular matrix-like properties and high permeability to nutrients and soluble factors [35]. Although promising, tissue-engineered auricles remain largely experimental, and further work is needed to ensure durable shape retention, cell viability, vascularized soft-tissue coverage, and regulatory feasibility. To address these mechanical and functional limitations, recent breakthroughs have utilized stress-relaxing HATG-Alg bioinks combined with growth factor-controlled chondrogenesis to engineer functional elastic cartilage. These 3D-bioprinted constructs achieve near-native mechanical properties, such as a compressive modulus of  $1.1 \pm 0.03$  MPa, and maintain structural integrity during *in vivo* evaluation [36] (Figure 2.).

## Middle Ear Reconstruction: Personalized Ossicular Prostheses and Acoustic Optimization

The middle ear transmits airborne sound to the cochlear fluid



**Figure 2.** Engineering and evaluation of elastic cartilage-mimetic auricular grafts.

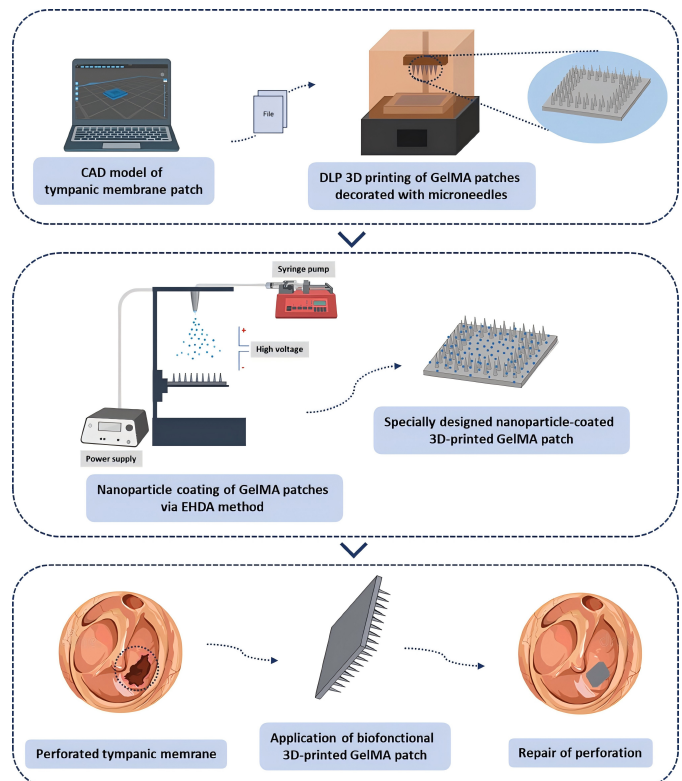
(A) Comparison of fibro- and elastic cartilage. (B) Bioink design featuring HATG and alginate crosslinking. (C-E) Strategies for promoting chondrogenic redifferentiation and tissue maturation via growth factors and LOX/Cu-mediated crosslinking. (F) Comprehensive workflow from primary cell isolation and bioprinting to *in vivo* subcutaneous rat model evaluation [36]. Copyright 2024 P. Fisch et al.

through the tympanic membrane and ossicular chain. Damage to the malleus, incus, or stapes caused by chronic otitis media, cholesteatoma, trauma, congenital anomalies, or surgery can disrupt sound conduction and result in conductive hearing loss. Ossicular chain reconstruction is therefore a key surgical strategy for restoring middle-ear function. However, conventional ossicular prostheses are usually produced in limited standard sizes and often require intraoperative trimming or adjustment. This may cause suboptimal prosthesis length, angular mismatch, poor fixation, displacement, extrusion, or unsatisfactory hearing outcomes [37]. Because the middle ear is a small and highly sensitive mechanical system, even minor deviations in prosthesis geometry or contact angle may affect acoustic transmission.

3D printing provides a strategy for patient-specific ossicular reconstruction. High-resolution computed tomography can be used to reconstruct the tympanic membrane, ossicular remnants, middle-ear cavity, ligaments, and surrounding structures [38]. Based on these data, customized ossicular prostheses can be designed to better fit the available anatomical space and intended reconstruction pathway. More importantly, finite element analysis can be integrated into this workflow to simulate displacement, stress, strain, and frequency response under sound stimulation [39]. This allows different prosthesis materials, shapes, lengths, and contact configurations to be evaluated before fabrication. In this sense, 3D printing is not only a manufacturing method but also part of an engineering-assisted platform for anatomical matching and acoustic optimization.

The design, 3D printing, coating, and application of GelMA patches are also currently trending technologies. Material selection remains central to the success of 3D-printed ossicular

prostheses. Titanium alloys are widely used in ossiculoplasty because of their favorable biocompatibility, mechanical strength, low weight, and acoustic performance. Recent evidence suggests that titanium total ossicular replacement prostheses may achieve better postoperative air-bone gap improvement than autologous materials in total ossicular chain reconstruction [40]. 3D printing may further improve titanium-based prostheses by enabling individualized geometry, lightweight structures, and more stable coupling with residual middle-ear components. Nevertheless, 3D-printed ossicular prostheses have not yet become routine in clinical practice. Key barriers include high-resolution segmentation, manufacturing standardization, sterilization validation, cost control, and long-term clinical outcome evaluation. Future studies should integrate anatomical fit, acoustic simulation, and postoperative hearing outcomes to clarify which patient groups may benefit most from personalized 3D-printed ossicular reconstruction. Beyond ossicular chain repair, 3D printing is significantly advancing the treatment of tympanic membrane (TM) perforations. For instance, biofunctional GelMA-based patches featuring microneedles for enhanced adhesion have been fabricated using Digital Light Processing (DLP) technology. These patches can be further functionalized with antimicrobial nanoparticles via electrohydrodynamic atomization (EHDA) to prevent infection while promoting tissue regeneration, offering a highly personalized and effective approach for TM repair [41] (Figure 3).



**Figure 3.** Fabrication and application of 3D-printed GelMA patches for tympanic membrane repair [41]. Copyright 2025 T.Bedir, D. Uzun

### Inner Ear Applications: Cochlear Implantation Assistance and Local Drug Delivery

The inner ear is anatomically confined, functionally delicate,



and difficult to repair once damaged [20]. Sensorineural hearing loss remains challenging to treat because hair cells and spiral ganglion neurons have limited regenerative capacity. Hearing aids are useful for mild to moderate hearing loss, whereas cochlear implants remain the most effective intervention for severe to profound sensorineural hearing loss by electrically stimulating residual spiral ganglion neurons [42]. However, cochlear implantation requires highly precise electrode placement, and preservation of residual hearing has become increasingly important as implantation indications expand. Individual variations in cochlear size, curvature, duct length, and internal anatomy may influence electrode selection, insertion depth, scalar position, and the risk of intracochlear trauma.

3D printing can support cochlear implantation by converting patient-specific imaging data into physical cochlear models for preoperative planning, electrode insertion training, and device evaluation [18]. Compared with conventional two-dimensional imaging, 3D-printed models provide a more intuitive understanding of cochlear geometry and electrode trajectory. Recent smart cochlear models with real-time feedback can further simulate electrode insertion and detect technical errors, such as excessive force, retraction, or bending [43]. One study reported that training with a smart 3D-printed cochlear model improved novice surgeons' electrode insertion depth by 11.8% and reduced retraction and bending errors by 60.3% and 54.9%, respectively [43]. These findings suggest that 3D printing may contribute not only to anatomical visualization but also to procedural standardization and skills assessment in cochlear implantation.

Another promising application is inner-ear drug delivery. Systemic administration is limited by the blood-labyrinth barrier, whereas intratympanic injection still depends on passive diffusion across the round window membrane, making local drug concentration and exposure duration difficult to control [44]. 3D-printed microneedle-based delivery systems may provide a more precise and minimally invasive solution. Using high-resolution methods such as two-photon polymerization, micron-scale trans-tympanic or round-window devices can be fabricated to create controlled pathways for drug entry. Veit et al. developed several 3D-printed trans-tympanic delivery devices with adjustable release durations ranging from days to months [45]. These platforms may improve the delivery of ototherapeutics, including macromolecular drugs, gene therapy vectors, and neurotrophic factors. Nevertheless, inner-ear applications require strict safety evaluation because any device interacting with the round window membrane or cochlea must minimize trauma, inflammation, fibrosis, infection, and long-term hearing damage [46]. Future studies should therefore focus on biocompatibility, reversibility, delivery accuracy, and functional auditory outcomes.

### Temporal Bone Models: Surgical Planning and Simulation-Based Education

Temporal bone surgery requires precise three-dimensional anatomical understanding and refined operative skills within a narrow surgical field [47]. The facial nerve, ossicles, labyrinth, sigmoid sinus, jugular bulb, carotid canal, and dura, are densely packed within a limited bony space. Traditional training has long relied on cadaveric temporal bone dissection, which remains valuable but is constrained by specimen scarcity, ethical considerations, high cost, storage requirements, non-reusability,

and potential biosafety risks [49-50]. These limitations have created a strong demand for alternative platforms for preoperative planning and surgical education.

3D-printed temporal bone models have emerged as practical tools to address this need. By converting high-resolution CT data into physical models, 3D printing can reproduce patient-specific temporal bone anatomy, including complex pathological variations. In clinical practice, such models help surgeons visualize spatial relationships beyond conventional two-dimensional imaging and rehearse key steps before surgery [12], particularly in complex cases such as lateral skull base lesions, facial nerve decompression, congenital malformations, or revision procedures. In education, 3D-printed temporal bones provide a safe, reproducible, and cost-effective supplement to cadaveric specimens. They can be produced in large numbers, tailored to different levels of difficulty, and repeatedly used for structured training. Several studies have shown that these models provide acceptable anatomical fidelity and realistic depth perception, while the SAPIENS model has further demonstrated value in otologic simulation and resident training [15, 51].

The utility of these models is further enhanced by advances in imaging, such as ultra-high-resolution CT and photon-counting computed tomography, which provide more detailed datasets for model reconstruction [52-53]. Nevertheless, 3D-printed temporal bone models cannot fully replace cadaveric dissection. Current limitations include incomplete simulation of soft tissue, variable drilling feedback, limited bleeding simulation, and differences in tactile realism compared with human bone. Therefore, their most appropriate role is as part of a complementary training system: 3D-printed models for early anatomical learning, repeated procedural rehearsal, and patient-specific preoperative planning, and cadaveric bones for advanced tissue handling and higher-fidelity surgical experience [53]. Such an integrated strategy may improve the accessibility, standardization, and effectiveness of otologic surgical training.

## Conclusion

3D printing technology is profoundly transforming traditional models of otological diagnosis and treatment, and its unique value in precise restoration and functional reconstruction has been widely recognized. Based on the systematic review presented in this paper, research progress and future directions in this field can be summarized as follows:

At the technological foundation level, advances in imaging technology have provided high-precision data sources for 3D printing, and the application of HRCT has enabled the clear visualization of micrometer-scale ossicular chain structures; Various printing technologies each have their specific applications: SLA excels at high-precision preoperative models; SLM is the preferred process for titanium alloy implants; FDM, with its cost advantage, serves educational purposes and the preparation of biodegradable scaffolds; and 3D bioprinting offers entirely new possibilities for tissue-engineered auricles; material selection strategies are becoming increasingly sophisticated.

In terms of morphological reconstruction, 3D printing technology has achieved comprehensive coverage from the outer ear



to the middle ear. In the field of outer ear reconstruction, advancements range from Kelly's 3D template model in 1993 to today's personalized titanium alloy scaffolds; in middle ear reconstruction, personalized ossicular prostheses have resolved the core issue of size mismatch in traditional prostheses through precise anatomical alignment; and temporal bone anatomical models have opened new avenues for resident training and preoperative simulation of complex cases, effectively addressing the shortage of cadaveric specimens.

However, the clinical translation of 3D printing technology in otology still faces multiple challenges. In terms of materials science, existing materials struggle to simultaneously meet the dual requirements of mechanical support and biocompatibility; regarding regulatory frameworks, the approval pathway for personalized implants remains unclear, and quality control standards have yet to be established; in terms of clinical adoption, printing costs and time efficiency remain significant barriers to widespread application. Resolving these issues requires the deep integration of materials science, biomedical engineering, clinical medicine, and regulatory science.

Looking ahead, with the development of cutting-edge technologies such as 4D printing, multi-material printing, and organ-on-a-chip, 3D printing in otology will continue to evolve toward intelligence, functionality, and personalization. The application of shape-memory materials holds promise for the development of smart implants that automatically unfold after minimally invasive implantation; multi-material gradient printing can construct functional scaffolds with gradually varying mechanical properties and bioactivity; and the combination of organoid technology with 3D printing may offer entirely new therapeutic strategies for intractable diseases such as inner ear hair cell regeneration. From the first 3D-printed ear template in 1993 to today's bioprinted auricles [3], over three decades of development have witnessed this technology's transformation from a "supporting tool" to a "therapeutic modality." We have every reason to believe that, with the deepening advancement of interdisciplinary collaboration, 3D printing technology will inevitably propel otological diagnosis and treatment into a new era of personalized precision medicine that unifies form and function.

## Abbreviations

3D: Three-Dimensional; 4D: Four-Dimensional; CAD: Computer-Aided Design; CT: Computed Tomography; DICOM: Digital Imaging and Communications in Medicine; DLP: Digital Light Processing; ECM: Extracellular Matrix; EHDA: Electrohydrodynamic Atomization; FDM: Fused Deposition Modeling; FGF-2: Fibroblast Growth Factor 2; FXIII: Factor XIII; GelMA: Gelatin Methacryloyl; HATG: Heparin-Conjugated Thrombin-Activatable Transglutaminase; HRCT: High-Resolution Computed Tomography; LOX: Lysyl Oxidase; MRI: Magnetic Resonance Imaging; PCL: Polycaprolactone; PLA: Polylactic Acid; SLA: Stereolithography; SLM: Selective Laser Melting; STL: Standard Tessellation Language; TGF- $\beta$ 3: Transforming Growth Factor Beta 3.

## Author Contributions

HYB and ZHY supervised the study and critically revised the

manuscript. All authors read and approved the final manuscript.

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Not applicable.

## Competing Interests

The authors declare no competing interests.

## Data Availability

All data generated or analyzed during this study are included in this published article.

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# Reconstruction of Vertical Hemilaryngeal Defect using Vascularized Tracheal Cartilage with Pectoralis Major Flap

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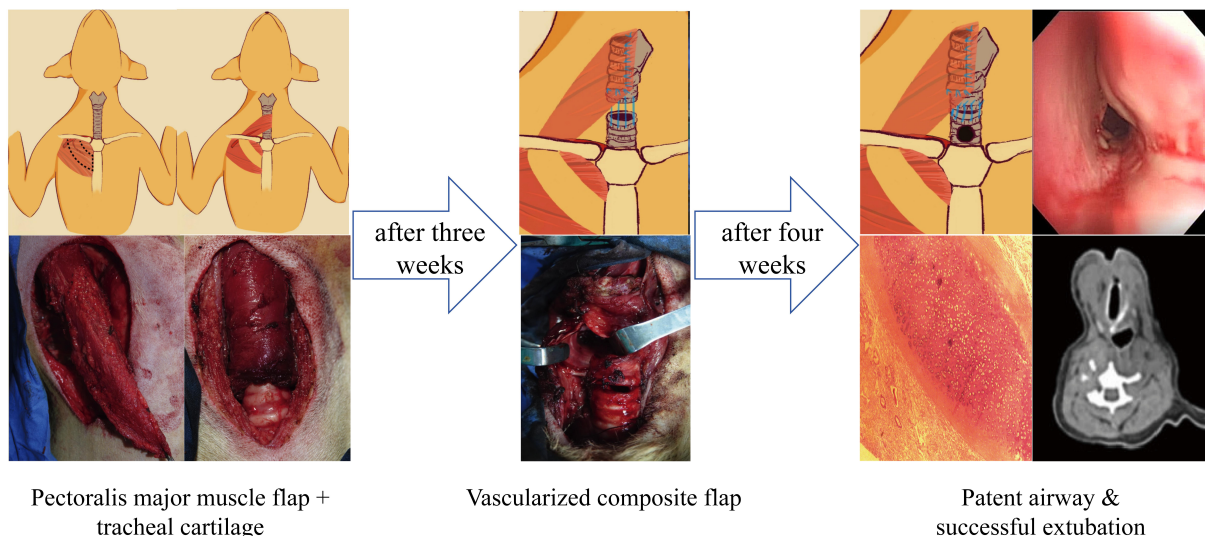
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## Graphical Abstract

### Reconstruction of Vertical Hemilaryngeal Defect using Vascularized Tracheal Cartilage with Pectoralis Major Flap



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# Reconstruction of Vertical Hemilaryngeal Defect using Vascularized Tracheal Cartilage with Pectoralis Major Flap

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## Abstract

**Objectives:** This study aims to explore the feasibility of utilizing tracheal cartilage vascularized with a pectoralis major muscle flap for the reconstruction of vertical hemilaryngeal defects and to provide an experimental foundation for such reconstructions.

**Methods:** Initially, the tracheal cartilages of nine Beagle canines were vascularized using pectoralis major muscle flaps. Subsequently, a defect model was established by vertically resecting the entire hemilarynx, and the vertical hemilaryngeal defect was reconstructed using the vascularized tracheal cartilage. Laryngeal computed tomography (CT) and video laryngoscopy were conducted at the following time points: pre-operation, immediately post-operation, and 28 days post-operation. At 28 days post-operation, the experimental dogs were euthanized after the completion of the examination. The reconstructed larynx was sectioned and subjected to hematoxylin and eosin (HE) staining.

**Results:** Eight dogs were successfully extubated and could consume normal food. Immediate postoperative video laryngoscopy indicated a well-sutured trachea, mucosa with normal color, and a restored and unobstructed laryngeal airway. Twenty-eight days post-operation, video laryngoscopy revealed a slightly edematous tracheal mucosa, a well-supported laryngeal cavity without collapse, routine healing of the tracheal suture, half-reconstruction of the laryngeal defect, and no obvious granulositis. Additionally, laryngeal CT demonstrated that the reconstructed laryngeal cavity was unobstructed and that the transplanted tracheal cartilage had maintained its position. Hematoxylin and eosin (HE) staining showed favorable survival of the pectoralis major composite flap and the transplanted tracheal cartilage.

**Conclusion:** This preliminary investigation demonstrates that short-term restoration of laryngeal structure and swallowing/respiratory function can be achieved through the reconstruction of vertical hemilaryngeal defects (including involvement of the unilateral cricoid cartilage) using tracheal cartilage vascularized with a pectoralis major flap.

**Keywords:** laryngotracheal stenosis; vertical hemilaryngeal defect; laryngotracheal reconstruction; vascularized tracheal cartilage; pectoralis major flap

## Introduction

Laryngeal trauma and tumor excision are prevalent causes of laryngeal defects. Firstly, laryngeal trauma is a frequent occurrence owing to the prominent position of the larynx on the central surface of the neck. The crux of the surgical management of laryngeal trauma lies in the prompt removal of necrotic lesions and the repair of laryngeal defects to reinstate the physiological functions of the larynx [1]. When laryngeal trauma is accompanied by infection, functional reconstruction of the larynx is contraindicated in the initial stage and can be carried out after the infection has been treated [2]. Secondly, laryngectomy for laryngeal tumors frequently leads to a laryngeal defect [3]. The repair and functional reconstruction of the laryngeal defect, in conjunction with the complete resection

of the tumor, can preserve laryngeal functions such as respiration, articulation, and protection of the lower airway, thereby enhancing the patients' quality of life [4].

Autologous tissue presents the merits of straightforward sampling, a high survival rate, and non-immunogenicity; consequently, it is being increasingly employed in laryngeal functional reconstruction [4-6]. At present, the pectoralis major flap, supraclavicular artery island flap, and free tissue transfer are among the autologous materials commonly utilized for repairing laryngeal defects [3, 7-11]. These restorative materials possess their respective indications and are regarded as suitable for the repair of various partial laryngeal defects, such as the repair of subtotal laryngeal defects, as well as horizontal and vertical hemilaryngeal defects. Despite continuous progress, the current clinical laryngeal reconstruction methods still de-

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pend on the posterior plate of the entire cricoid cartilage, and there remains no satisfactory surgical approach for an entire half-laryngeal defect (including the cricoid cartilage on one side) resulting from trauma or radical tumor resection [12]. The mucosal epithelium of the larynx exhibits structural and functional similarities to that of the trachea, which, in theory, renders the tracheal mucosal epithelium the most appropriate tissue for laryngeal defect reconstruction [13]. Nevertheless, there are few relevant experimental and clinical investigations in this domain. It is well-established that free tracheal cartilage lacks blood supply and is highly prone to ischemic necrosis. To utilize tracheal cartilage for repairing a laryngeal defect, a tissue flap with an ample blood supply should first be employed to establish the blood supply to the tracheal cartilage. The pectoralis major flap is one of the most frequently used tissue flaps for laryngeal defect reconstruction. This flap has the advantage of an abundant blood supply and a large tissue volume. It is also in close proximity to the myocutaneous flap of the neck and can offer a favorable vascular bed. However, to the best of our knowledge, there are no reports on the repair of horizontal or vertical hemilaryngeal defects using tracheal cartilage vascularized with the pectoralis major flap. In this study, we experimentally repaired a vertical hemilaryngeal defect in an animal model using tracheal cartilage vascularized with a pectoralis major flap. A positive outcome would provide an experiment-based approach to laryngeal defect reconstruction.

## Methods

Nine Beagle dogs (male, approximately 2 years old, and weighing approximately 15 kg) were provided by the Animal Center of the Second Military Medical University [license number: SCXK (Shanghai) 2012-0007]. The ethics committee of Changhai Hospital approved the experiment. The dogs were individually housed in standard cages under controlled temperature (20-22 °C), with a constant 12-hour light/dark cycle and unrestricted access to food and water.

### Preparation for Animal Surgery

All procedures were carried out under general anesthesia (1 ml/kg 3% sodium pentobarbital, i.v.). Oxygen was administered via a face mask, and the animals were monitored with pulse oximetry. The neck and chest were depilated with a 10% NaS solution. All microsurgical dissections were performed under  $\times 2.5$  magnification loupes and an operating microscope.

### Vertical Hemilaryngeal Defect Reconstruction Using Pectoralis Major Flap-Vascularized Tracheal Cartilage

#### First-stage prefabrication of vascularized tracheal cartilage

After disinfection and draping, a radial incision was made along the midline of the neck from the lower margin of the thyroid cartilage to the suprasternal fossa. The strap muscles were then separated from the midline to expose the larynx and trachea, while preserving the blood vessels and nerves. A diagonal chest incision was made from the intersection of the right midclavicular line and the lower edge of the clavicle to expose the pectoralis major muscle, which was dissected along the side of the sternum and retracted laterally to create a pectoralis major muscle flap approximately 5 cm wide and 25 cm long. The pedicled pectoralis major muscle flap was tunneled

through the subclavian region to the inferior border of the first tracheal ring, wrapped around the 3rd–5th tracheal cartilages to cover an approximately 4-cm tracheal segment, and secured to the tracheal membrane with 3-0 absorbable sutures as a vascular carrier (Figure 1A, B). Finally, the surgical cavity was sequentially irrigated, a negative pressure tube was inserted, and the wound was closed.

### Second-stage creation of the vertical hemilaryngeal defect

After the three-week prefabrication step to vascularize the tracheal cartilage, vertical hemilaryngeal defect reconstruction was performed. The preoperative preparation was the same as that for the first-stage surgery. The cervical skin was incised, and the pedicled pectoralis major muscle-tracheal cartilage flap was cut from the lower edge of the first tracheal ring to the 4th or 5th tracheal rings, producing a flap length of approximately 4 cm (Figure 1C). The right hemilarynx, including the thyroid cartilage plate, arytenoid cartilage, and periglottic space, was removed vertically to prefabricate a hemilaryngeal defect model (Figure 1D).

### Reconstruction of the vertical hemilaryngeal defect

An end-to-end anastomosis of the remaining trachea was performed, and a tracheostomy was fixed to the skin below the anastomosis. The trachea with the muscle flap was sutured to the remaining left part of the larynx, and the lower edge was sutured to the first tracheal ring (Figure 1E, F). The surgical cavity was irrigated routinely, and a disposable suction bulb was inserted in the neck. The cervical skin incision was closed, and a 14 mm metal tracheal cannula was inserted into the tracheostomy.

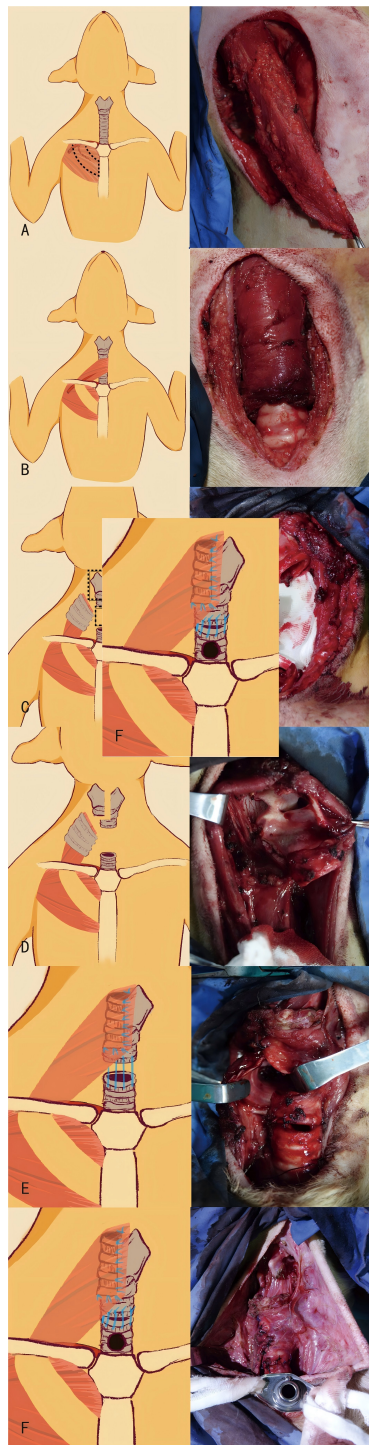
### Postoperative Care

Following the surgical procedure, the canine subject was maintained in a thermally regulated environment and permitted to regain consciousness spontaneously upon the dissipation of the anesthetic effect. To mitigate the risk of infection throughout the experimental period, all animals received a course of antibiotic therapy (penicillin, 1.6 million units per day) for a duration of one week. An anti-bite collar was affixed around the dog's cervical region to preclude any interference with the surgical wound, and the metal inner housing was subjected to daily cleaning and disinfection using sputum aspiration techniques. Subsequent to the initial stage of surgery, the dogs were provided with a standard diet, and enteral nutrition suspension was administered via a nasogastric tube subsequent to the second stage of the surgical intervention.

### Postoperative Monitoring and Assessment

#### Definition of Primary Endpoints

Successful extubation was defined as tracheostomy tube occlusion for 48 h without dyspnea, stridor, or desaturation ( $\text{SpO}_2 \geq 95\%$  on room air), according to standard veterinary tracheostomy decannulation criteria [14-15] and previous canine laryngeal reconstruction studies [16]. Normal feeding was defined as the ability to consume solid kibble without coughing, aspiration, or leakage from the tracheostomy site for 3 consecutive days, consistent with established postoperative functional assessment in canine head and neck models [16].



**Figure 1. Surgical procedure for vertical hemilaryngeal defect reconstruction with vascularized tracheal cartilage using a pectoralis major flap.** (A) The pectoralis major muscle and the thoraco-acromial artery. (B) The pedicled pectoralis major muscle flap wrapped around the tracheal cartilage surface. (C) The trachea was cut from the lower edge of the first tracheal ring to the 4th or 5th rings to create a pedicled pectoralis major muscle-tracheal cartilage flap. (D) The right hemilarynx was resected vertically to create a hemilaryngeal defect model. (E) The trachea with the muscle flap was sutured to the remaining left part of the larynx. (F) End-to-end anastomosis of the remaining trachea was performed, and a lower tracheostomy was established. PMF:pectoralis major flap;TC:tracheal cartilage.

### Evaluation of swallowing and respiratory function

Seven days after surgery, we gave fluid feedings to check for any food exudation in the tracheotomy hole as well as to check for the presence of coughing during drinking and eating. We recorded recovery based on the time required for swallowing function and changed to normal feeds if there was no choking or pharyngeal fistula. Fourteen days post-operatively, the tracheal tubes were occluded for 2 days and removed if no difficulty in deglutition or breathing was noted.

### Computed tomography examination of the larynx and video laryngoscopy examination

Computed tomography (CT) and video laryngoscopy were performed under general anesthesia at the following time points: preoperatively, immediately postoperatively, and 28 days post-operatively. CT scans were obtained in 1-mm slices. After each iteration of the laryngeal CT scan, a video laryngoscope was advanced through the glottis via the direct laryngoscope to observe the new half-laryngeal mucosa, laryngeal and tracheal cavities, suture healing, and surface color and luster.

### Histologic evaluation

The experimental dogs were euthanized by intravenous injection of KCl after anesthesia, and the reconstructed laryngeal tissue specimens were paraffin-embedded and subjected to hematoxylin-eosin (HE) staining.

## Results

### Postoperative survival and functional recovery

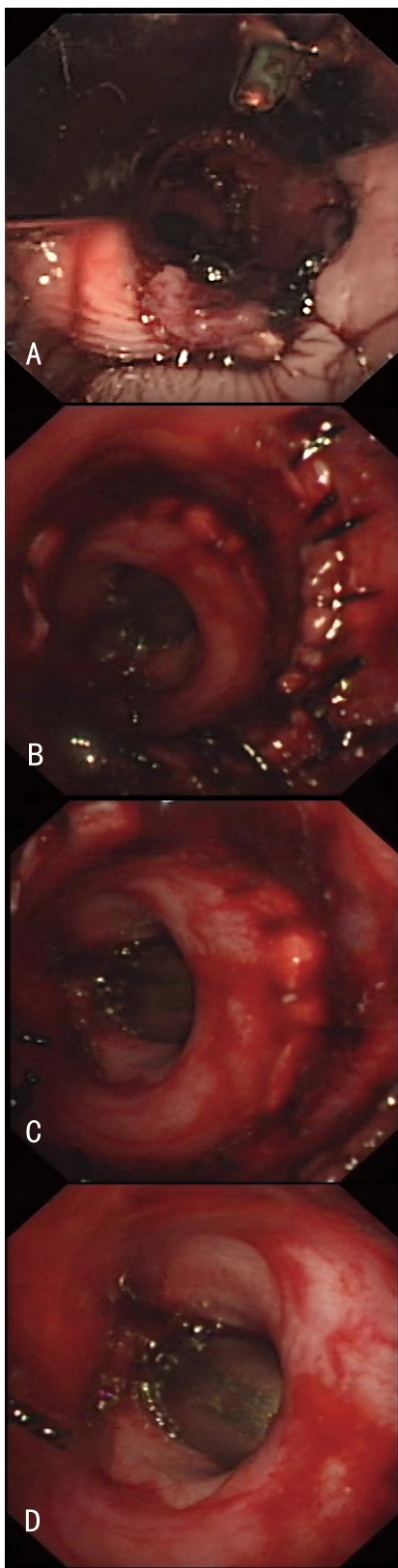
Table 1 shows the normal feeding time, postoperative survival time, and extubation time of the nine experimental dogs. All dogs survived the surgeries, but dog #7 developed a postoperative infection, resulting in a local neck abscess. The abscess was treated with incision and drainage, followed by wound dressings, and eventually healed completely. All dogs were observed and evaluated for 28 days after the surgery. Among the surviving animals, the median time to resumption of normal feeding was 16 days (range: 14-17 days), and the median time to successful decannulation was 18 days (range: 16-21 days) (Table 1). Their swallowing difficulties were relieved at different time points after the surgery, and they were successfully extubated to resume normal feeding. No respiratory distress after extubation in all dogs except dog #7.

**Table 1. The normal feeding time, postoperative survival time and extubation time of the experimental dogs.**

| Serial number | Weight (Kg) | Survival time | Postoperative extubation time | Normal feeding time after surgery |
|---------------|-------------|---------------|-------------------------------|-----------------------------------|
| 1             | 14.5        | 28 d          | 18 d                          | 16 d                              |
| 2             | 16.0        | 28 d          | 17d                           | 16 d                              |
| 3             | 15.0        | 28 d          | 20 d                          | 16 d                              |
| 4             | 17.0        | 28 d          | 21 d                          | 17 d                              |
| 5             | 16.0        | 28 d          | 17 d                          | 15 d                              |
| 6             | 16.5        | 28 d          | 16 d                          | 14 d                              |
| 7             | 13.5        | 28 d          | *                             | 18 d                              |
| 8             | 15.5        | 28 d          | 18 d                          | 17 d                              |
| 9             | 15.0        | 28 d          | 17 d                          | 15 d                              |

Note: \*: No extubation



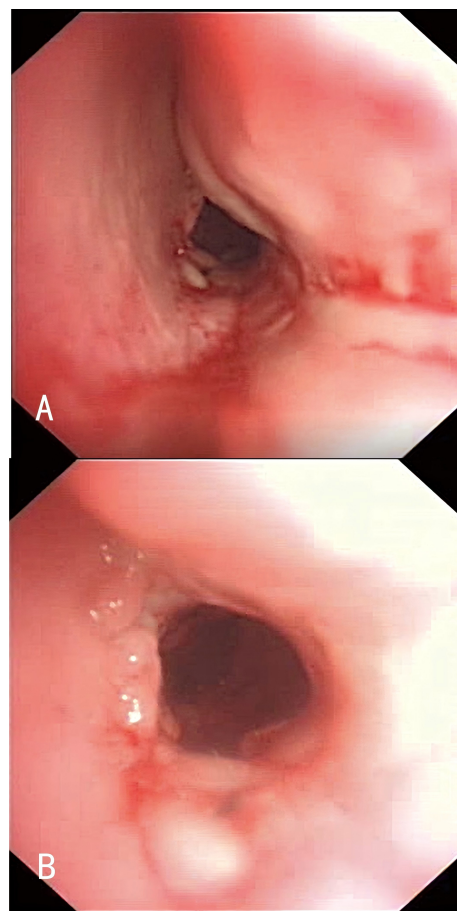


**Figure 2. Immediate postoperative video laryngoscopy.** (A) Immediate postoperative video laryngoscopy findings. (B) Glottic inlet and suture line (white arrow). (C) Anastomosis at the upper margin of the transplanted tracheal cartilage (white arrow). (D) Anastomosis at the lower margin of the tracheal cartilage and the end-to-end tracheal anastomosis.

Dog #7 developed a localized cervical abscess on postoperative day 10, which was managed with incision and drainage plus daily dressing changes. Histology showed excessive scar proliferation and poor cartilage survival on the operative side, leading to persistent airway stenosis and failed extubation. This case highlights that postoperative infection is a critical risk factor for graft failure; strict aseptic technique, prolonged antibiotic prophylaxis, and tension-free anastomosis are recommended to minimize this complication.

#### Video laryngoscopy findings

One day after surgery, video laryngoscopy revealed complete removal of the right vocal cord. Furthermore, we could see that the tracheal wall was convex outwardly and had a smooth mucosa. It was noted that the larynx and the lower edge of the first tracheal ring were well anastomosed, and the metal tracheal tube was in place (Figure 2). On postoperative day 28, video laryngoscopy confirmed normal movement of the left arytenoid cartilage. In addition, the right tracheal mucosa was pale pink in appearance, and a well-supported and intact laryngeal chamber was noted. There was a small amount of white secretion adhering to the mucosal surface, and the laryngeal and tracheal cavities showed no obvious granulation hyperplasia (Figure 3).

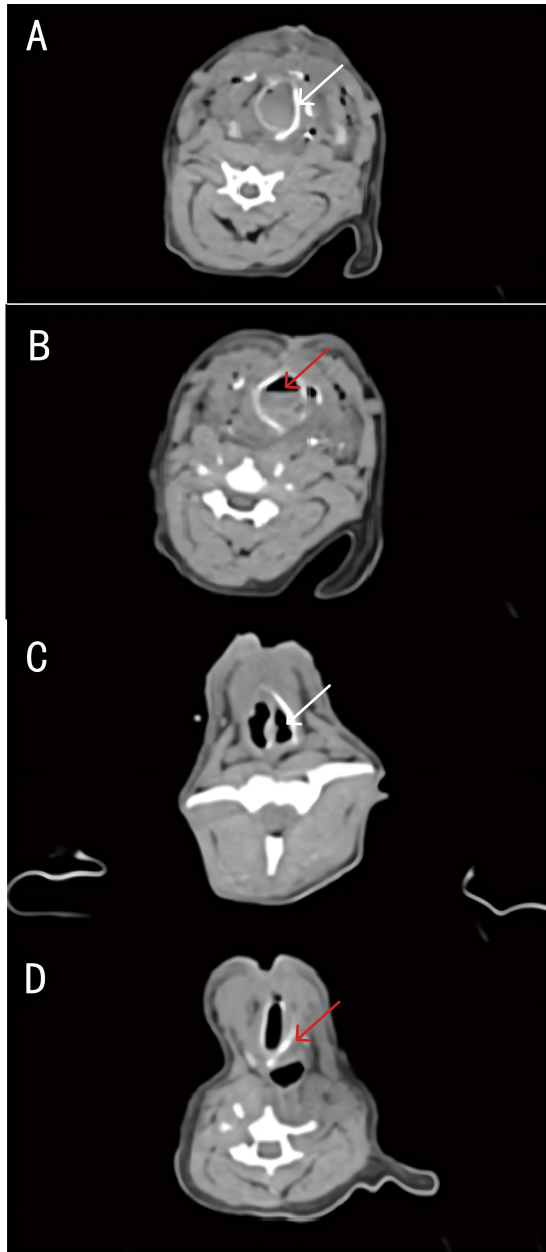


**Figure 3. Video laryngoscopy findings at 28 days postoperatively.** (A) Glottic entrance. (B) Laryngotracheal anastomosis site. Video picture indicates that slightly edematous mucosa, a well-supported laryngeal cavity without collapse, and well-healed anastomosis without obvious granulation.



### CT examination results and analysis of the larynx and trachea

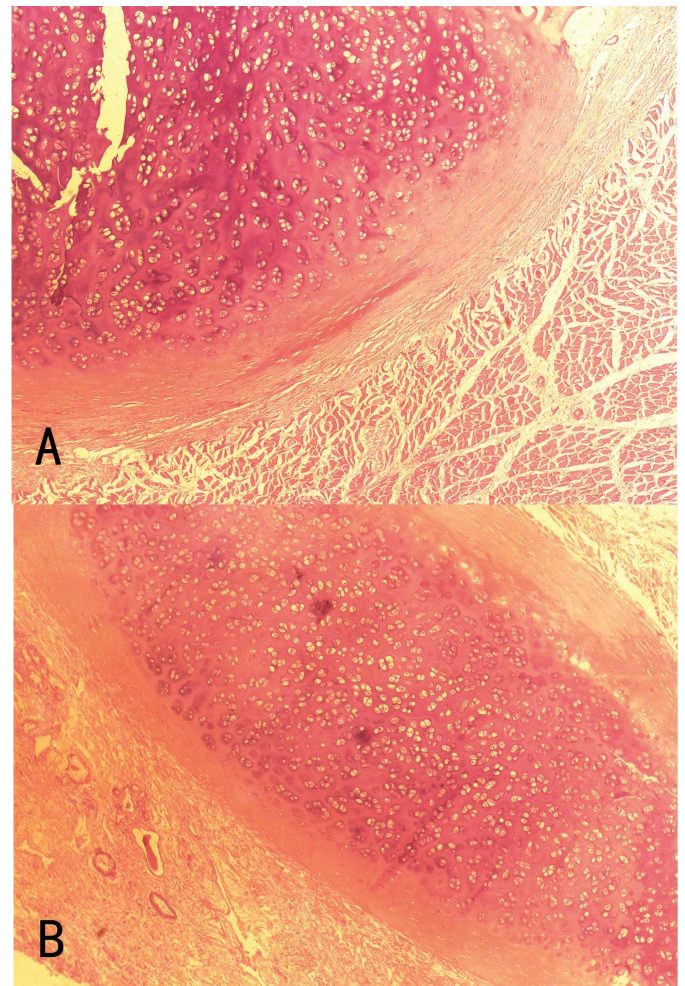
In this case, CT images of the larynx taken immediately post-operatively showed the semicircular tracheal cartilage of the right graft segment as well as the anastomosis with the left larynx. Post-operatively, an excessive fluid density shadow was observed in the laryngeal cavity, indicating the formation of a gas-liquid level (Figure 4A, B). Next, a CT scan of the larynx 28 days post-operatively showed an unobstructed laryngeal cavity and well-supported right reconstructed laryngeal cartilage (Figure 4C, D).



**Figure 4. Postoperative computed tomography (CT) images of the larynx.** (A, B) Immediate postoperative CT images showing the semi-circular transplanted tracheal cartilage segment (white arrow) and a gas-fluid level in the laryngeal cavity (red arrow). (C, D) CT images at 28 days postoperatively demonstrating a patent laryngeal cavity (white arrow) and well-supported reconstructed laryngeal cartilage on the right side (red arrow).

### Histological results and analysis

Eight dogs were extubated, and complete histologic data at 28 days were available for all eight dogs. The histologic findings for the reconstructed hemilarynx of the eight dogs showed restoration of good tracheal cartilage structure and mucosal structure on the operative side of the hemilarynx, which did not appear significantly different from the laryngeal cartilage on the normal side (Figure 5A, B). However, extubation failed in one dog (dog 7), and this dog had more scar and connective tissue, significantly poor healing, and poor cartilage structure on the operative side of the larynx.



**Figure 5. Hematoxylin and eosin (H&E) staining of the laryngeal tissue (28 days postoperatively).** (A) Normal side of the hemilarynx. (B) Operative (reconstructed) side of the hemilarynx, showing restored tracheal cartilage and mucosal structures with no significant difference from the normal side.

### Discussion

Adequate cartilaginous support of the airway is essential for the repair of laryngeal defects, and a relatively complete posterior plate of cricoid cartilage is essential to avoid uncompensated aspiration. Despite recent advances in the field of laryngotracheal reconstruction, current clinical approaches in this area require a complete posterior plate of cricoid cartilage [12]. Therefore, there is still no satisfactory repair approach for

a trauma- or radical tumor treatment-induced entire hemi-laryngeal defect that includes the cricoid cartilage on one side. For cartilaginous tissue reconstruction, the costal cartilage is clinically the most widely used tissue [17-19]; however, its application in laryngeal defect reconstruction is limited by some of its characteristics, such as difficulties in vascularization and its long and narrow shape. Instead, the tracheal cartilage adjacent to the larynx, which has a support resembling a C-shaped hose ring, is well suited for laryngeal defect reconstruction. Because of the similarity between its internal epithelium and the laryngeal epithelium and the ease of epithelialization, the likelihood of granulation hyperplasia formation is reduced [13]. The shape, structure, and function of the airway and the internal mucosal epithelium are theoretically identical, making the trachea vascularized by a pectoralis major flap the most suitable option for reconstructing laryngeal defects and ensuring blood supply to the reconstructed tissue; however, this is supported by few relevant experimental and clinical studies. The main reason for this gap in the literature is that the reconstructed laryngeal tissue after tracheal cartilage transfer to the larynx requires a vascularized tissue flap to provide blood supply. Some researchers have reported the use of free omentum to wrap the trachea and reconstruct laryngeal defects, followed by using small blood vessel anastomosis to provide blood supply to the reconstructed tissue. However, the free-flap technique is complex, and the chances of complications further limit its application. The pectoralis major flap is among the most commonly used tissue flaps for laryngeal defect reconstruction [10]. The advantages of this flap include large tissue volume and abundant blood supply, and its proximity to the neck, which can provide an ideal vascular bed for the tracheal cartilage [20]. The experimental design of this project aimed to solve the problems of vascularization and epithelialization of the graft. Therefore, we chose to use tracheal cartilage vascularized with the pectoralis major flap for laryngeal reconstruction.

According to our experimental animal model using Beagle dogs, we observed that eight of nine dogs were successfully extubated and their dysphagia gradually resolved over time, allowing them to eat normally. The experimental results suggested a high success rate for the surgical procedure. On day 7, we attempted to feed the dogs a liquid diet to exercise and restore swallowing function, but some dogs experienced varying degrees of coughing and leakage of liquid food from the fistula tube. However, they were typically able to eat normally at an average of 16 days after surgery, and the tracheal tube could be removed at an average of 18 days. Immediate electronic laryngoscopy after surgery showed a smooth laryngo-tracheal cavity after reconstruction, with normal mucosal color and well-sutured anastomosis between the residual larynx and the transplanted trachea. Long-term postoperative findings also showed normal color of the mucosal epithelium of the tracheal cartilage in the right graft segment, consistent with the surrounding laryngeal tissue, and no obvious granulation formation. In addition, the laryngeal cavity was unobstructed, indicating sufficient mucosal blood supply and well-preserved mucosal clearance function. CT scans of the throat showed that in the early postoperative period, the transplanted tracheal cartilage segment had a semi-arc shape, causing an enlarged laryngeal cavity and an increased risk of aspiration during swallowing. In the later period, the tracheal cartilage of

the transplanted segment formed a good laryngeal support structure with the left residual thyroid cartilage and cricoid cartilage, reconstructing a smooth laryngeal cavity. The curvature of the transplanted tracheal cartilage segment decreased with compensatory movement of the left arytenoid cartilage, resulting in a reduced laryngeal cavity and decreased risk of aspiration, leading to the restoration of swallowing function. In this study, dog #7 developed a localized neck abscess postoperatively. Although it healed completely with drainage and dressings. This dog had more scar and connective tissue, significantly poorer healing, and poor cartilaginous structure on the surgical side of the larynx, which eventually led to failed extubation. Therefore, postoperative anti-infective treatment should be intensified, and tension on the anastomosis should be reduced.

Based on the above results, when reconstructing and repairing a vertical hemilaryngeal defect, the structure and function of the larynx can be satisfactorily restored using the pectoralis major flap in combination with tracheal cartilage. The following aspects should be considered when performing the operation: First, a period of 3 weeks was required for complete vascularization of the tracheal cartilage after the first-stage operation. Second, in the first-stage operation, the pectoralis major flap must be wrapped downward from the first tracheal ring. With a low wrapping position, the pectoralis major flap may not be long enough for the second-stage reconstruction, causing excessive anastomotic tension in the larynx. Third, the trachea must be appropriately trimmed according to the size of the defect to achieve an adequate laryngeal cavity. Fourth, during reconstruction, every step should be taken to avoid infection and inflammatory reaction, so that fibrosis or necrosis of the transplanted larynx can be avoided.

However, this study has several limitations. First, the small sample size ( $n=9$ ) and absence of a control group (e.g., rib cartilage reconstruction comparator) limit the strength of conclusions. Second, the 28-day follow-up only reflects short-term feasibility, and long-term outcomes ( $\geq 6$  months) regarding cartilage resorption, airway stability, and laryngeal phonatory function remain unknown. Third, swallowing and respiratory function were assessed via qualitative observation rather than validated scoring systems (e.g., Penetration-Aspiration Scale). Future studies with larger cohorts, longer follow-up, and objective functional assessments are needed to validate these preliminary findings.

Future clinical translation of this technique warrants caution. While the approach theoretically expands indications for organ-preserving surgery in select advanced laryngeal cancers, the current 3-week prefabrication interval conflicts with standard oncologic principles requiring immediate tumor resection. Potential solutions include simultaneous neck dissection with induction chemotherapy during the prefabrication stage, or adopting single-stage designs with prevascularized tissue-engineered grafts. These hypotheses require validation in tumor-bearing animal models prior to human trials. We believe that future breakthroughs in laryngeal defect repair technology will come from advanced techniques such as 3D printing of the repair laryngeal cartilage [6, 21]. Joanna F et al. fabricated a polycaprolactone scaffold using 3D printing technology and coated it with porcine-derived small intestinal submucosa extracellular matrix. The resulting bioengineered graft was transplanted into experimental pigs to replace a 2-cm segment of

the trachea. The experimental results showed that loading the small intestinal submucosa extracellular matrix on the surface of the graft could promote a good integration of the graft with the natural organ [22]. This exploration of constructing multifunctional organ prostheses using the scaffold-free approach holds great promise for bringing about a major breakthrough in the field of biological reconstruction of the laryngeal defect [23]. These will provide new ways to reconstruct laryngeal mucosal tissue and help restore laryngeal function by reconstructing the laryngeal structure. Certainly, there is still a long way to go before these advances are realized, as there are many technical problems that need to be solved.

In conclusion, this pilot study demonstrates that vertical hemilaryngeal defects (including unilateral cricoid cartilage defects) can be successfully reconstructed using pectoralis major flap-vascularized tracheal cartilage, with satisfactory short-term restoration of laryngeal structure and swallowing/respiratory function. The technique does not require a laryngeal mould due to the spacious postoperative airway, and offers a high success rate of extubation. Further studies are warranted to validate long-term outcomes and expand the clinical applicability of this approach.

## Conclusion

This preliminary investigation demonstrates that short-term restoration of laryngeal structure and swallowing/respiratory function can be achieved through the reconstruction of vertical hemilaryngeal defects (including involvement of the unilateral cricoid cartilage) using tracheal cartilage vascularized with a pectoralis major flap.

## Abbreviations

3D: Three-dimensional; CT: Computed tomography; HE: Hematoxylin and eosin; i.v.: Intravenous; KCl: Potassium chloride; NaS: Sodium sulfide; SCXK: Laboratory Animal Production License; SpO<sub>2</sub>: Peripheral capillary oxygen saturation.

## Author Contributions

Fangzheng Yi: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. Mengjie Chen: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. Jing Song: Writing - original draft, Writing - review & editing. Guanghao Zhu: Investigation, Writing - original draft. Tianxiang Zhu, Haopu Li, Haojun Yu: Data curation, software. Hongliang Zheng & Minhui Zhu: Conceptualization, Funding acquisition, Methodology, Resources, Supervision. All authors read and approved the final manuscript.

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## Ethics Approval and Consent to Participate

This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th edition, USA). The animal experiments were approved by the Ethics Committee of Changhai Hospital. The Beagle dogs were provided by the Animal Center of the Second Military Medical University [license number: SCXK (Shanghai) 2012-0007]. All procedures were performed under general anesthesia to minimize animal suffering. The study did not involve human participants; therefore, informed consent was not applicable.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

Not Applicable.

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# Multilayered Regulation of SLC7A11 in Cancer: From Expression Control to Functional Ferroptosis Adaptation

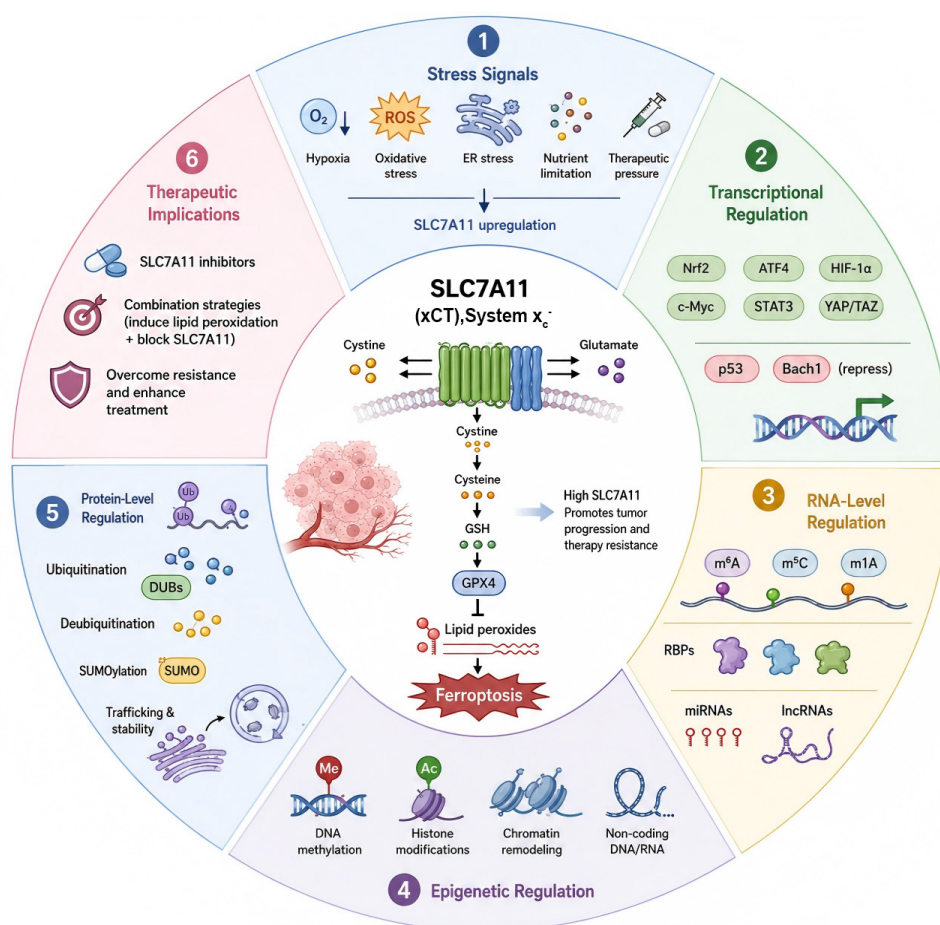
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## Graphical Abstract



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# Multilayered Regulation of SLC7A11 in Cancer: From Expression Control to Functional Ferroptosis Adaptation

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## Abstract

SLC7A11, also known as xCT, is the functional light-chain subunit of system xc<sup>-</sup> and mediates extracellular cystine uptake in exchange for intracellular glutamate. By supplying cystine for glutathione (GSH) biosynthesis and sustaining glutathione peroxidase 4 (GPX4)-dependent detoxification of lipid peroxides, SLC7A11 occupies a central position in ferroptosis control. In cancer, SLC7A11 is not merely an amino acid transporter, but a stress-adaptive hub through which tumor cells convert hypoxia, oxidative stress, endoplasmic reticulum stress, nutrient limitation, and therapeutic pressure into antioxidant defense and ferroptosis resistance. This review summarizes the multilayered regulation of SLC7A11 expression in cancer. We first introduce the structural and functional basis of SLC7A11 and its biological significance in ferroptosis and tumor progression. We then discuss its epigenetic regulation, transcriptional regulatory network, post-transcriptional and translational control-including RNA modifications, RNA-binding proteins, and non-coding RNA networks-and protein homeostasis and post-translational regulation. Finally, we highlight therapeutic strategies centered on SLC7A11 and propose that effective ferroptosis-based cancer therapy may require simultaneous induction of lipid peroxidation stress and blockade of adaptive SLC7A11 upregulation or stabilization.

**Keywords:** SLC7A11; xCT; system xc<sup>-</sup>; ferroptosis; cystine transport; glutathione; RNA modification; ubiquitination; cancer therapy

## Introduction

Ferroptosis is a regulated form of cell death driven by iron-dependent lipid peroxidation. Unlike apoptosis or necrosis, ferroptosis is defined by a specific metabolic vulnerability, namely the failure to detoxify phospholipid hydroperoxides in cellular membranes. Since its initial description by Dixon et al., ferroptosis has become a major conceptual framework for understanding tumor biology, therapy resistance, metabolic stress, and redox homeostasis [1-2]. The importance of SLC7A11 is particularly evident in cancer. Tumor cells are frequently exposed to persistent oxidative stress, hypoxia, nutrient limitation, endoplasmic reticulum stress, and therapy-induced damage. These conditions would, in principle, render malignant cells highly vulnerable to ferroptosis. However, many cancers acquire robust antioxidant programs that enable survival under such hostile conditions, and SLC7A11 is a key determinant of this adaptive phenotype. High SLC7A11 expression has been associated with tumor growth, metastasis, stemness, radioresistance, chemoresistance, and reduced sensitivity to ferroptosis-inducing therapies [3-5]. In addition, SLC7A11 reshapes amino acid metabolism, glutamine dependence, and redox balance, thereby creating both survival advantages and exploit-

able metabolic liabilities [3, 5]. Conversely, tumor suppressive pathways, especially p53, can repress SLC7A11 transcription and lower the threshold for ferroptosis [6].

SLC7A11 is one of the most important determinants of this adaptive state. SLC7A11, also known as xCT, is the light-chain subunit of system xc<sup>-</sup> and usually functions as a heterodimer with the heavy-chain subunit SLC3A2 [1-2]. This transporter imports extracellular cystine into the cell in exchange for intracellular glutamate. After entering the reducing intracellular environment, cystine is converted to cysteine, which is then used for glutathione (GSH) synthesis. GSH provides reducing equivalents for glutathione peroxidase 4 (GPX4), the key enzyme that detoxifies lipid peroxides and suppresses ferroptosis [3, 7-8]. Thus, the SLC7A11-cystine-GSH-GPX4 axis forms a core metabolic defense system against lipid peroxidation.

Accumulating evidence indicates that aberrant SLC7A11 expression in cancer is not governed by a single regulatory pathway. Rather, it is sustained by a multilayered regulatory network involving epigenetic remodeling, stress-responsive transcription factors, RNA modifications, RNA-binding proteins, non-coding RNAs, ubiquitination, deubiquitination, SUMOylation, and membrane trafficking [9-22]. Accordingly, understanding how SLC7A11 expression is regulated has become

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increasingly important, not only for clarifying the molecular basis of tumor stress adaptation, but also for identifying therapeutic vulnerabilities that may be exploited to sensitize tumors to ferroptosis-based treatment.

This review focuses on the expression regulation of SLC7A11 in cancer. Rather than listing individual regulators in isolation, we organize current evidence into four mechanistic layers: chromatin and epigenetic regulation, transcriptional control, RNA-level regulation, and protein-level regulation. We emphasize that SLC7A11 functions as a stress-adaptive hub through which cancer cells convert adverse microenvironmental and therapeutic signals into ferroptosis resistance. This framework also highlights a therapeutic principle: simply increasing oxidative stress may be insufficient if tumor cells simultaneously activate SLC7A11-dependent defenses. More effective strategies may require inducing ferroptotic pressure while blocking adaptive SLC7A11 upregulation or stabilization. This review is a narrative review that aims to synthesize and discuss the multilayered regulation of SLC7A11 in cancer, with a focus on its role in ferroptosis adaptation. As such, it does not follow a systematic search strategy or predefined inclusion criteria.

### **Structural and Functional Basis of SLC7A11 and Its Role in Tumorigenesis**

SLC7A11 is the substrate-selective light-chain component of system  $x_c^-$  and typically forms a disulfide-linked heterodimer with the heavy-chain subunit SLC3A2 [1-2]. Within this transporter complex, SLC3A2 mainly contributes to membrane trafficking and structural stabilization, whereas SLC7A11 confers the transport specificity required for cystine/glutamate exchange [1-2]. SLC7A11 is a 12-transmembrane-domain protein with both its N- and C-terminal domains localized to the cytoplasm, whereas SLC3A2 is a single-pass transmembrane protein with an intracellular N-terminus and a heavily glycosylated extracellular C-terminus [23]. This structural arrangement underlies the functional output of system  $x_c^-$  at the plasma membrane.

SLC7A11 is positioned upstream of one of the most important anti-ferroptotic metabolic axes in cancer cells. By mediating cystine uptake, SLC7A11 ensures the intracellular supply of cysteine, a rate-limiting precursor for GSH biosynthesis. GSH then supports GPX4-dependent reduction of lipid hydroperoxides, thereby preventing lethal membrane damage. Inhibition of system  $x_c^-$  by genetic depletion of SLC7A11 or pharmacological blockade reduces cystine availability, decreases GSH synthesis, impairs GPX4 activity, and drives the accumulation of lipid peroxides [3, 7-8].

This metabolic role explains why SLC7A11 is frequently associated with therapy resistance. Many anticancer treatments increase reactive oxygen species, perturb mitochondrial metabolism, or induce lipid peroxidation. Tumor cells with high SLC7A11 expression are better equipped to buffer these pressures. In this setting, SLC7A11 acts as a key buffer, allowing cancer cells to sustain redox homeostasis despite ongoing oxidative injury [3-5]. However, this protection comes with trade-offs. Because system  $x_c^-$  exports glutamate while importing cystine, strong SLC7A11 activity can influence glutamate metabolism and create specific nutrient dependencies. These metabolic liabilities may be exploited therapeutically, particularly in tumors that are highly dependent on cystine uptake and GSH synthesis.

SLC7A11 also functions as a readout of stress-adaptive transcriptional activity. Hypoxia, oxidative stress, endoplasmic reticulum stress, nutrient stress, inflammatory cues, and oncogenic signaling can all converge on SLC7A11 induction [9-16, 24-30]. The resulting increase in cystine uptake allows tumor cells to transform potentially damaging stress signals into antioxidant capacity. In this sense, high SLC7A11 expression should not be interpreted only as a static biomarker. It represents a dynamic adaptive state maintained by coordinated transcriptional, post-transcriptional, and post-translational regulation.

### **Epigenetic Regulation of SLC7A11: Chromatin Remodeling and Histone Modifications**

In addition to being regulated by stress-responsive transcription factors, the expression of SLC7A11 is subject to epigenetic control at the level of chromatin architecture and histone modification. These mechanisms shape the accessibility of the SLC7A11 locus and the permissiveness of its transcriptional activation without altering the underlying DNA sequence.

Transcription of SLC7A11 depends on a permissive chromatin environment. ATP-dependent chromatin remodeling is an important component of this process. In addition to binding the promoter, NRF2 can recruit SNF2L, the ATPase subunit of the ISWI chromatin remodeling complex, to the SLC7A11 locus [31]. SNF2L promotes nucleosome repositioning and increases local chromatin accessibility, thereby facilitating transcriptional activation. This finding suggests that NRF2 functions not only as a transcription factor but also as a coordinator of chromatin remodeling.

The SWI/SNF complex also contributes to SLC7A11 regulation. Its subunit ARID1A binds the SLC7A11 promoter and supports transcriptional activation. Notably, although ARID1A is widely regarded as a tumor suppressor and is frequently mutated in cancer, its loss reduces rather than increases SLC7A11 expression, in contrast to the effects of p53 or BAP1 deficiency [32]. This highlights the context dependence of epigenetic regulators. Because ARID1A-deficient cells exhibit reduced SLC7A11 expression and impaired glutathione metabolism, they may be particularly vulnerable to therapies targeting redox homeostasis [32].

Histone modifications provide an additional layer of control. In breast cancer, HNF4 $\alpha$  recruits p300/CBP to the SLC7A11 promoter, promotes histone acetylation, and activates SLC7A11 transcription, thereby suppressing ferroptosis [33]. By contrast, the repressive mark H3K27me3 inhibits SLC7A11 expression. A recent study showed that m6A-dependent regulation of KDM6B and GATA3 reduces KDM6B-mediated H3K27me3 demethylation and weakens GATA3-driven transactivation, resulting in coordinated suppression of SLC7A11 [34]. This mechanism illustrates how RNA modification, histone regulation, and transcription factor activity can converge on the same locus. Overall, chromatin remodeling and histone modification collectively determine the accessibility and transcriptional permissiveness of the SLC7A11 locus, and thereby set the baseline upon which stress-responsive transcription factors act.

### **Transcriptional Regulatory Network of SLC7A11**

Transcriptional control constitutes the most direct mechanism by which cancer cells increase SLC7A11 expression in response to environmental and therapeutic stress. Multiple

signaling pathways converge on the SLC7A11 locus, forming an integrated regulatory network that determines ferroptosis sensitivity.

### KEAP1–NRF2 Signaling as a Central Antioxidant Axis

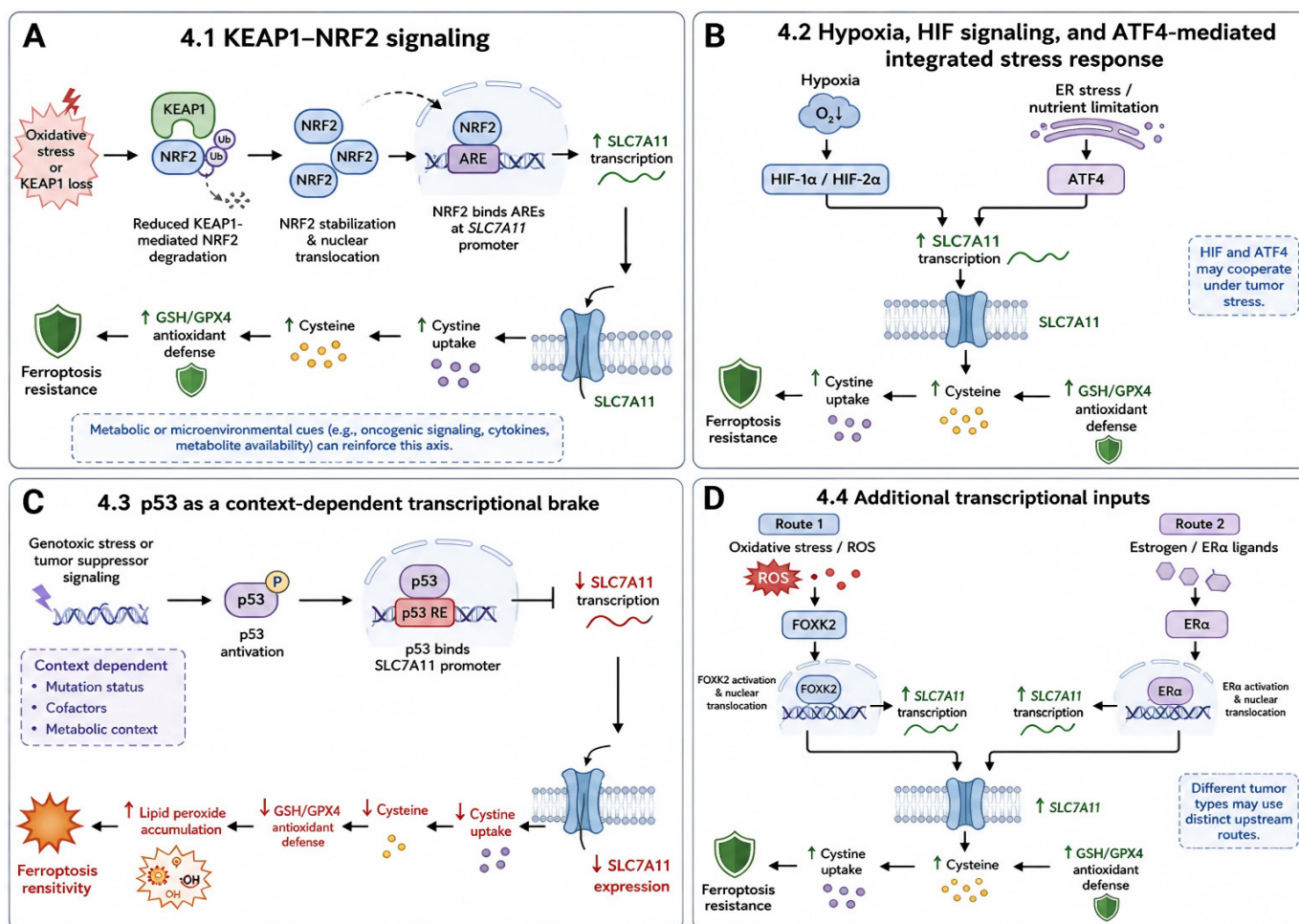
Among all transcriptional regulators, the KEAP1–NRF2 pathway is one of the most prominent drivers of SLC7A11 expression. Under basal conditions, KEAP1 promotes NRF2 degradation, whereas oxidative stress or KEAP1 dysfunction stabilizes NRF2 and enables its nuclear translocation. Activated NRF2 binds antioxidant response elements and induces expression of a wide range of cytoprotective genes, including SLC7A11 [24]. In many cancers, persistent NRF2 activation enhances cystine uptake, promotes GSH production, and suppresses ferroptosis, thereby facilitating tumor survival under oxidative stress.

Recent studies further indicate that the NRF2–SLC7A11 axis is shaped by metabolic and microenvironmental cues. In triple-negative breast cancer, cysteine availability itself can promote NRF2 activation, forming a feed-forward survival mechanism [25]. In gastric cancer, TMEM160 suppresses ferroptosis and induces chemoresistance by inhibiting KEAP1 and thereby activating NRF2-dependent SLC7A11 expression [26]. Lipid

metabolism also intersects with this pathway, as SCD1 inhibition in lung adenocarcinoma blocks AKT–NRF2–SLC7A11 signaling and primes cells for ferroptosis [27]. Moreover, immune-metabolic signals from the tumor microenvironment can enhance ferroptosis resistance through SLC7A11-associated antioxidant programs [28]. These findings suggest that NRF2-dependent induction of SLC7A11 is not a simple generic oxidative stress response, but rather part of a broader adaptive network integrating metabolism, redox regulation, and therapy pressure [24–30] (Figure. 1A).

### Hypoxia, HIF signaling, and ATF4-Mediated integrated stress response

Hypoxia is a hallmark of solid tumors and an important driver of SLC7A11 transcription. HIF-1 can promote glutathione synthesis and ferroptosis resistance by inducing SLC7A11-associated transcriptional programs, thereby contributing to therapy-resistant cancer stem-like phenotypes [9]. HIF-2 may also mediate ferroptosis resistance in a tumor type-specific manner, as reported in pancreatic cancer [10]. Although the relative contribution of HIF-1 versus HIF-2 may vary across contexts, these studies support the general concept that hypoxia-induced SLC7A11 expression is an important component of tu-



**Figure 1. Transcriptional regulation of SLC7A11.**

**A**, KEAP1 loss or oxidative stress stabilizes NRF2, promoting SLC7A11 transcription and ferroptosis resistance. **B**, hypoxia, ER stress and nutrient limitation activate HIFs or ATF4 to induce SLC7A11 expression. **C**, p53 represses SLC7A11 transcription, reducing cystine uptake and increasing ferroptosis sensitivity. **D**, ROS–FOXK2 and estrogen–ERα signaling provide additional routes for SLC7A11 activation.



mor adaptation.

HIF signaling also cooperates with oncogenic metabolic pathways. In ovarian cancer, YY1-induced USP43 stabilizes FASN, which subsequently activates SLC7A11 expression through HIF-1 $\alpha$  stabilization [11]. This cascade illustrates how lipid metabolism and deubiquitinating pathways can converge on hypoxia-responsive transcriptional control. Thus, SLC7A11 upregulation under hypoxia reflects an active integration of oxygen sensing, transcription factor stability, and metabolic rewiring rather than a passive consequence of low oxygen tension.

Endoplasmic reticulum stress and nutrient limitation provide an additional route to SLC7A11 induction via ATF4, a central transcription factor of the integrated stress response. ATF4 directly activates SLC7A11 transcription in hepatocellular carcinoma and colorectal cancer, thereby promoting cystine uptake and GSH synthesis under adverse conditions [12–13]. Pharmacological evidence further supports this pathway, as dihydroartemisinin induces ferroptosis in hepatocellular carcinoma partly by suppressing the ATF4–xCT axis [14]. Given that hypoxia can simultaneously elevate reactive oxygen species and trigger endoplasmic reticulum stress, HIF and ATF4 pathways may cooperate to establish a robust stress-adaptive program sustaining SLC7A11 expression in solid tumors (Figure. 1B).

### p53 as a Context-Dependent Transcriptional Brake

In contrast to the activating pathways described above, p53 serves as a major transcriptional repressor of SLC7A11. p53 directly binds the SLC7A11 promoter and represses its transcription, thereby reducing cystine uptake, limiting GSH synthesis, and sensitizing cells to ferroptosis [6]. This mechanism links tumor suppressor activity to ferroptosis regulation and is distinct from the classical roles of p53 in cell-cycle arrest, apoptosis, and senescence.

Importantly, the p53–SLC7A11 axis is highly context dependent. Its output may be influenced by p53 mutation status, polymorphisms, post-translational modifications, interacting cofactors, and tumor-specific metabolic context. For example, the African-specific P47S variant of p53 exhibits impaired tumor suppressor function and altered ferroptosis regulation [17]. ACSL1 loss in clear cell renal cell carcinoma modifies ferroptosis resistance in a manner connected to lipid metabolism [18]. In ovarian cancer, TOP2A knockdown reverses cisplatin resistance through the TP53/GPX4/SLC7A11 axis, whereas in breast cancer FXR deficiency induces ferroptosis by modulating CBP-dependent p53 acetylation [19–20]. Additional regulators such as CAPG and lncRNA-HMG further reshape p53-mediated ferroptosis through broader signaling and non-coding RNA networks [21–22]. These findings indicate that p53 is best viewed as a context-sensitive ferroptosis gatekeeper rather than a universal binary switch (Figure. 1C).

### Additional Transcriptional Inputs

In addition to canonical stress pathways, several other mechanisms contribute to SLC7A11 transcriptional activation. Under oxidative stress, ROS can trigger an oxidation–O-GlcNAcylation cascade that promotes FOXK2 nuclear translocation and SLC7A11 transcription, representing an NRF2-independent route by which oxidative injury is converted into antioxidant adaptation [15]. In estrogen receptor-positive breast cancer, es-

trogen receptor  $\alpha$  enhances system xc<sup>−</sup> activity and SLC7A11 expression, thereby linking hormone signaling to ferroptosis resistance and endocrine responsiveness [16]. These findings broaden the conceptual framework of SLC7A11 regulation, indicating that distinct tumor types may use different upstream routes to converge on the same anti-ferroptotic effector.

Taken together, the transcriptional regulatory network of SLC7A11 integrates oxidative stress, hypoxia, endoplasmic reticulum stress, nutrient availability, hormonal cues, and tumor suppressor pathways to determine whether tumor cells remain resistant or become vulnerable to ferroptosis (Figure. 1D).

### Post-Transcriptional and Translational Regulation of SLC7A11

After transcription, SLC7A11 expression is further refined at the RNA level through RNA modifications, RNA-binding proteins, non-coding RNAs, and alternative splicing. This layer of regulation allows cells to rapidly and reversibly adjust SLC7A11 output in response to environmental fluctuation and therapeutic challenge.

#### RNA modifications and mRNA fate

Among epitranscriptomic modifications, m6A is the most extensively studied in the context of SLC7A11. METTL3-mediated m6A modification of SLC7A11 enhances radioresistance in nasopharyngeal carcinoma by inhibiting ferroptosis [35]. In non-small cell lung cancer, IGF2BP2 recognizes m6A-modified SLC7A11 mRNA and promotes its stability, thereby supporting proliferation and ferroptosis resistance [36]. METTL5 likewise promotes cervical cancer progression through m6A-dependent suppression of ferroptosis involving SLC7A11 [37]. In hepatocellular carcinoma, inhibition of METTL3 enhances radiosensitivity by downregulating SLC7A11, while IGF2BP3 stabilizes SLC7A11 in gastric cancer and antagonizes ferroptosis [38–39]. Conversely, RNA demethylases can reduce SLC7A11 expression and increase ferroptosis sensitivity. ALKBH5 suppresses non-small cell lung cancer progression by facilitating ferroptosis through m6A demethylation of SLC7A11 [40]. FTO inhibition decreases the SLC7A11/GPX4 axis and induces ferroptotic death in colorectal cancer [41]. These observations emphasize that the effect of m6A on SLC7A11 is highly context dependent and may vary according to the modified site, reader proteins, tumor lineage, and surrounding stress environment.

Other RNA modifications also participate in SLC7A11 control. NSUN2-mediated m5C modification affects SLC7A11 mRNA stability and translation, and NSUN2 knockdown promotes ferroptosis in colorectal cancer [42]. NAT10-mediated ac4C modification increases SLC7A11 protein expression and contributes to sorafenib resistance in nasopharyngeal carcinoma [43]. Together, these studies identify SLC7A11 as an important effector transcript in the broader field of epitranscriptomic regulation of ferroptosis (Figure. 2A).

#### RNA-Binding Proteins and 3'UTR-Mediated Regulation

The stability and translational efficiency of SLC7A11 mRNA are also strongly influenced by RNA-binding proteins interacting with cis-regulatory elements, particularly within the 3' untranslated region (3'UTR). In bladder cancer, PHGDH promotes malignant progression by upregulating SLC7A11, at least partly through stabilization of the RNA-binding protein PCBP2

[44]. In colon adenocarcinoma, HnRNP stabilizes the mRNAs encoding both SLC7A11 and SLC3A2, suggesting coordinated control of the two subunits of system xc<sup>-</sup> [45]. ALYREF similarly promotes SLC7A11 mRNA stability and facilitates lung adenocarcinoma growth and metastasis [46].

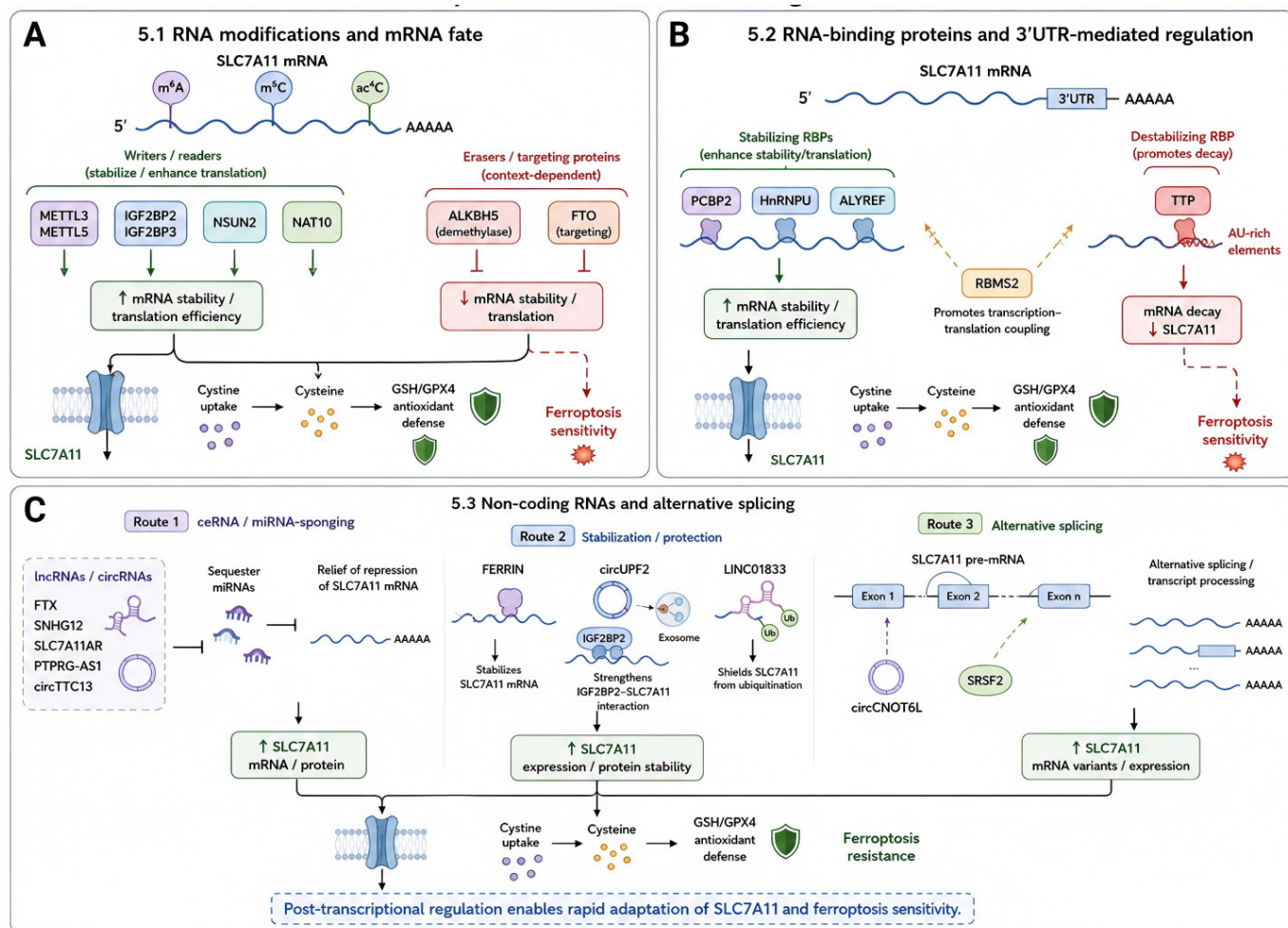
By contrast, destabilizing RNA-binding proteins can decrease SLC7A11 levels and sensitize cells to ferroptosis. In KRAS<sup>G12C</sup>-mutant lung cancer, TTP binds AU-rich elements in the SLC7A11 3'UTR and accelerates mRNA decay, thereby enhancing sensitivity to KRAS<sup>G12C</sup> inhibitors [47]. RBMS2 has also been reported to regulate SLC7A11 transcription-translation coupling in colorectal cancer [48]. These studies underscore that the 3'UTR of SLC7A11 functions as a regulatory platform where oncogenic signaling, metabolic status, and therapy response are integrated (Figure. 2B).

### Non-Coding RNAs and Alternative Splicing

Non-coding RNAs constitute another major post-transcriptional regulatory layer upstream of SLC7A11. Many long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) act through competitive endogenous RNA mechanisms, typically by sequestering microRNAs that would otherwise repress SLC7A11. In colorectal cancer, lncRNA FTX promotes radioresistance

through the miR-625-5p/SLC7A11 axis [49]. In lung cancer, small extracellular vesicle-associated SNHG12 enhances metastasis through the miR-326/SLC7A11 axis, and lncRNA SLC7A11AR promotes lung adenocarcinoma progression by increasing SLC7A11 expression [50-51]. In breast cancer, POU2F2-induced PTPRG-AS1 inhibits ferroptosis through the miR-376c-3p/SLC7A11 axis [52]. In hepatocellular carcinoma, circTTC13 promotes sorafenib resistance through the miR-513a-5p/SLC7A11 axis [53].

Notably, non-coding RNAs can also regulate SLC7A11 through mechanisms beyond classical miRNA sponging. LINC01833 protects SLC7A11 from WWP1-mediated ubiquitination and promotes gemcitabine resistance in non-small cell lung cancer [54]. LINC01088 enhances SLC7A11 expression in glioblastoma through the HLT/USP7 axis [55]. Under glutamine starvation, the lncRNA FERRIN suppresses ferroptosis by stabilizing SLC7A11 mRNA [56]. Exosome-derived circUPF2 enhances resistance to targeted therapy in hepatocellular carcinoma by strengthening the interaction between IGF2BP2 and SLC7A11 mRNA [57]. In prostate cancer, circNOT6L modulates alternative splicing of SLC7A11 pre-mRNA through SRSF2, thereby extending post-transcriptional regulation from mRNA abundance to transcript processing [58].



**Figure 2. Post-transcriptional and translational regulation of SLC7A11.**

**A**, RNA modifications regulate SLC7A11 mRNA stability and translation, thereby altering ferroptosis sensitivity. **B**, RNA-binding proteins control SLC7A11 mRNA stability through 3'UTR-dependent mechanisms. **C**, non-coding RNAs and alternative splicing modulate SLC7A11 expression, protein stability and ferroptosis resistance.



Collectively, these findings indicate that non-coding RNAs influence SLC7A11 at multiple levels, including transcript stability, protein turnover, and RNA processing, and that post-transcriptional and translational regulation provides a rapid and versatile means for cancer cells to fine-tune SLC7A11 expression and dynamically adjust ferroptosis sensitivity. (Figure. 2C).

### Protein Homeostasis and Post-Translational Regulation of SLC7A11

Although transcriptional and RNA-level mechanisms determine SLC7A11 mRNA output, the ultimate anti-ferroptotic function of SLC7A11 depends on protein abundance, stability, membrane localization, and transporter complex assembly.

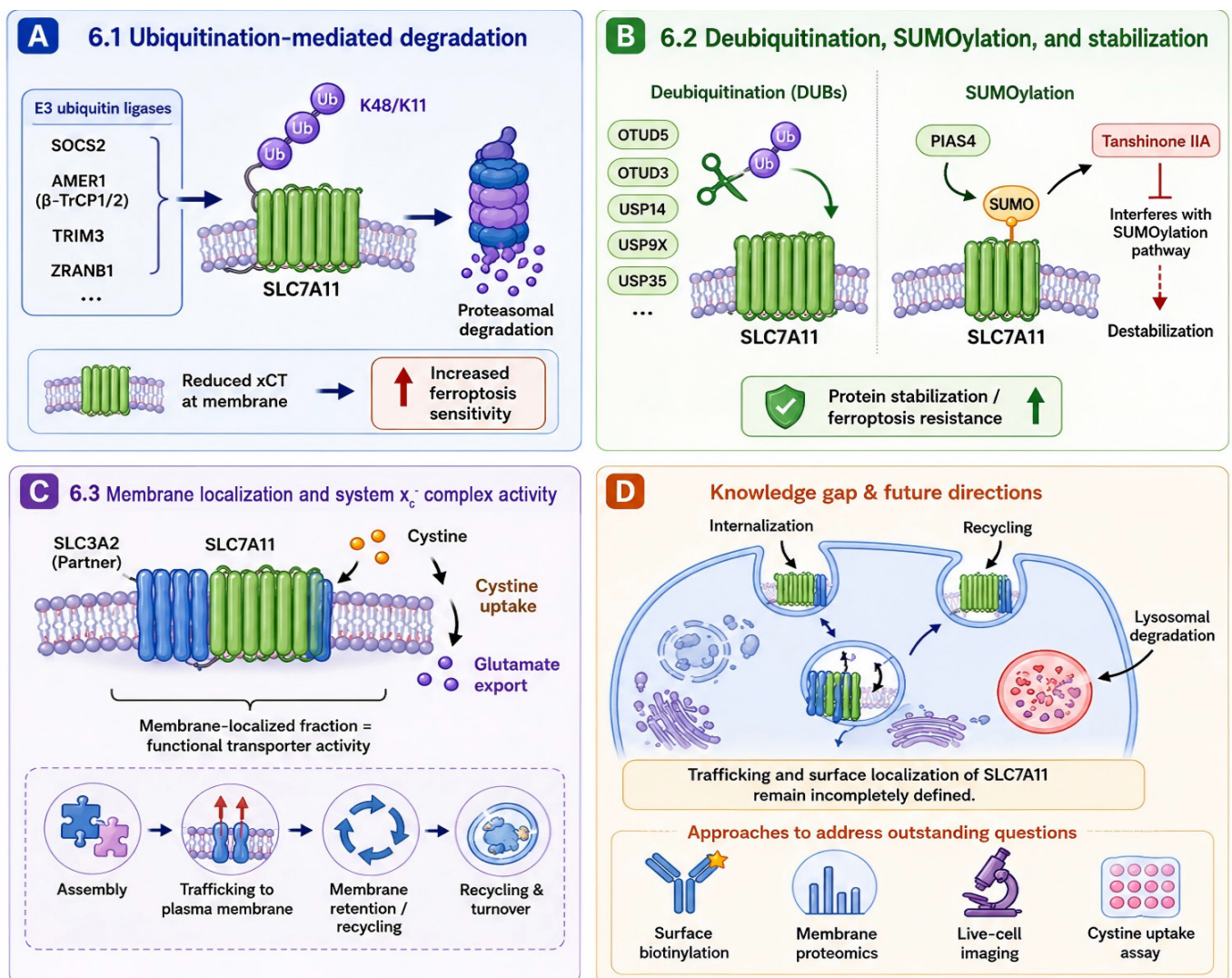
#### Ubiquitination-mediated degradation

The ubiquitin–proteasome system is a major mechanism governing SLC7A11 protein turnover. Several E3 ubiquitin ligases promote SLC7A11 degradation and thereby enhance

ferroptosis sensitivity. In hepatocellular carcinoma, SOCS2 induces K48-linked polyubiquitination of SLC7A11 by recruiting the Elongin B/C complex, leading to its degradation and consequent radiosensitization [59]. In colorectal cancer, AMER1 recruits  $\beta$ -TrCP1/2 to target SLC7A11 and FTL, thereby promoting ferroptosis and suppressing metastasis [60]. TRIM3 facilitates K11-linked ubiquitination and degradation of SLC7A11 in non-small cell lung cancer [61]. ZRANB1 has also been reported to act as an E3 ligase for SLC7A11 and to regulate ferroptotic resistance [62]. These studies indicate that distinct tumors may employ different ubiquitin ligases and linkage types to regulate SLC7A11 stability (Figure. 3A).

#### Deubiquitination, SUMOylation, and Protein Stabilization

Deubiquitinating enzymes (DUBs) can counteract ubiquitin-mediated degradation and maintain SLC7A11 abundance. OTUD5 removes ubiquitin chains from SLC7A11 in triple-negative breast cancer, promoting progression and reducing paclitaxel



**Figure 3. Protein-level regulation of SLC7A11.**

**A**, E3 ligases mediate SLC7A11 ubiquitination and degradation, reducing membrane xCT and increasing ferroptosis sensitivity. **B**, DUBs and SUMOylation stabilize SLC7A11, promoting ferroptosis resistance. **C**, SLC3A2-assisted membrane localization enables system  $x_c^-$  activity, cystine uptake and glutamate export. **D**, SLC7A11 trafficking, recycling and lysosomal degradation remain key unresolved mechanisms for future study.

sensitivity [63]. OTUD3 stabilizes SLC7A11 and contributes to sunitinib resistance in clear cell renal cell carcinoma [64]. In multiple myeloma, HSPA9 enhances USP14-mediated SLC7A11 deubiquitination and suppresses ferroptosis [65]. In hepatocellular carcinoma, EFNA4 recruits USP9X to stabilize SLC7A11, linking extracellular signaling to intracellular ferroptosis resistance [66]. USP35 promotes growth of estrogen receptor-positive breast cancer through a BRD4-SLC7A11-dependent anti-ferroptotic mechanism [67] (Figure. 3B).

SUMOylation is another important post-translational mechanism. PIAS4-mediated SUMOylation stabilizes SLC7A11 in breast cancer, whereas tanshinone IIA destabilizes SLC7A11 by interfering with KDM1A–PIAS4-dependent SUMOylation and thereby promotes ferroptosis [68]. These results suggest that protein stabilization pathways may sustain SLC7A11 expression even when transcriptional activation is only moderate, making them attractive therapeutic targets.

### Membrane localization and system xc<sup>-</sup> complex activity

The functional output of SLC7A11 depends not only on total protein abundance but also on its localization to the plasma membrane and its proper assembly with SLC3A2 [1-2]. SLC3A2 is required for transporter stability and trafficking, whereas SLC7A11 provides the substrate-selective transport function. Coordinated regulation of both subunits may therefore be necessary for maximal cystine uptake. For example, HnRNPU stabilizes both SLC7A11 and SLC3A2 mRNAs, supporting the idea that the entire system xc<sup>-</sup> complex can be co-regulated [45] (Figure. 3C).

Compared with transcriptional, RNA-level, and ubiquitin-dependent regulation, mechanisms governing SLC7A11 trafficking, membrane retention, internalization, recycling, and lysosomal degradation remain less clearly defined. This represents an important knowledge gap, because only the membrane-localized fraction of SLC7A11 is functionally active. Future studies integrating surface biotinylation, membrane proteomics, live-cell imaging, and cystine uptake assays will be needed to distinguish total SLC7A11 protein from surface-functional SLC7A11 under stress and treatment conditions (Figure. 3D). Representative regulators across different regulatory layers of SLC7A11 are summarized in Table 1.

### Therapeutic implications: Inducing Ferroptotic Pressure while Blocking Adaptive Defense

The regulatory network described above has an important therapeutic implication: ferroptosis-inducing therapy may fail if tumor cells simultaneously activate SLC7A11-dependent adaptive defenses. Many treatments increase oxidative stress, hypoxia, lipid peroxidation, endoplasmic reticulum stress, or metabolic pressure. These same stimuli can activate NRF2, HIF, ATF4 [9-14, 24-27], RNA stabilization programs [35-48], and protein stabilization mechanisms [59-68] that restore or further increase SLC7A11 expression. As a result, treatment-induced stress may paradoxically select for SLC7A11-high, ferroptosis-resistant cancer cell populations [3-5, 24].

A more effective therapeutic principle may be to combine stress induction with adaptation blockade. The first component increases lipid peroxidation pressure through radiotherapy, chemotherapy, targeted therapy, GPX4 inhibition, system xc<sup>-</sup> inhibition, or agents that perturb lipid metabolism. The second component blocks the compensatory SLC7A11 response at one or more regulatory levels. Potential targets include NRF2 or KEAP1-dependent transcriptional activation, HIF-driven hypoxia adaptation, ATF4-mediated integrated stress response, m6A writer-reader modules, stabilizing RNA-binding proteins, oncogenic non-coding RNAs, DUBs, and SUMOylation enzymes. Regarding the first component, several clinically available agents can, in principle, serve as the ferroptosis-inducing arm of such combination regimens. Sorafenib, a multikinase inhibitor approved for hepatocellular carcinoma, renal cell carcinoma, and thyroid cancer, induces ferroptosis partly through system xc<sup>-</sup> suppression and GSH depletion [69]. By contrast, therapeutic strategies targeting the second component—namely, blockade of adaptive SLC7A11 upregulation or stabilization—currently lack clinical-stage compounds and remain largely at the preclinical or target-validation stage. Bridging this gap will require not only the development of selective and pharmacologically tractable inhibitors, but also biomarker-driven patient selection strategies to identify tumors in which SLC7A11-dependent adaptive defense is the dominant resistance mechanism. Whether such combination approaches are ultimately feasible will need to be validated in further preclinical and clinical studies.

This strategy also requires biomarker development. SLC7A11 expression alone may be insufficient to stratify patients be-

**Table 1. Representative regulators of SLC7A11 expression and functional consequences.**

| Regulatory layer                         | Representative regulators                                                                  | Mechanism and functional consequence                                                                                                | Key References   |
|------------------------------------------|--------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|------------------|
| Transcriptional control                  | NRF2, HIFs, ATF4, p53, ERα, FOXK2                                                          | Stress-responsive transcriptional activation or repression of SLC7A11; balances antioxidant adaptation and ferroptosis sensitivity. | [6, 9-16, 24-30] |
| Chromatin and epigenetic control         | SNF2L, ARID1A, HNF4α-p300/CBP, KDM6B/GATA3                                                 | Controls promoter accessibility, histone acetylation, chromatin state, and sustained transcriptional output.                        | [31-34]          |
| RNA modifications                        | METTL3, METTL5, IGF2BP2/3, ALKBH5, FTO, NSUN2, NAT10                                       | Regulates SLC7A11 mRNA stability, translation efficiency, and therapy-associated ferroptosis resistance.                            | [35-43]          |
| RNA-binding proteins and non-coding RNAs | PCBP2, HnRNPU, ALYREF, TTP, RBMS2, FTX, SNHG12, SLC7A11AR, circUPF2, circCNOT6L, circTTC13 | Controls mRNA decay, stabilization, miRNA-dependent repression, scaffold functions, and alternative splicing.                       | [44-58]          |
| Protein-level regulation                 | SOCS2, AMER1, TRIM3, ZRANB1, OTUD5, OTUD3, USP14, USP9X, USP35, PIAS4                      | Determines SLC7A11 degradation, stabilization, SUMOylation, membrane availability, and functional anti-ferroptotic output.          | [59-68]          |



cause its activity is shaped by upstream drivers and post-transcriptional regulators. A more informative biomarker panel may include SLC7A11 protein level, membrane localization, SLC3A2 co-expression, KEAP1/NRF2 status, p53 status, ATF4/HIF activity, GSH abundance, lipid peroxide burden, and expression of key RNA or protein stabilizers. Such integrated profiling could help identify tumors that are both dependent on SLC7A11 and vulnerable to combined ferroptosis-based interventions.

Several challenges remain. First, SLC7A11 regulation is highly context dependent [3, 6, 17]. The same regulator may have different effects depending on tumor lineage, genetic background, nutrient availability, and treatment exposure. Second, systemic inhibition of cystine uptake or antioxidant defense may cause toxicity in normal tissues [2-3]. Third, ferroptosis does not occur in isolation. It interacts with apoptosis, immune signaling, metabolic remodeling, and the tumor micro-environment [7-8, 28]. Therefore, future therapeutic studies should incorporate in vivo models, patient-derived organoids, immune-competent systems, single-cell omics, and spatial analysis to determine when SLC7A11-targeted strategies are most likely to succeed.

## Conclusions and Future Perspectives

SLC7A11 is a central regulator of ferroptosis sensitivity in cancer. By importing extracellular cystine and supporting GSH-GPX4-dependent lipid peroxide detoxification, it allows tumor cells to maintain redox homeostasis under hostile micro-environmental and therapeutic conditions. Current evidence indicates that high SLC7A11 expression is not produced by a single pathway. It is maintained by coordinated regulation at the transcriptional, chromatin, RNA, and protein levels.

At the transcriptional level, NRF2, HIFs, ATF4, hormone receptors, FOXK2, and oncogenic metabolic pathways activate SLC7A11 in response to oxidative stress, hypoxia, endoplasmic reticulum stress, and metabolic remodeling. In contrast, p53 functions as a context-dependent transcriptional brake that links tumor suppression to ferroptosis sensitivity. At the chromatin level, remodeling factors, histone acetylation programs, and chromatin-associated transcriptional coactivators shape accessibility of the SLC7A11 locus. At the RNA level, m6A, m5C, ac4C, RNA-binding proteins, non-coding RNAs, and splicing regulators determine mRNA stability, translation, and isoform output. At the protein level, ubiquitination, deubiquitination, SUMOylation, and membrane localization determine whether SLC7A11 becomes a functional transporter at the cell surface. Notably, total SLC7A11 expression does not necessarily reflect membrane-localized transporter activity, and the mechanisms governing this gap remain an important open question for future investigation.

The central message is that SLC7A11 acts as a stress-adaptive hub. Tumor cells use this hub to transform oxidative stress, hypoxia, nutrient stress, and therapy-induced damage into antioxidant defense and ferroptosis resistance. This concept also reframes therapy. Rather than relying only on agents that increase oxidative or lipid peroxidation stress, future strategies may need to pair ferroptosis induction with blockade of SLC7A11 adaptation. Such approaches may include targeting upstream transcriptional activators, RNA-stabilizing machinery,

non-coding RNA networks, DUBs, SUMOylation pathways, or transporter membrane function.

Future research should move beyond single-regulator studies and define the hierarchy, timing, and cooperativity of SLC7A11 regulatory modules in specific tumor contexts. Integrating single-cell transcriptomics, spatial proteomics, metabolomics, functional genetic screening, and patient-derived models will be essential for identifying actionable SLC7A11-high states. A deeper understanding of this multilayered regulatory architecture may provide a foundation for more precise and durable ferroptosis-targeted cancer therapies.

## Abbreviations

3'UTR: 3' Untranslated Region; ac4C: N4-Acetylcytidine; ACSL1: Acyl-CoA Synthetase Long Chain Family Member 1; AKT: AKT Serine/Threonine Kinase; ALKBH5: AlkB Homolog 5; ALYREF: Aly/REF Export Factor; AMER1: APC Membrane Recruitment Protein 1; ARID1A: AT-Rich Interaction Domain 1A; ATF4: Activating Transcription Factor 4; BAP1: BRCA1 Associated Protein 1; BRD4: Bromodomain Containing 4; CAPG: Capping Actin Protein, Gelsolin Like; CBP: CREB Binding Protein; circCNOT6L: Circular RNA from CNOT6L Gene; circRNA: Circular RNA; circTTC13: Circular RNA from TTC13 Gene; circUPF2: Circular RNA from UPF2 Gene; DUB: Deubiquitinating Enzyme; ERα: Estrogen Receptor Alpha; FOXK2: Forkhead Box K2; FTL: Ferritin Light Chain; FTO: Fat Mass and Obesity-Associated Protein; FXR: Farnesoid X Receptor; GATA3: GATA Binding Protein 3; GPX4: Glutathione Peroxidase 4; GSH: Glutathione; H3K27me3: Histone H3 Lysine 27 Trimethylation; HIF: Hypoxia-Inducible Factor; HIF-1: Hypoxia-Inducible Factor 1; HIF-2: Hypoxia-Inducible Factor 2; HLTF: Helicase-Like Transcription Factor; HNF4α: Hepatocyte Nuclear Factor 4 Alpha; HnRNPU: Heterogeneous Nuclear Ribonucleoprotein U; HSPA9: Heat Shock Protein Family A Member 9; IGF2BP2: Insulin Like Growth Factor 2 mRNA Binding Protein 2; IGF2BP3: Insulin Like Growth Factor 2 mRNA Binding Protein 3; KDM6B: Lysine Demethylase 6B; KEAP1: Kelch Like ECH Associated Protein 1; LINC01088: Long Intergenic Non-Protein Coding RNA 1088; LINC01833: Long Intergenic Non-Protein Coding RNA 1833; lncRNA: Long Non-Coding RNA; m5C: 5-Methylcytosine; m6A: N6-Methyladenosine; METTL3: Methyltransferase Like 3; METTL5: Methyltransferase Like 5; miRNA: MicroRNA; NAT10: N-Acetyltransferase 10; NRF2: Nuclear Factor Erythroid 2-Related Factor 2; NSUN2: NOP2/Sun RNA Methyltransferase 2; OTUD3: Ovarian Tumor Deubiquitinase 3; OTUD5: Ovarian Tumor Deubiquitinase 5; p300: E1A Binding Protein p300; PCBP2: Poly(rC) Binding Protein 2; PHGDH: Phosphoglycerate Dehydrogenase; PIAS4: Protein Inhibitor of Activated STAT 4; POU2F2: POU Class 2 Homeobox 2; PTPRG-AS1: PTPRG Antisense RNA 1; RBMS2: RNA Binding Motif Single Stranded Interacting Protein 2; ROS: Reactive Oxygen Species; SCD1: Stearoyl-CoA Desaturase 1; SLC3A2: Solute Carrier Family 3 Member 2; SLC7A11: Solute Carrier Family 7 Member 11; SNF2L: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A-Like 1; SNHG12: Small Nucleolar RNA Host Gene 12; SOCS2: Suppressor of Cytokine Signaling 2; SRSF2: Serine and Arginine Rich Splicing Factor 2; SUMOylation: Small Ubiquitin-Like Modifier Conjugation; SWI/SNF: Switch/Sucrose Non-Fermentable; system xc<sup>-</sup>: Cystine/

Glutamate Antiporter System; TMEM160: Transmembrane Protein 160; TOP2A: DNA Topoisomerase II Alpha; TP53: Tumor Protein p53; TRIM3: Tripartite Motif Containing 3; TTP: Tristetraprolin; USP14: Ubiquitin Specific Peptidase 14; USP35: Ubiquitin Specific Peptidase 35; USP43: Ubiquitin Specific Peptidase 43; USP7: Ubiquitin Specific Peptidase 7; USP9X: Ubiquitin Specific Peptidase 9 X-Linked; WDR74: WD Repeat Domain 74; WWP1: WW Domain Containing E3 Ubiquitin Protein Ligase 1; xCT: Cystine/Glutamate Antiporter; ZRANB1: Zinc Finger RANBP2-Type Containing 1;  $\beta$ -TrCP1/2: Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase 1/2.

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ZHY conceptualized, researched, wrote, and edited the entire manuscript.

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We declare that our manuscript is not published elsewhere. All co-authors had substantial contribution to the content and creation of the manuscript. All authors approved the final version of the submitted paper.

## Competing Interests

The authors declare that they have no existing or potential commercial or financial relationships that could create a conflict of interest at the time of conducting this study.

## Data Availability

Not Applicable.

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# Association of Bedtime Milk Consumption with Adenoid Size and Tissue Microbiota Profiles in Children with Adenoid Hypertrophy

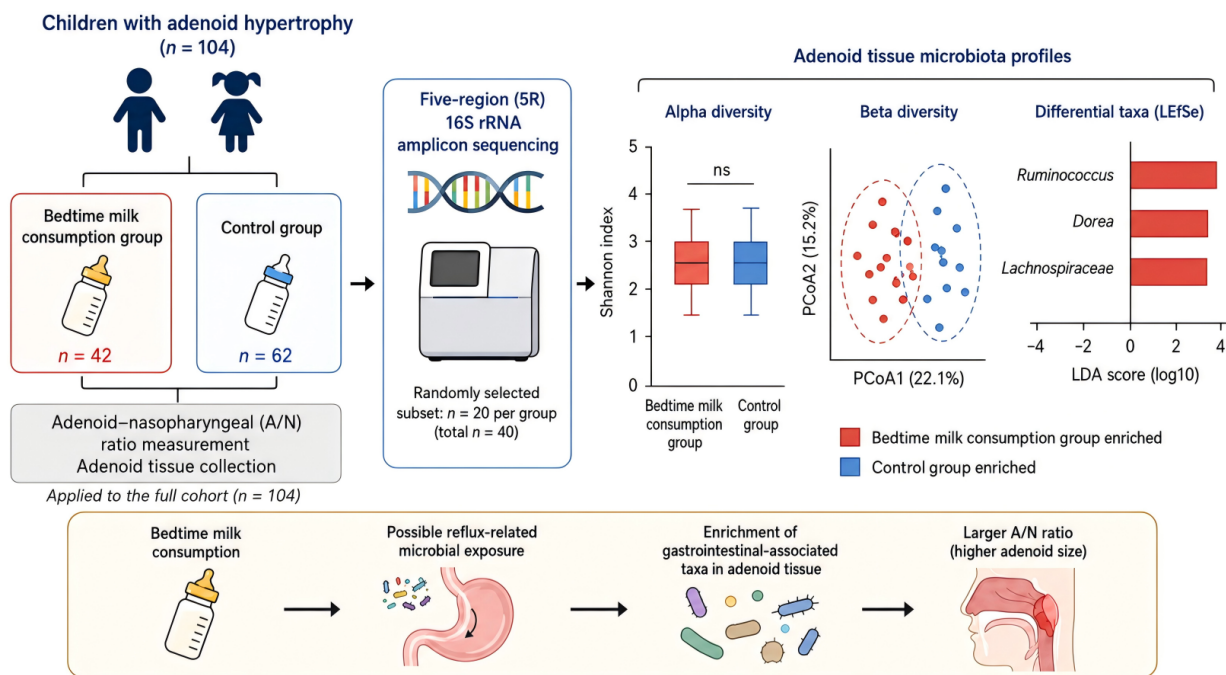
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## Graphical Abstract



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# Association of Bedtime Milk Consumption with Adenoid Size and Tissue Microbiota Profiles in Children with Adenoid Hypertrophy

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## Abstract

**Background:** Adenoid hypertrophy is a common and frequently occurring disease in children, and the surgical rate is relatively high. Currently, the etiology is not yet clear, and it is of great significance to explore its pathogenesis and new treatment methods. To investigate the effect of milk consumption before bedtime on adenoid size and the tissue microbiota in children.

**Methods:** A total of 104 children who underwent surgical treatment for adenoid hypertrophy (AH) at our hospital between March 2023 and January 2024 were divided into a bedtime milk consumption group (n = 42) and a control group (n = 62) according to their milk consumption before bedtime. And the adenoid-nasopharyngeal (A/N) ratio was measured. The A/N ratio was measured, and adenoid tissue samples from a randomly selected subset of 20 participants in each group were subjected to five-region (5R) 16S rRNA amplicon sequencing.

**Results:** The average A/N ratio in the experimental group was  $0.808 \pm 0.070$ , vs  $0.736 \pm 0.037$  in the control group, showing a significant difference ( $P < 0.001$ ). There was no significant difference in total microbial diversity or dominant bacterial community distribution in adenoid tissue between the two groups. However, comparison revealed the experimental group's adenoid tissue was enriched in Ruminococcus, Dorea, and Lachnospiraceae, common in the gastrointestinal tract.

**Conclusions:** Bedtime milk consumption was associated with a larger adenoid size and altered adenoid tissue microbiota profiles in children with AH. The enrichment of gastrointestinal-associated taxa suggests a potential link with reflux-related microbial exposure, which warrants further validation.

**Keywords:** milk drinking; adenoid hypertrophy; gastroesophageal reflux; microorganisms

## Introduction

Adenoids, also known as pharyngeal tonsils, are located between the pharyngeal recesses on both sides at the junction of the roof and posterior wall of the nasopharynx. Adenoids are part of Waldeyer's ring and play an important role in immune system development. Adenoids are also the first organ of the immune system in contact with respiratory tract and digestive tract antigens and serve as a collective defense barrier against infection. Studies have shown that adenoids perform the earliest immune function in nasal-associated lymphoid tissue (NALT). Immune activity is strongest in the adenoids at 4-10 years of age in humans and begins to regress after puberty [1]. The number of children diagnosed with adenoid hypertrophy (AH) is increasing annually [2]. AH in children is typically

accompanied by a series of clinical manifestations, such as nasal obstruction, oral breathing, snoring, hearing impairment, and changes in the structure and appearance of the cranio-facial region [3]. Although these symptoms can be treated surgically, surgery has a psychological impact on children and parents and potentially results in complications, such as bleeding. These negative effects of surgical treatment have prompted doctors to investigate alternative nonsurgical means of treatment. However, the development of new diagnostic and treatment strategies has been challenging. One recent study indicated that drug therapy does not reduce the surgical rate in AH children. Nonsurgical treatment methods, such as the use of leukotriene receptor antagonists, nasal steroids, and/or oral antihistamines, are not directly linked to a decrease in the number of surgeries performed [4]. Although the causes of AH in children are incompletely understood, existing research sug-

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gests that AH may be related to infections, immune dysregulation, environmental exposure, hormonal factors, and genetic susceptibility [5-7]. Adenoid tissue is exposed to both inhaled and swallowed microorganisms and may therefore reflect microbial signals from the upper airway and digestive tract. Recent microbiome studies have suggested that AH is associated with altered local or intestinal microbial communities, but the relationship between daily feeding habits, reflux-related exposure, and adenoid tissue microbiota remains poorly understood. Because adenoid tissue contains relatively low microbial biomass, careful contamination control and negative controls are essential for reliable microbial profiling.

In clinical practice, bedtime milk consumption is common in children and may be associated with AH. Therefore, we divided children with AH who underwent surgical treatment into groups according to bedtime milk consumption, measured the adenoid-nasopharyngeal (A/N) ratio, and performed five-region (5R) 16S rRNA amplicon sequencing on adenoid tissue samples to explore the association between bedtime milk consumption, adenoid size, and local microbial profiles.

## Materials and Methods

### Participant recruitment

The participants in this study comprised 104 children who underwent surgical treatment for AH at the Department of Otolaryngology-Head and Neck Surgery of the Affiliated Bozhou Hospital of Anhui Medical University between March 2023 and January 2024. The participants included 60 males and 44 females aged 3-10 ( $6.38 \pm 1.81$ ) years. All participants (legal guardians) provided written consent (provided by legal guardians and children over 7 years old) for their participation. The diagnosis of AH was achieved through nasal endoscopy and nasopharynx imaging. The enrolled children with AH had over 75% obstruction in their nasal airways, as confirmed by nasal endoscopy. Imaging was also performed to confirm an adenoid-nasopharyngeal (A/N) ratio greater than or equal to 0.7.

### CT examination and measurement of the A/N ratio

Imaging was performed on the nasopharynx with the participants remaining calm and adopting a supine position. Computer software was used for data acquisition and analysis. In the A/N ratio, the adenoid size A is the vertical distance from the outermost point of convexity of the adenoid shadow to the tangent of the basiocciput, and the nasopharyngeal size N is the distance between the anterior edge of the sphenobasioccipital synchondrosis and the posterior edge of the hard palate. The A/N ratio was obtained by dividing two of the measured values. All measurements were obtained by the imaging department using professional software.

### Inclusion, exclusion, and grouping criteria

Enrolled children with AH were surveyed on their bedtime milk consumption behavior. Children who consumed > 100 ml of milk 1 h before bedtime for more than 5 days per week were assigned to the experimental group. Children who did not consume milk before bedtime or consumed any other type of food 1 h before bedtime were grouped into the control group. The exclusion criteria were as follows: a) the presence of other potential diseases, such as cancer, gastrointestinal diseases, or

inflammatory diseases; b) the use of antibiotics and probiotics in the month before the start of the study; c) surgical treatment within three months before the study; and d) incomplete clinical information.

Of the 104 patients selected for the study, 42 were grouped into the bedtime milk consumption group, including 24 males and 18 females aged 3-10 ( $6.19 \pm 1.95$ ) years. The control group comprised 62 patients, including 36 males and 26 females aged 3-9 ( $6.50 \pm 1.72$ ) years. The final grouping was 42 children in the bedtime milk consumption group and 62 children in the control group.

### Sample collection and sequencing

The clinical symptoms of the children with AH were observed and recorded. Fresh tissue samples were collected from every child during surgery and stored at  $-80^{\circ}\text{C}$  for subsequent experimental use. The following procedures were adopted: the nasopharynx of the children with AH was rinsed with saline water three times before adenoidectomy, and fresh adenoid tissue samples were collected during surgery. The samples were mung bean-sized with a length, width, and height of  $\leq 0.5$  cm. The samples were immediately washed with  $1\times$  PBS or saline prepared with sterile, nuclease-free water precooled to  $2-6^{\circ}\text{C}$  to remove any blood or stains from their surface. The samples were then dried with sterile and lint-free paper towels and quickly placed in precooled, sterile, and nuclease-free cryogenic vials with threaded caps that can withstand temperatures of  $-192^{\circ}\text{C}$ . The vials were frozen in liquid nitrogen for 1 h and stored at  $-80^{\circ}\text{C}$  for later use. Repeated freeze-thaw cycles were avoided before DNA extraction. Due to the low bacterial content in the tissue samples and the difficulty in avoiding external bacterial contamination during sampling, negative control samples were collected along with the sequencing samples. More specifically, contaminating bacteria in the sampling environment were collected in a 1.5 mL sterile centrifuge tube. The tube was opened at the beginning of collection and closed at the end. Five negative control samples were collected to ensure the accurate identification of contaminating bacteria.

### 5R 16S rRNA amplicon sequencing

5R 16S rRNA amplicon sequencing, 20 children were randomly selected from the bedtime milk consumption group and 20 children were randomly selected from the control group. In this study, 5R refers to the parallel amplification and sequencing of five 16S rRNA amplicon regions spanning the following hypervariable segments: V2, V3, V4, V6-V7, and V8-V9. This approach differs from conventional single-region 16S rRNA sequencing, which usually relies on one amplicon, such as V3-V4 or V4, to profile microbial communities. By integrating sequence information from several non-contiguous 16S regions, the 5R approach provides broader taxonomic information for microbial community profiling. The short multiple regions framework (SMURF) [8] was then used to integrate reads from the five amplified regions and perform taxonomic identification of bacteria. Due to the low microorganism content in the tissue samples collected, any microorganisms from the environment introduced during sample collection, DNA extraction, and PCR could cause significant interference in the identification of microorganisms already present in the sample. Hence, a filtration step to remove contaminating microorganisms (negative controls) introduced from the above sources was performed

[9]. Finally, bacterial data collected after the filtration step were used to analyze the intra- and intergroup bacterial diversity and perform comparisons among the bacterial communities. 5R 16S rRNA amplicon sequencing was performed by Hangzhou Lianchuan Biotechnology Co., Ltd.

### Comparison of bacterial communities

Five-region 16S rRNA sequencing data were pooled and analyzed with SMURF to identify the bacterial communities in the sample and calculate the relative abundance. Specifically, SMURF uses the expectation-maximization algorithm to compile the sequencing data from multiple short, amplified regions and reconstruct them to find the most likely set of 16S sequences. Bacterial taxonomy identification was then performed, and the relative abundance was calculated. The optimized Greengenes (May 2013 version) database was used. To mitigate the impact of low-abundance noise on subsequent analysis, samples with a total number of reads < 1,000 (including negative controls) and bacterial data with a relative abundance of <  $10^{-4}$  were removed. The number of reads was normalized for each sample. Bacterial contaminants introduced during sampling and experimentation were identified according to the prevalence of bacterial communities in the negative controls. In this study, a threshold of 50% prevalence was set for identifying bacterial contaminants. The bacterial communities remaining in the sample after removal of the contaminating bacteria were considered those present in adenoid tissue. Analyses of alpha diversity, beta diversity, and dominant bacterial communities and comparisons of bacterial communities were subsequently conducted using these data.

### Statistical analysis

For the comparison of the A/N ratio between groups, the mean difference and 95% confidence interval (CI) were calculated using Welch's method and reported to improve statistical transparency.

## Results

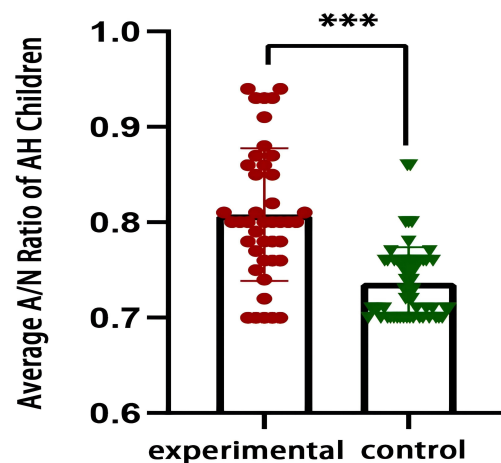
### Essential clinical traits of participants with AH

A total of 104 children with AH fulfilled the inclusion and exclusion criteria described in the Materials and Methods section. Forty-two of them (40.4%) who regularly consumed milk 1 h before bedtime were included in the bedtime milk consumption group, including 24 males and 18 females aged 3-10 (6.19

$\pm 1.95$ ) years. Sixty-two children (59.6%) who did not regularly consume milk before bedtime, including 36 males and 26 females aged 3-9 years ( $6.50 \pm 1.72$ ), were included in the control group. No significant differences in sex, age, body mass index (BMI), or disease onset time were observed between the two groups (Table 1).

### The A/N ratio was higher in children with bedtime milk consumption behavior

A higher average A/N ratio ( $0.808 \pm 0.070$ ) was observed in children who consumed milk before bedtime, whereas those who did not had an average A/N ratio of  $0.736 \pm 0.037$ . The mean difference between groups was 0.072 (95% CI, 0.048 to 0.096), and the intergroup difference was significant ( $P < 0.001$ ) (Figure 1).



**Figure 1. Comparison of the A/N ratio between the bedtime milk consumption group and the control group.**

Children in the bedtime milk consumption group had a higher average A/N ratio than those in the control group. The mean difference was 0.072 (95% CI, 0.048 to 0.096). \* $P < 0.001$ .

### Comparison of total bacterial diversity in adenoid tissue between children with AH with and without bedtime milk drinking behavior

Alpha diversity is an indicator of diversity within a particular environment or ecosystem and is primarily used to reflect species richness and evenness and sequencing depth. Various alpha diversity metrics, such as the Chao1, Good's coverage, observed species, Shannon, and Simpson indices (Figure 2A-

**Table 1. Clinical characteristics of children with adenoidal hypertrophy (AH).**

| Clinical characteristic                              | AH children participating in the study<br>(n = 104) |                           |                  |       |
|------------------------------------------------------|-----------------------------------------------------|---------------------------|------------------|-------|
|                                                      | Bedtime milk consumption group<br>(n = 42)          | Control group<br>(n = 62) | t/X <sup>2</sup> | P     |
| Gender (male/female)                                 | 24/18                                               | 36/26                     | 0.009            | 0.926 |
| Age (years, mean $\pm$ SD)                           | 6.19 $\pm$ 1.95                                     | 6.50 $\pm$ 1.72           | 0.853            | 0.395 |
| BMI ( $\text{kg}\cdot\text{m}^{-2}$ , mean $\pm$ SD) | 16.50 $\pm$ 2.82                                    | 17.21 $\pm$ 3.47          | 1.147            | 0.254 |
| Disease onset time<br>(years, mean $\pm$ SD)         | 1.65 $\pm$ 1.36                                     | 1.42 $\pm$ 0.90           | -1.028           | 0.307 |

Note: Continuous variables are expressed as the means  $\pm$  standard deviations. Categorical variables are expressed as percentages. Continuous variables were compared using Student's t test, and categorical variables were compared using the chi-square test.

E), were used to quantify species richness and evenness in this study. Beta diversity measures the species diversity between different ecological communities. Together, alpha and beta diversity constitute the total species diversity or biological heterogeneity of a certain ecological community. Analysis of the data obtained from the two groups of children with AH revealed no significant difference in the total bacterial diversity in their adenoid tissue regardless of bedtime milk consumption behaviors.

#### Analysis of dominant bacterial communities in the adenoid tissue of AH children in the experimental and control groups

The distribution of bacterial communities in the adenoid tissue of the two groups of children with AH was analyzed at the domain, phylum, class, order, family, genus, and species levels. Figure 3 shows the results obtained at the phylum and genus levels. Figure 3A-B shows the overall distribution of bacteria in the adenoid tissue of the two groups of children at the phylum level and the intergroup comparisons. At the phylum level, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria were the dominant bacteria in the adenoid tissue of both groups. No significant difference in the distribution of dominant bacterial communities was observed between the experimental and control groups. Figure 3C-D shows the total

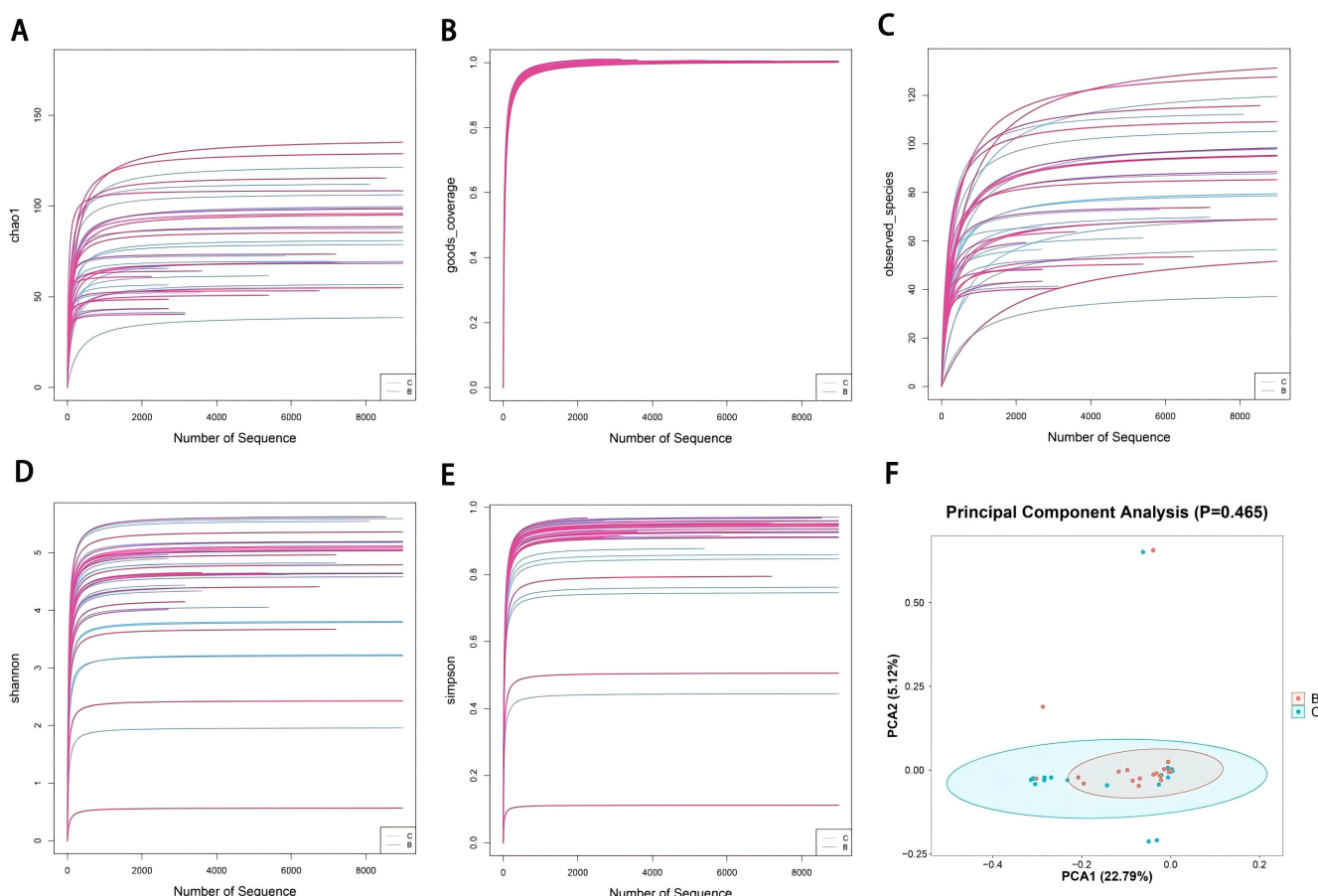
bacterial distribution in the adenoid tissue of the two groups at the genus and intergroup levels. At the genus level, Streptococcus, Vibrio, Pseudoalteromonas, Fusobacterium, Pseudomonas, and Corynebacterium were the dominant bacteria in the adenoid tissue of both groups. No significant difference was detected in the distribution of the dominant bacterial communities between the experimental and control groups.

#### Comparison of bacterial communities in the adenoid tissue of the two groups of children with AH

Linear discriminant analysis effect size (LEfSe) was used to analyze the differences in the bacterial communities between the two groups. The threshold value was set at LDA > 3 and P < 0.05. Compared to those in the control group, the adenoid tissue of children with AH who consumed bedtime milk was enriched in *Ruminococcus*, *Mycobacterium*, *Dorea* (a member of the Lachnospiraceae family), *Lachnospiraceae*, *Peptidophilus*, and *Massilia* (Figure 4). *Ruminococcus*, *Dorea*, and *Lachnospiraceae* commonly colonize the gastrointestinal tract.

## Discussion

In recent years, there has been a steady increase in cases of



**Figure 2. Characteristics of the total bacterial diversity in the adenoid tissue of the bedtime milk consumption group and the control group.** (A-E) Alpha diversity indices (Chao1, Good's coverage, observed species, Shannon index, and Simpson index) showing the differences between the bedtime milk consumption group and the control group. Red lines indicate the bedtime milk consumption group, and blue lines indicate the control group. (F) Principal component analysis (PCA) showing the intragroup similarity and intergroup differences between the bedtime milk consumption group and the control group using biological replicates (P = 0.465; red: bedtime milk consumption group; blue: control group).





could be related to various diseases in the human body [12], and scientists have previously investigated the relationship between microorganisms and AH [13-14]. In this study, 5R 16S rRNA amplicon sequencing was performed on adenoid tissue from children with AH. The dominant bacterial communities in adenoid tissue at the phylum level were Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria. This result is consistent with the distribution of nasopharyngeal bacterial communities identified by Bogaert et al. [15]. At the genus level, *Streptococcus*, *Vibrio*, *Pseudoalteromonas*, *Fusobacterium*, *Pseudomonas*, and *Corynebacterium* were the dominant bacterial communities in adenoid tissue. Previous microbial analysis of adenoid tissue in children by Brook et al. also suggested a similar bacterial distribution [16]. The unique microbiology of AH and the interactions among various organisms revealed in this study provide new directions for antimicrobial therapy for AH.

In clinical practice, bedtime milk consumption is common among children and may be associated with AH. In this study, children in the bedtime milk consumption group had a higher average A/N ratio than those in the control group ( $0.808 \pm 0.070$  vs.  $0.736 \pm 0.037$ ; mean difference, 0.072; 95% CI, 0.048 to 0.096;  $P < 0.001$ ), suggesting an association between bedtime milk consumption and larger adenoid size. 5R 16S rRNA amplicon sequencing further showed that adenoid tissue from children in the bedtime milk consumption group was enriched in *Ruminococcus*, *Mycobacterium*, *Dorea*, *Lachnospiraceae*, *Pep-toniphilus*, and *Massilia*. Among these taxa, *Ruminococcus*, *Dorea*, and *Lachnospiraceae* are commonly associated with the gastrointestinal tract [17-19]. Their enrichment in adenoid tissue may therefore reflect reflux-related microbial exposure, microbial translocation along the aerodigestive tract, or broader host-microbiome interactions involving mucosal immunity, diet, and local inflammatory status.

Previous studies have reported an association between GERD and AH. Niu et al. [20] reported a significant correlation between AH and GERD, and another study suggested that upper airway obstruction may be associated with increased GERD occurrence through changes in intrathoracic pressure [21]. Crapko et al. [22] also reported possible reflux-related inflammatory responses in adenoid tissue. Consistent with these findings, our results showed that children in the bedtime milk consumption group had larger adenoids and a higher abundance of gastrointestinal-associated taxa in adenoid tissue. However, these observations do not establish causality, and reflux involvement can only be inferred indirectly. This study was limited by its single-center observational design, the relatively small number of sequenced samples, the absence of direct reflux-related assessments such as pH/impedance monitoring or pepsin detection, potential unmeasured dietary confounders, and the lack of longitudinal follow-up.

In summary, bedtime milk consumption was associated with larger adenoid size and altered adenoid tissue microbiota profiles in children with AH. The enrichment of gastrointestinal-associated taxa may be linked to reflux-related microbial exposure; however, direct reflux-related examinations were not performed, and further prospective studies incorporating pH/impedance monitoring or pepsin detection are needed to clarify this association.

## Abbreviations

5R: Five-region; 16S rRNA: 16S ribosomal RNA; A/N: Adenoid-nasopharyngeal ratio; AH: Adenoid hypertrophy; BMI: Body Mass Index; CI: Confidence Interval; CT: Computed Tomography; DNA: Deoxyribonucleic Acid; GERD: Gastroesophageal Reflux Disease; GSA: Genome Sequence Archive; LDA: Linear Discriminant Analysis; LEfSe: Linear Discriminant Analysis Effect Size; NALT: Nasal-associated Lymphoid Tissue; PBS: Phosphate-buffered Saline; PCA: Principal Component Analysis; PCR: Polymerase Chain Reaction; SD: Standard Deviation; SMURF: Short Multiple Regions Framework.

## Author Contributions

W.C. and L.W. contributed equally to this work. W.C. and L.W.: Conceptualization, methodology, formal analysis, data interpretation, and writing of the original draft. W.C.: Supervision, project administration, critical manuscript revision, and final approval of the manuscript. W.W. and C.F.: Investigation, methodology, data curation, formal analysis, and writing review and editing. M.H., J.J., and M.Z.: Sample collection, laboratory experiments, data curation, and result interpretation. All authors reviewed and approved the final manuscript.

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## Ethics Approval and Consent to Participate

The study was performed in accordance with relevant guidelines and regulations, as well with the principles of the Declaration of Helsinki. Ethical approval was obtained from the People's Hospital of Bozhou Medical Ethics Committee. Informed consent was obtained from the parents/legal guardians before their children participated in the study, and assent was obtained from all children aged 7 years or older.

## Competing Interests

The authors declare no potential conflicts of interest.

## Data Availability

The raw sequence data have been deposited in the Genome Sequence Archive (GSA: CRA017958) and can be accessed at <https://bigd.big.ac.cn/gsa/browse/CRA017958>.

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# Transcriptomics Identify KCNJ2 as a Potential Novel Biomarker for the Postoperative Recurrence in Chronic Sinusitis

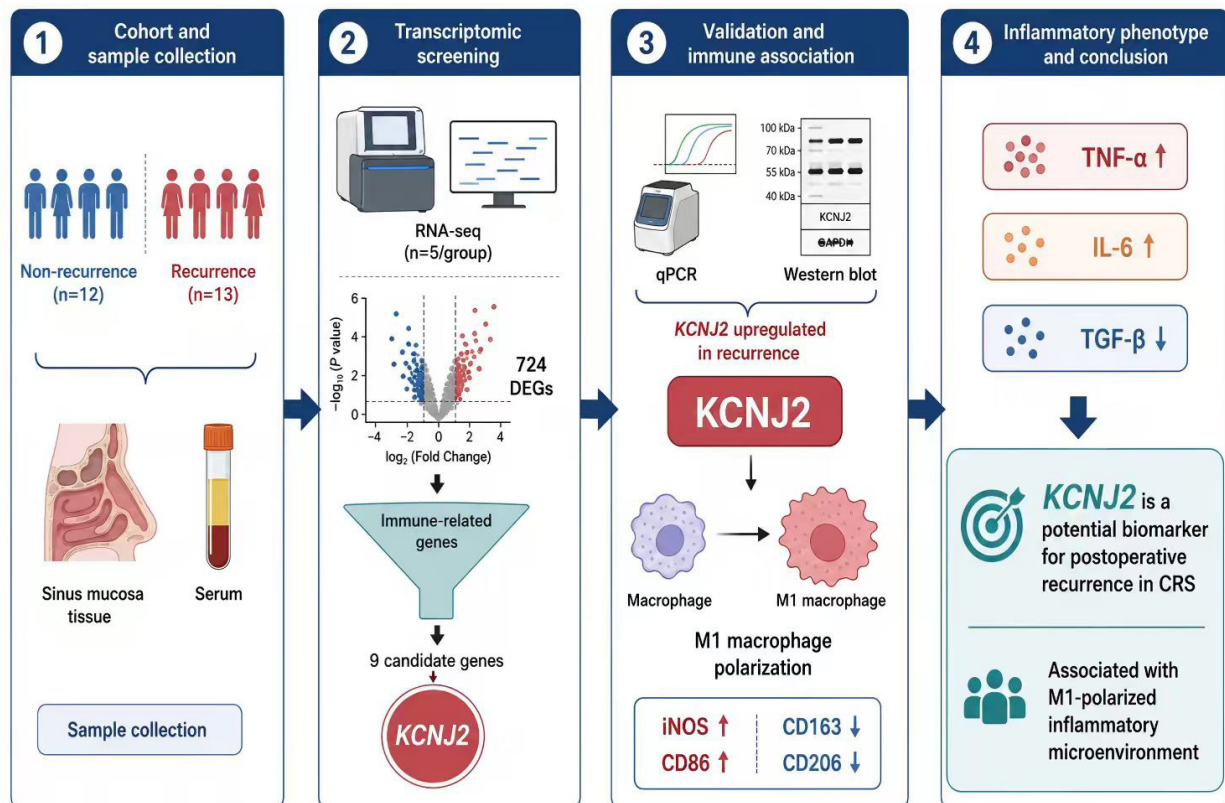
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## Graphical Abstract



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# Transcriptomics Identify KCNJ2 as a Potential Novel Biomarker for the Postoperative Recurrence in Chronic Sinusitis

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## Abstract

Chronic rhinosinusitis (CRS) is a prevalent inflammatory disease of the upper respiratory tract with a relatively high postoperative recurrence rate. Transcriptomic profiling may facilitate the identification of biomarkers associated with disease recurrence. This study aimed to characterize the transcriptomic features of postoperative recurrent CRS and identify potential recurrence-associated biomarkers. A total of 25 patients with CRS were enrolled, including 13 patients with postoperative recurrence and 12 without recurrence. Nasal mucosal tissues and serum samples were analyzed using RNA sequencing, RT-qPCR, Western blotting, and ELISA. KCNJ2 expression was significantly upregulated in the postoperative recurrence group. Immune infiltration analysis revealed a positive correlation between tissue KCNJ2 mRNA expression and estimated M1 macrophage infiltration. Further validation confirmed that KCNJ2 mRNA and protein levels were significantly increased in the recurrence group and positively correlated with M1 macrophage markers. In addition, serum cytokine levels were measured using ELISA. The serum levels of TNF- $\alpha$  and IL-6 were significantly increased in the postoperative recurrence group, whereas the serum level of TGF- $\beta$  was significantly decreased compared with the non-recurrence group. These cytokine alterations were significantly correlated with tissue KCNJ2 expression. These findings suggest that elevated KCNJ2 expression is associated with postoperative recurrence and an M1-skewed inflammatory microenvironment in CRS. KCNJ2 may therefore represent a candidate recurrence-associated biomarker, although prospective validation is required.

**Keywords:** Chronic rhinosinusitis; RNA-seq; Macrophages; KCNJ2; Recurrence

## Introduction

CRS is a common upper respiratory tract disease, mainly caused by infections, allergies, or immune system abnormalities, and is characterized by chronic inflammation of the sinus mucosa [1-2]. The typical pathological changes of this disease include inflammatory cell infiltration, mucosal edema, glandular hyperplasia, and secretion retention [3-4]. Patients usually present with symptoms such as nasal congestion, rhinorrhea, hyposmia, and headache, which significantly impair their quality of life [5-6]. Epidemiological studies have shown that the prevalence of chronic sinusitis in China is approximately 8% and is increasing year by year [7]. Currently, the main treatment methods include nasal irrigation with normal saline and local steroid therapy [8-9]. If the therapeutic effect of drugs is poor, functional endoscopic sinus surgery (FESS) can be considered, which can effectively improve clinical symptoms [10]. However, studies have demonstrated that the postoperative recurrence rate remains relatively high [11], which not only

increases the disease burden on patients but also imposes significant social and economic pressure. Therefore, identifying biomarkers related to postoperative recurrence is of great significance for in-depth understanding of the disease mechanism and improvement of prognosis management.

Transcriptomics, as an important tool for exploring disease pathogenesis, has been widely applied in the research of chronic sinusitis and its related disorders. For instance, Liu et al. identified four potential diagnostic biomarkers associated with nasal epithelial cell dysfunction by integrating multiple transcriptomic datasets of chronic sinusitis with nasal polyps (CRSwNP) [12]. Wang et al. combined single-cell sequencing with transcriptomic sequencing and found that dendritic cells are the key immune cell subset in CRSwNP; they further identified three hub genes, namely NR4A1, CLEC4G, and CD163, suggesting that these genes may serve as disease biomarkers and potential therapeutic targets [13]. Additionally, analysis based on transcriptomic data indicated that SLC17A4 was significantly downregulated in patients with CRS, and this find-

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ing was verified in clinical samples, rendering it a promising biomarker and therapeutic intervention target [14]. However, current studies focusing on analyzing the postoperative recurrence mechanism of CRS based on transcriptomic characteristics remain relatively limited.

The immune microenvironment plays a significant role in various inflammatory diseases. As key effector cells of the immune system, macrophages are crucial for regulating inflammation and maintaining the immune homeostasis of the nasal mucosa [15]. Under different microenvironmental stimuli, macrophages can differentiate into M1 and M2 macrophages. Upon activation, M1 macrophages secrete large amounts of pro-inflammatory factors, thereby promoting the inflammatory phenotype, while M2 macrophages are mainly involved in anti-inflammatory responses and tissue repair [16-17]. Studies have demonstrated that the infiltration of both M1 and M2 macrophages is significantly increased in patients with CRS. Hypoxia-inducible factor 1 $\alpha$  (HIF1- $\alpha$ ) can improve CRS by downregulating transferrin receptor (TFRC) expression and inhibiting M1 macrophage polarization. In patients with CRSwNP, the infiltration of M2 macrophages is more prominent [18-19], indicating the crucial role of macrophages in driving the progression of CRS. However, the mechanism underlying the role of macrophage polarization in the pathogenesis of CRS remains unclear. Therefore, it is essential to deeply understand the differentiation, phenotype, and function of macrophages in CRS.

This study aims to screen and identify key molecular markers closely associated with postoperative recurrence using transcriptome sequencing technology, followed by verification in clinical tissue samples. Based on these findings, it will further explore the intrinsic correlation between these markers and macrophage polarization in the immune microenvironment, thereby providing key scientific evidence for elucidating the pathological mechanism of postoperative recurrence, improving patient prognosis, and enhancing their quality of life.

## Materials and Methods

### Patient and Sample Collection

All participants provided written informed consent. The diagnosis of CRS was based on the criteria outlined in the European Position Paper on Rhinosinusitis and Nasal Polyps 2020 (EPOS 2020). The exclusion criteria are as follows: (1) Fungal sinusitis, allergic fungal sinusitis or malignant tumors; (2) Autoimmune diseases and other allergic or eosinophilic diseases; (3) Received immunotherapy, antibiotics, nasal/systemic steroids or anti-allergy drugs within 4 weeks before recruitment. A total of 25 patients with CRS were enrolled, including 12 patients without postoperative recurrence and 13 patients with recurrence. All participants provided written informed consent. Five samples from each group were randomly selected for RNA-seq. Initial RT-qPCR and Western blotting validation was performed using three biological samples per group, whereas expanded RT-qPCR, macrophage-marker analysis, and ELISA were performed using ten patients per group. The remaining samples were not included in specific assays because of insufficient sample volume or failure to meet assay-specific quality requirements. The RNA-seq samples were independent of the expanded validation cohort.

### RNA-seq

Total RNA was extracted from human nasal mucosal tissue samples using TRIzol Reagent (Life Technologies, California, USA) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). A total amount of 1  $\mu$ g RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using Hieff NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasten Biotechnology (Shanghai) Co., Ltd.) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was synthesized and second strand cDNA synthesis was subsequently performed. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. The library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3  $\mu$ l USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The libraries were sequenced on an Illumina NovaSeq platform to generate 150 bp paired-end reads, according to the manufacturer's instructions.

### Bioinformatics analysis

Differential expression analysis of two conditions/groups was performed using the DESeq2. DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value < 0.01 & Fold Change  $\geq$  2 found by DESeq2 were assigned as differentially expressed.

### Enrichment analysis

To identify candidate genes by crossing DEG and immune-related gene sets, The VennDiagram was drawn using the "Venn diagram" package. Enrichment analysis of these candidate genes was conducted using the ClusterProfiler software package (v3.18.1) in R, including the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [20].

### Immune infiltration analysis

Based on the transcriptome data, the immune infiltration was quantified using the "CIBERSORT" software package according to the CIBERSORT algorithm, which yielded the relative abundance of 22 immune cell types. The correlation between the target gene and these immune cells was then calculated via Spearman correlation analysis, and the lollipop plots was generated using the "ggplot2" package.

## RT-qPCR

High-quality RNA was isolated from the sample to maintain both its integrity and purity. The extracted RNA was then converted into complementary DNA (cDNA) through reversed transcription using reverse transcriptase. Subsequently, targeted amplification of the cDNA was performed via PCR technology, utilizing appropriate primers and reaction conditions to acquire the desired fragment. The PCR process employed SYBR Premix Ex Taq™ (TaKaRa) and was carried out on the ABI StepOne™ Real-Time PCR System (Applied Biosystems, CA). The results were calculated using the 2-ΔΔCT method. GAPDH was used as the internal reference. The specific primers for qPCR are shown in Table 1.

**Table 1. List of Primer Sequences.**

| Primer   | Sequences(5'-3')       |
|----------|------------------------|
| KCNJ2 F  | TTCAGTCACAATGCCGTGATT  |
| KCNJ2 R  | GCTTTTCCGAAGATTGCCCA   |
| KCNMB2 F | GAGGACCGAGCTATTCTCCTG  |
| KCNMB2 R | TGTTTCCGTGATGGACGCATT  |
| SLC4A4 F | TGATCGGGAGGCTTCTTCTCT  |
| SLC4A4 R | GGACCGAAGGTTGGATTCTTG  |
| GNA15 F  | CCAGGACCCCTATAAAGTGACC |
| GNA15 R  | GCTGAATCGAGCAGGTGGAAT  |
| APLNR F  | CTCTGGACCGTGTTCGGAG    |
| APLNR R  | GGTACGTGTAGGTAGCCACA   |
| EDN1 F   | AGAGTGTGTCTACTTCTGCCA  |
| EDN1 R   | CTTCCAAGTCCATACGGAACAA |

## Western Blotting

Sinus mucosal tissues were lysed in RIPA lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF; Bioteke Co., Wuxi, China). Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked at room temperature for 1 hour in TBST buffer containing 5% skimmed milk. Then, the membranes were incubated overnight at 4°C with anti-KCNJ2 (DF8117, Affinity, UK), and anti-GAPDH (60004-1-Ig, Proteintech, China). The next day, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (abs20040, absin, China) or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (abs20039, absin) at room temperature for 1 hour. Then, the membranes were washed 3 times with TBST. The enhanced chemiluminescence (ECL) detection kit was used to detect protein bands and quantitative analysis was performed using ImageJ software.

## ELISA

Blood samples were collected and homogenized at room temperature for 30 minutes. The samples were then centrifuged at 2000×g for 10 minutes at 4°C. The supernatant obtained after centrifugation was collected, aliquoted, and stored at temperatures below -20°C for subsequent analysis. Commercial ELISA kits were used to measure the serum concentrations of TNF-α (EH111776, BYabsience, China), IL-6 (EH110377, BYabsience), and TGF-β (EH1100532, BYabsience) following the manufacturer's instructions.

## Statistical analysis

Data are presented as the mean ± standard deviation. Normality and homogeneity of variance were assessed using the Shapiro–Wilk test and Levene's test, respectively. Comparisons between two groups were performed using an unpaired two-tailed Student's t-test when these assumptions were met. Welch's t-test or the Mann–Whitney U test was used when appropriate. Categorical variables were compared using Fisher's exact test. Correlations were evaluated using Spearman's rank correlation analysis. All data points represent independent patients. Exact P values were reported where feasible, and P < 0.05 was considered statistically significant.

## Result

### Clinical characteristics of the subjects

A total of 25 patients were enrolled in this study, including 12 in the CRS non-recurrence group and 13 in the postoperative recurrence group. The baseline characteristics of the two groups are presented in Table 2. In the non-recurrence group, there were 7 males and 5 females, whereas the recurrence group consisted of 11 males and 2 females. There was no significant difference in sex distribution between the two groups (Fisher's exact test, P = 0.020). Regarding age, the non-recurrence group had a mean age of (39 ± 14) years, while the recurrence group had a mean age of (32 ± 12) years (P = 0.071). For body mass index (BMI), the non-recurrence group had an average of (25 ± 3) kg/m², compared with (28 ± 5) kg/m² in the recurrence group (P = 0.064). Statistical analysis revealed no statistically significant differences in age or BMI between (P>0.05).

**Table 2. Overall Patient Demographic Characteristics and Associated Clinical Features.**

|               | Non-recurrence group(n=12) | Recurrence group(n=13) | P value |
|---------------|----------------------------|------------------------|---------|
| Male / female | 7/5                        | 11/2                   | /       |
| Agr (years)   | 39±14                      | 32±12                  | 0.071   |
| BMI (kg/m²)   | 25±3                       | 28±5                   | 0.064   |

Note: P value for sex distribution was calculated using Fisher's exact test.

### Transcriptomic analysis reveals the specific transcriptome associated with the recurrence of CRS

To identify the biomarkers for the recurrence of chronic sinusitis after surgery, 5 samples were randomly selected from each of the two groups for transcriptome sequencing. The transcriptomic characteristics of the two groups of samples were shown in Figure 1. PCA revealed that the samples from the non-recurrence group and the recurrence group exhibited independent clustering trends, indicating significant differences in transcriptomic expression between the two groups (Figure 1A). Volcano plot analysis showed that a total of 724 differentially expressed genes (DEGs) were identified, among which 543 genes exhibited significantly upregulated expression and 181 genes exhibited significantly downregulated expression (Figure 1B-C). Subsequently, we performed GO enrichment analysis on the DEGs, and the results showed that these DEGs

were mainly enriched in processes related to ciliary structural integrity, motor function, or ciliogenesis and ciliary structure and dysfunction are also one of the pathophysiological characteristics of chronic sinusitis (Figure 1D) [21]. Further KEGG pathway enrichment analysis revealed that the DEGs were mainly involved in immune inflammation-related pathways and the PPAR signaling pathway (Figure 1E), suggesting that they might participate in the recurrence process of sinusitis after surgery by regulating these pathways.

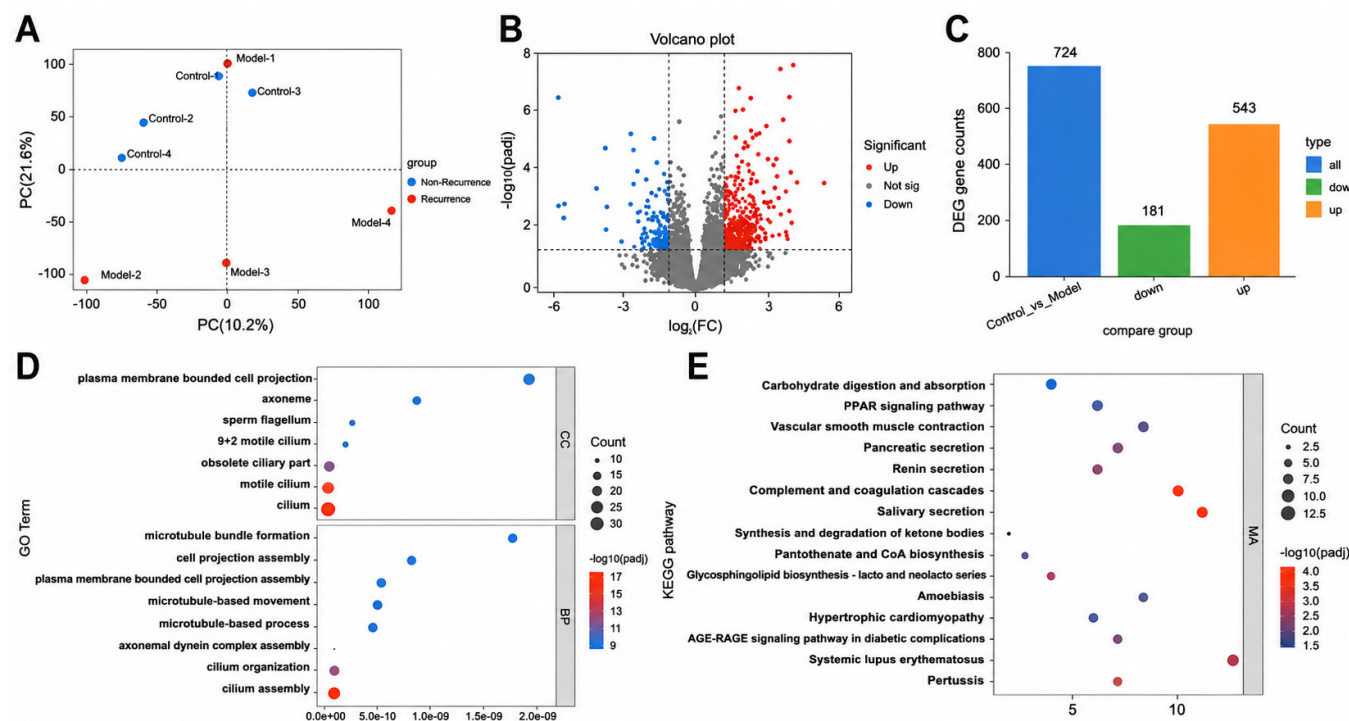
### KCNJ2 was significantly elevated in the recurrence group and was associated with M1 macrophages

Given that the immune microenvironment plays an important role in CRS, we downloaded immune-related gene sets from the database and took the intersection with the DEGs obtained by RNA-seq. As shown in Figure 2A, 9 DEGs were finally obtained. The expression heatmap showed that EDN1 and APLNR were downregulated in the postoperative recurrence group, while GNA15, KCNJ2, CXCL6, CCL20, CSF3, KCNMB2, and SLC4A4 were upregulated (Figure 2B). Among these 9 DEGs, CXCL6, CCL20, and CSF3 have been previously reported in relevant studies [22-23]. Subsequently, GO and KEGG enrichment analyses were performed on these 9 genes, and the results showed that these 9 genes were mainly enriched in biological processes such as neuroelectrophysiological regulation, immune cell chemotactic response, and ion channel

function, and were involved in the activation and regulation of immune inflammatory responses, such as classic inflammatory pathways including IL-17, TNF, and cytokine interaction (Figure 2C-D). This indicated that these DEGs might play key roles in biological processes related to inflammatory diseases or immune disorders. Therefore, we further performed immune infiltration analysis of these six previously unreported DEGs, and only KCNMB2 and KCNJ2 were significantly positively correlated with M1 macrophage infiltration (Figure 3). To further validate the results obtained from RNA-seq, we performed qPCR verification using clinical samples; the results indicated that only the expression level of KCNJ2 was significantly increased in the recurrence group (Figure 4E). After expanding the sample size for detection, the mRNA and protein expression levels of KCNJ2 were still significantly elevated, which were consistent with our previous transcriptomic and differential analysis results (Figure 4G-H). These findings collectively suggest that KCNJ2 may serve as a candidate biomarker associated with postoperative recurrence of CRS.

### Macrophage M1 polarization associates with KCNJ2 expression in recurrent CRS

To clarify whether abnormal macrophage polarization occurs during the recurrence of chronic sinusitis after surgery and whether it is related to KCNJ2 expression, we evaluated the expression of M1 and M2 macrophage marker genes between



**Figure 1. RNA-seq identified a distinctive gene profile in CRS patients between the Recurrence and Non-recurrence groups.**

(A) PCA model of the transcriptomics data between the two groups. (B) Volcano plot showing genes upregulated and downregulated by RNA-seq. (C) Bar chart shows the number of DEGs between the two groups. (D) GO enrichment analysis. (E) KEGG enrichment analysis. Control, Non-recurrence; Model, Recurrence. n=5.



the recurrence and non-recurrence groups. As shown in Figure 5, the mRNA levels of CD86 and iNOS were significantly elevated in the recurrence group, whereas the expression levels of CD163 and CD206 were markedly reduced. Notably, the expression of CD86 and iNOS was positively correlated with KCNJ2, while the expression of CD163, CD206, and KCNJ2 was negatively correlated. ELISA analysis showed that serum TNF- $\alpha$  and IL-6 levels were significantly increased in the post-operative recurrence group, whereas serum TGF- $\beta$  levels were significantly decreased. TNF- $\alpha$  and IL-6 levels were positively correlated with tissue KCNJ2 expression, whereas TGF- $\beta$  levels were negatively correlated with KCNJ2 expression. Collectively, these findings indicated that KCNJ2 might promote M1 macrophage polarization, stimulate the secretion of pro-inflammatory cytokines, and thereby contribute to the postoperative recurrence of chronic sinusitis.

### Macrophage M1 polarization is associated with KCNJ2 expression in recurrent CRS

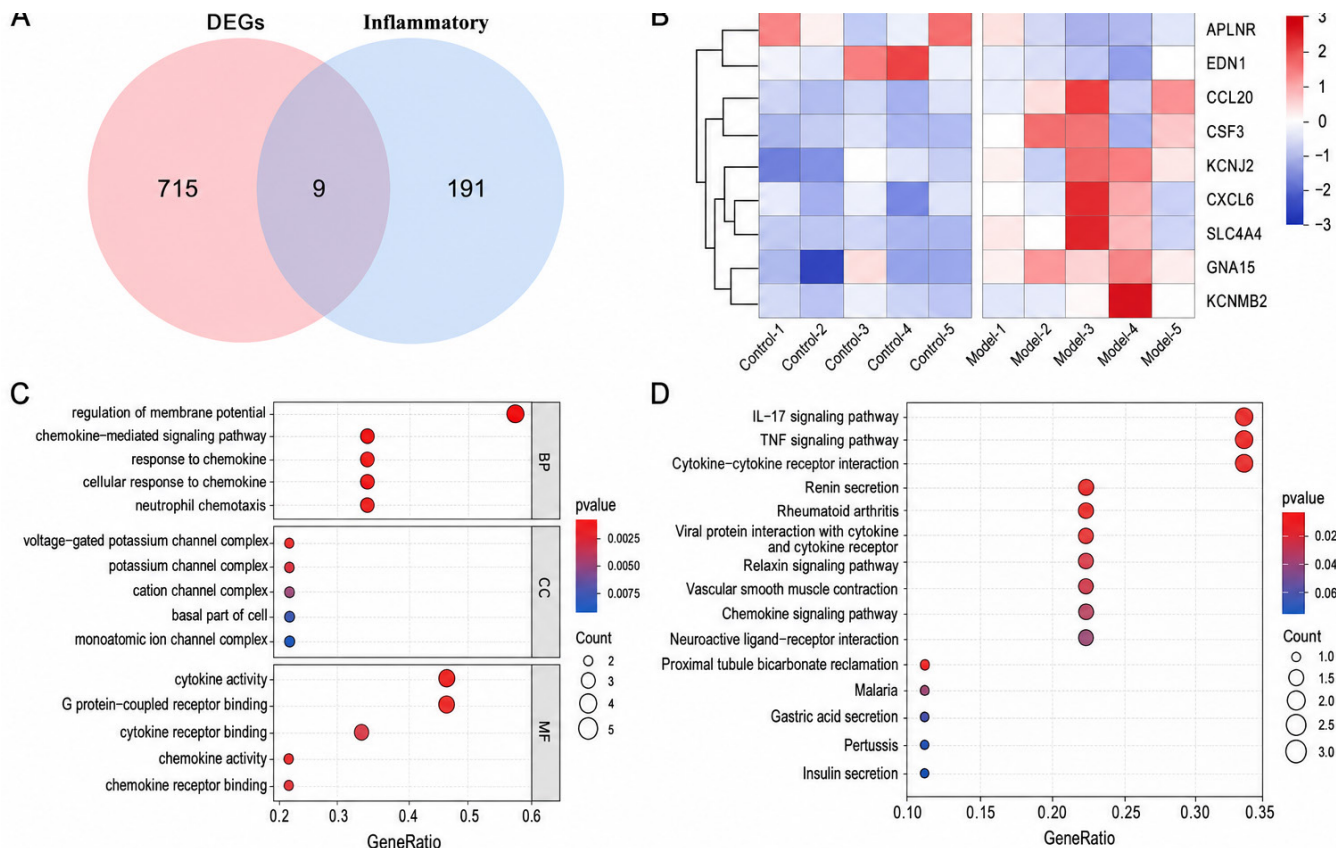
To determine whether macrophage polarization was altered in patients with postoperative recurrence of CRS and whether these changes were associated with KCNJ2 expression, we examined the expression of representative M1- and M2-associated macrophage markers. As shown in Figure 5A-D, the mRNA expression levels of the M1-associated markers CD86

and iNOS were significantly increased in the recurrence group, whereas the expression levels of the M2-associated markers CD163 and CD206 were significantly decreased. Spearman correlation analysis further showed that tissue KCNJ2 expression was positively correlated with CD86 and iNOS expression but negatively correlated with CD163 and CD206 expression (Figure 5E-H). These findings indicate that elevated KCNJ2 expression is associated with an M1-skewed macrophage-related inflammatory profile in recurrent CRS.

We subsequently measured serum inflammatory cytokine levels using ELISA. Serum IL-6 and TNF- $\alpha$  levels were significantly higher in the recurrence group than in the non-recurrence group, whereas serum TGF- $\beta$  levels were significantly lower (Figure 6A-C). Furthermore, tissue KCNJ2 expression was positively correlated with serum IL-6 and TNF- $\alpha$  levels but negatively correlated with serum TGF- $\beta$  levels (Figure 6D-F). Collectively, these results demonstrate that elevated tissue KCNJ2 expression is associated with increased systemic pro-inflammatory cytokine levels and reduced TGF- $\beta$  levels in patients with postoperative recurrence of CRS.

## Discussion

CRS is a highly heterogeneous disease with a high postopera-

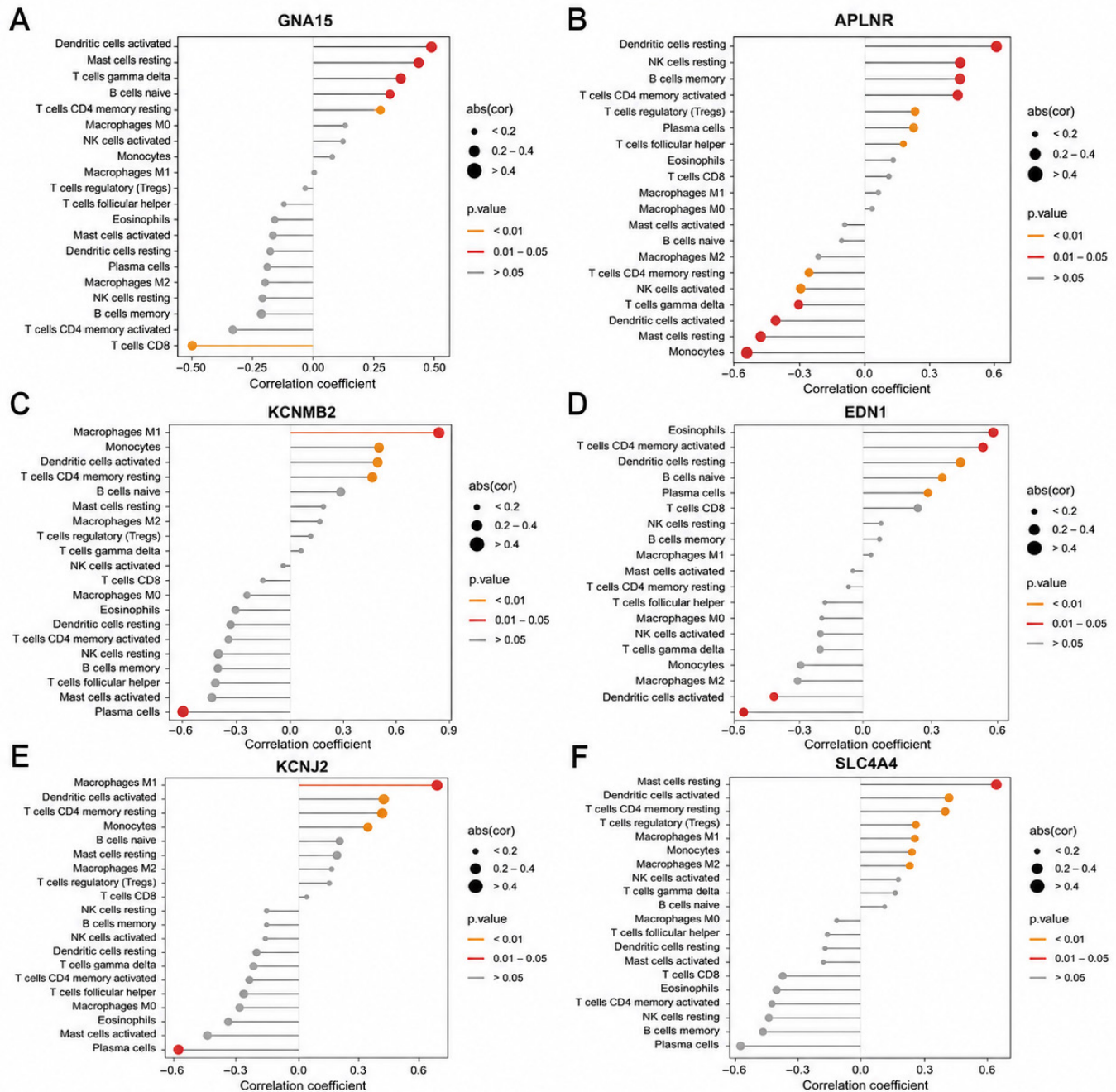


**Figure 2. Bioinformatics analysis identified 9 immune-related DEGs. (A)** The Venn diagram illustrated the intersection relationship between the DEGs in RNA-seq and the immune-related genes. **(B)** Heatmap of expression levels of candidate DEGs. **(C)** GO enrichment of the candidate DEGs. **(D)** KEGG enrichment of the candidate DEGs.

tive recurrence rate. Despite extensive research on the pathogenesis of CRS and its subtypes, no predictive model for CRS postoperative recurrence has been established to date. Studies have demonstrated that persistent inflammatory responses and immune dysregulation may be the core factors driving postoperative recurrence. Therefore, from the perspective of immune regulation, screening key biomarkers associated with CRS postoperative recurrence is of great significance for the

management of postoperative recurrence in patients, the reduction of recurrence rates, and the alleviation of burdens on patients and society.

RNA-seq, as one of the commonly used detection techniques, was often employed to identify disease markers. In this study, we sequenced the nasal sinus mucosal tissues of patients with postoperative recurrence and those without recurrence, and identified a total of 724 DEGs. These genes were enriched

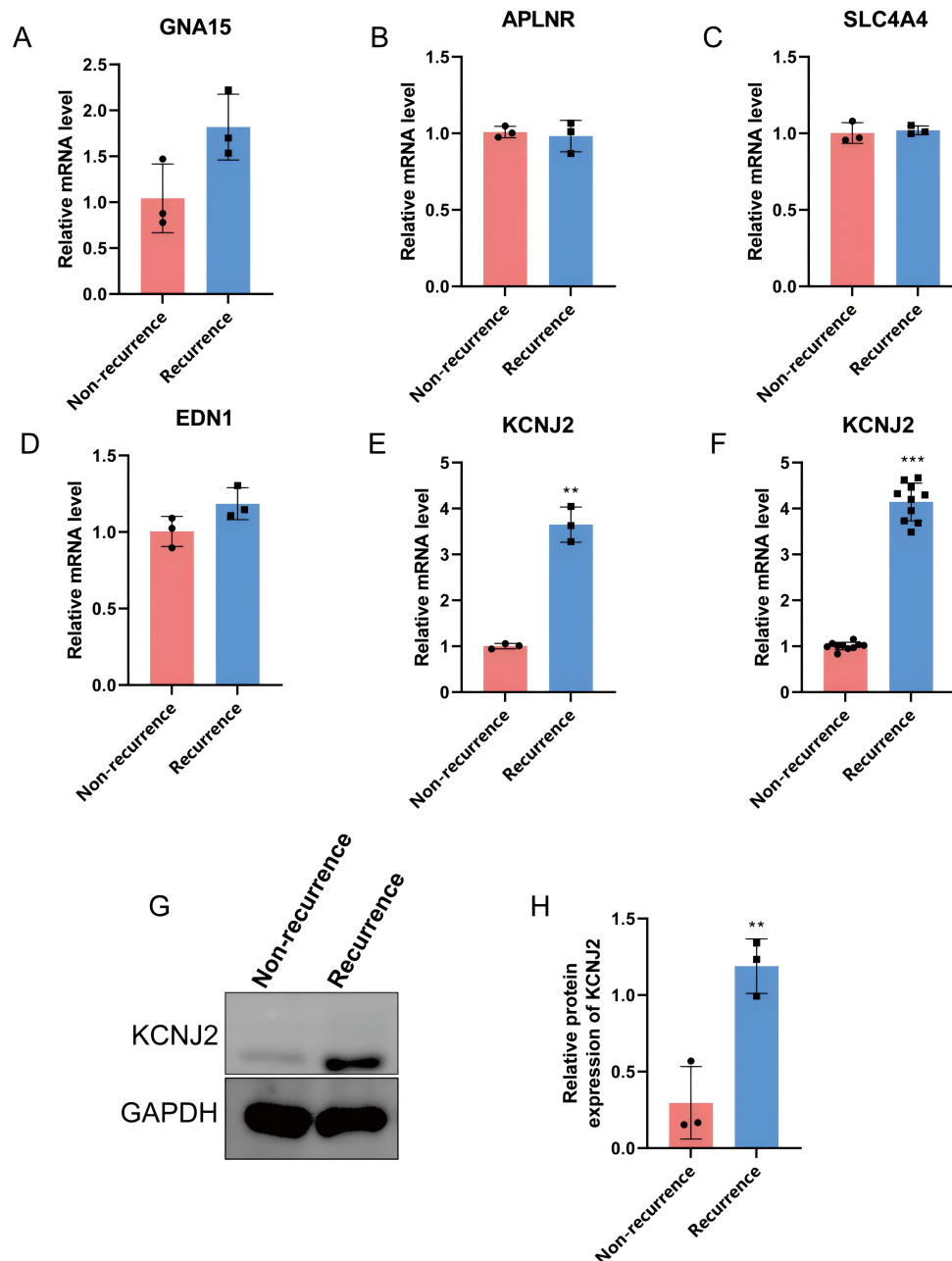


**Figure 3. Correlation analysis of DEGs expression and immune infiltration. (A) GNA15; (B) APLNR; (C) KCNMB2; (D) EDN1; (E) KCNJ2; (F) SLC4A4.  $P < 0.01$ .**

in immune inflammation-related pathways, suggesting that immune system dysregulation might play an important role in disease recurrence. Therefore, we downloaded immune inflammation-related gene sets from the database and performed intersection analysis with the sequenced differential gene set. A total of 9 DEGs were obtained. To further select the key genes, we combined it with immune infiltration analysis, and the results showed that only KCNJ2 was highly expressed in the disease and significantly positively correlated with M1 macrophages.

KCNJ2, as one of the members of the inward rectifying potassium ion channel subfamily J, is widely expressed in various cells such as neurons, skeletal muscles, cardiomyocytes, and

immune cells [24-25]. Studies have shown that phosphatidylinositol-4,5-bisphosphate, by binding to KCNJ2, can open the inward potassium ion channel Kir2.1, thereby regulating the membrane potential [26], providing a theoretical basis for the participation of KCNJ2 in cell function regulation. In recent years, numerous reports have indicated that ion channels are closely related to the regulation of the mucosal barrier and inflammatory cells [27-28]. As one of the most widespread ion channels in the human body, potassium ion channels are widely expressed in various cells [24]. Lin et al. discovered that knocking down KCa3.1 could reduce the number of goblet cells and mast cells in the nasal mucosa of allergic rhinitis mice, as well as the levels of inflammatory cytokines IL-4, IL-



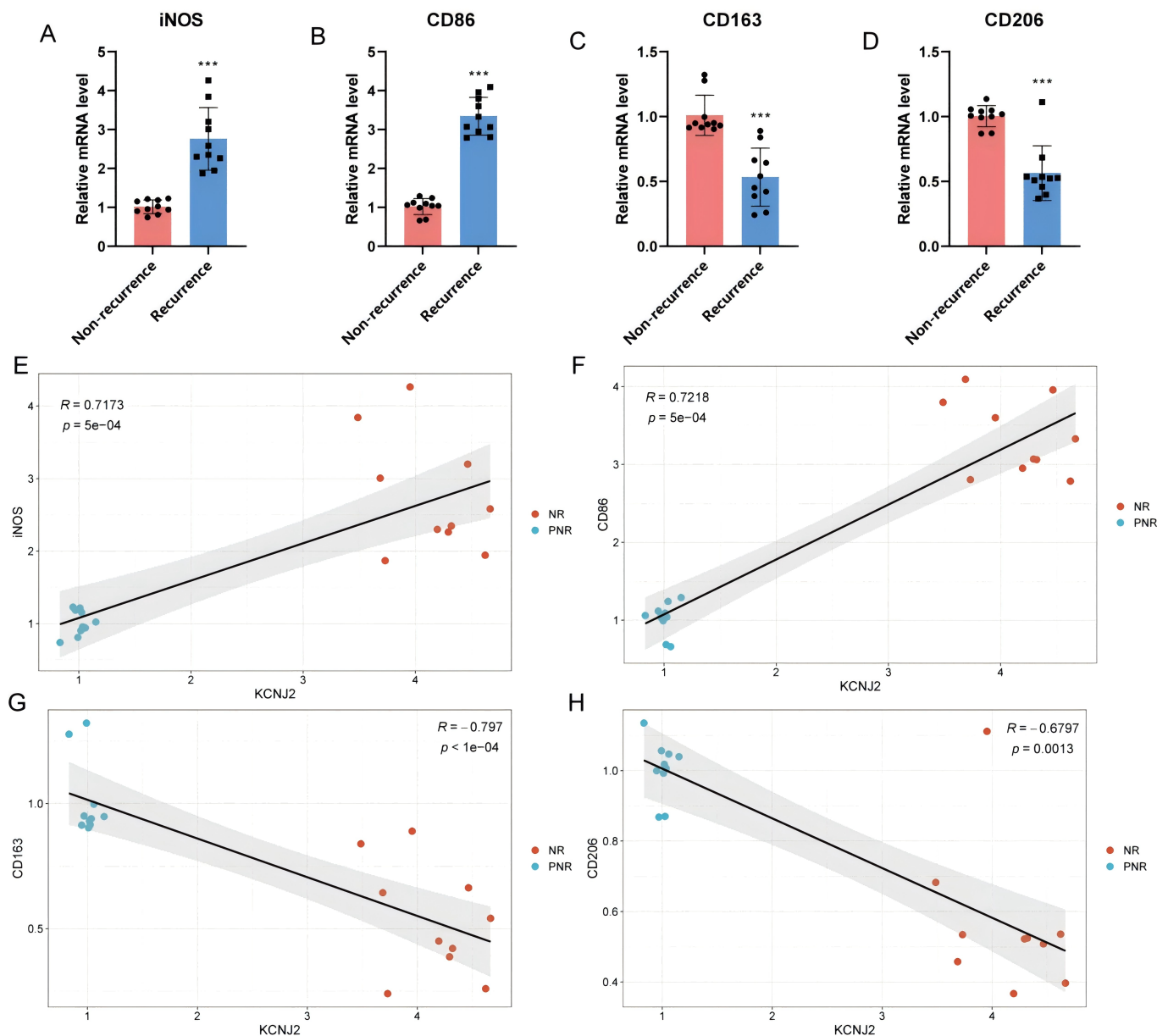
**Figure 4. Verify the expression levels of candidate DEGs in clinical samples. (A-E)** qPCR was performed to assess the mRNA expression levels of DEGs, n=3 per group. **(F)** RT-qPCR was performed to assess KCNJ2 mRNA expression, n = 10 per group. **(G-H)** Western blotting was performed to assess the protein expression levels of KCNJ2, n=3 per group. Data are presented as mean  $\pm$  SD, \*\*p < 0.01, \*\*\*p < 0.001.

9, and IL-17 in nasal lavage fluid [29]. The lack of KCa3.1 in T lymphocytes, B lymphocytes, mast cells, and bronchial smooth muscle cells has a protective effect against OVA-induced asthma [30], demonstrating the important role of potassium ion channels in regulating respiratory inflammatory diseases. The sequencing results of this study showed that the expression level of KCNJ2 in the sinus mucosa tissue of the recurrence group was significantly higher than that of the non-recurrence group. Combined with the regulatory role of potassium ion channels in respiratory inflammation, it suggests that KCNJ2 may be involved in the pathological process of sinusitis recurrence after surgery. Subsequently, to eliminate possible errors in the sequencing process, we collected 10 clinical samples for further verification. The results showed that the expression of KCNJ2 was significantly increased, consistent with the se-

quencing results.

The association between KCNJ2 and macrophage function has been documented previously. KCNJ2 is expressed in various macrophage populations, including microglia, bone marrow derived macrophages, and THP 1 macrophages [31-32]. Yu et al. demonstrated that KCNJ2 is essential for maintaining the electrophysiological properties of macrophages and positively regulates macrophage inflammatory activation in vitro [33]. Furthermore, Chen et al. reported that knockdown of KCNJ2 markedly suppressed LPS induced M1 macrophage polarization [34], suggesting that KCNJ2 contributes to M1 macrophage polarization.

In the present study, we observed that the expression of M1 macrophage markers iNOS and CD86 was significantly elevated in tissues from patients with postoperative recurrence,

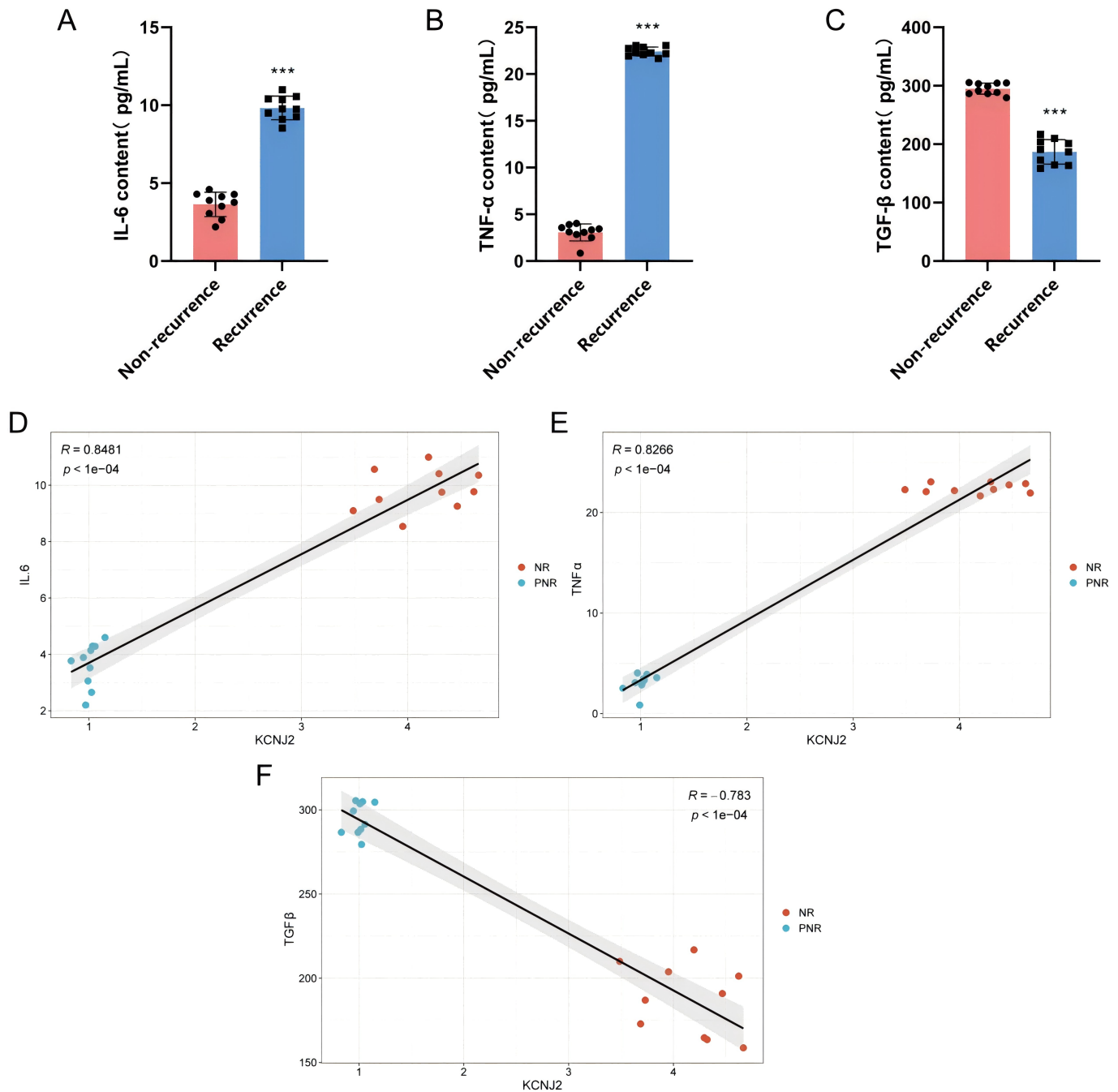


**Figure 5. The mRNA expressions of macrophage M1- and M2-associated macrophage markers in different groups.** (A-D) qPCR was used to detect the expression of M1 and M2 macrophage marker genes.  $n = 10$ , per group. Data are presented as mean  $\pm$  SD, \*\*\*P < 0.001. (E-H) Spearman correlation analysis of KCNJ2 expression with the marker genes, \*\*\*P < 0.001.



whereas the expression of M2 markers CD163 and CD206 was markedly reduced. Meanwhile, the levels of pro inflammatory cytokines TNF- $\alpha$  and IL 6 were increased, while the anti inflammatory factor TGF- $\beta$  was decreased. These findings were consistent with previous reports [35], further supporting that aberrant M1 macrophage polarization may contribute to postoperative recurrence of chronic sinusitis. Notably, studies on postoperative recurrence of CRSwNP often reported a predominant M2 macrophage phenotype, which differs partially from our observations [36-37]. We speculate that this discrepancy may arise from distinct pathological processes between

the two phenotypes. CRSwNP is characterized by nasal polyp formation, epithelial mesenchymal transition (EMT), and nasal mucosal remodeling [38]. M2 macrophages not only mediate anti inflammatory responses but also secrete cytokines that promote mucosal fibrosis, tissue remodeling, and EMT [39-41]. Thus, a certain level of M2 polarization is required to sustain these pathological features in CRSwNP. In contrast, the phenotype examined in this study was dominated by persistent local inflammatory activation, which favored a shift toward M1 macrophage polarization. Although our study identified concurrent upregulation of KCNJ2 and enhanced M1 macrophage polar-



**Figure 6. Altered serum cytokine levels in patients with postoperative recurrence of CRS.** (A-C) ELISA was used to determine serum IL-6, TNF- $\alpha$ , and TGF- $\beta$  levels in the non-recurrence and recurrence groups, with  $n = 10$  independent patients per group. (D-F) Spearman correlation analysis between tissue KCNJ2 expression and serum cytokine levels. \*\*\* $P < 0.001$ .

ization in patients with postoperative recurrence, the functional link between these two events remained unclear. We therefore performed correlation analysis. The results showed that KCNJ2 expression was significantly and positively correlated with M1 macrophage markers and pro inflammatory cytokines TNF $\alpha$  and IL 6, but negatively correlated with M2 macrophage markers. Collectively, based on our findings and previous literature, we propose that KCNJ2 may promote M1 macrophage polarization and the subsequent release of pro inflammatory mediators including TNF $\alpha$  and IL 6, thereby exacerbating local chronic inflammation in the nasal mucosa and contributing to the pathogenesis of postoperative recurrence of CRS.

In summary, our findings indicate that KCNJ2 may be involved in the pathogenesis of CRS and contribute to postoperative recurrence of CRS. Therefore, tissue KCNJ2 expression may serve as a candidate biomarker associated with postoperative recurrence of CRS. However, its predictive value requires validation in prospective cohorts using preoperative samples and formal discrimination analyses.

This study still has some limitations. First, the specific molecular mechanism by which KCNJ2 regulates macrophages remains unclear, and key downstream factors need further identification and verification. Second, due to time constraints, KCNJ2 as a recurrence marker has not been verified in a large number of clinical samples. In addition, CIBERSORT analysis and bulk-tissue expression of macrophage-associated markers cannot directly demonstrate macrophage polarization or confirm that KCNJ2 functions specifically within macrophages. Cell-type-specific localization and functional experiments using isolated macrophages or macrophage-specific models are required in future studies. Therefore, future studies should focus on investigating the molecular mechanism by which KCNJ2 regulates M1 macrophages. In addition, it is necessary to collect a large number of clinical samples and corresponding clinical data to comprehensively evaluate the effectiveness of KCNJ2 as a marker for postoperative recurrence.

## Conclusion

This study shows that the transcriptional profiles of patients with postoperative recurrence are significantly different from those of patients without recurrence. Among these potential DEGs, KCNJ2 shows potential predictive ability for recurrence. The clinical sample validation results indicate that the expression of KCNJ2 in postoperative recurrence patients is significantly higher than that in non-recurrence patients. Moreover, KCNJ2 expression was elevated in patients with established postoperative recurrence and was associated with M1 macrophage polarization. These findings support KCNJ2 as a candidate recurrence-associated biomarker rather than a prospectively validated predictive marker.

## Abbreviations

CRS: Chronic rhinosinusitis; CRSwNP: chronic sinusitis with nasal polyps; KCNJ2: potassium inwardly rectifying channel subfamily J member 2; RNA-seq: RNA sequencing; PCA: Principal Component Analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: Differentially expressed genes; GO: Gene

Ontology.

## Author Contributions

M.Z. and W.C.: Study conception and design, data analysis and interpretation, and manuscript drafting. H.H. and C.F.: Experimental design, key experiments, data collection and analysis, and manuscript drafting and revision. M.H. and J.J.: Sample collection and processing, laboratory experiments, data organization, and result interpretation. G.D.: Study design participation, experimental assistance, and manuscript feedback. Q.J.: Data collection and organization, figure and table preparation, and manuscript revision. D.L.: Study design, data interpretation, manuscript revision, and research guidance. W.C. supervised the study, provided project administration, critically revised the manuscript, and approved the final version. All authors read and approved the final manuscript.

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Not Applicable.

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## Ethics Approval and Consent to Participate

This study was approved by the Medical Ethics Committee of Bozhou People's Hospital (Approval No. 2023-228). Written informed consent was obtained from all participants before sample collection. The study was conducted in accordance with the Declaration of Helsinki.

## Competing Interests

The authors declare that they have no competing interests.

## Data Availability

The data generated or analyzed during this study are included in this article and its supplementary information files. Additional data are available from the corresponding author upon reasonable request.

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