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peng wang

wangpengpublic@163.com

Second Affiliated Hospital of Harbin Medical University

zuer liu

Second Affiliated Hospital of Harbin Medical University

shuo liu

Second Affiliated Hospital of Harbin Medical University

baiying liu

Second Affiliated Hospital of Harbin Medical University

xiang li

Second Affiliated Hospital of Harbin Medical University

yanan sun

Second Affiliated Hospital of Harbin Medical University

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GlycoRNA-associated miR-1228-3p promotes malignant progression of laryngeal squamous cell carcinoma by targeting SOX17

Peng Wang^{1#*}, Zuer Liu^{1#}, Shuo Liu^{1#}, Baiying Liu¹, Xiang Li¹, Yanan Sun^{1*}

¹The Department of Otolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Harbin Medical University, Harbin, 150000, China.

[#]These authors contributed equally: First author and Co-author

*Correspondence author:

Peng Wang, PhD. The Department of Otolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Harbin Medical University, No. 246, Xuefu Road, Nan'gang District, Harbin, Heilongjiang Province, 150000, China.

Tel: 15699610915; E-mail: wangpengpublic@163.com

Yanan Sun, PhD. The Department of Otolaryngology Head and Neck Surgery, The Second Affiliated Hospital of Harbin Medical University, No. 246, Xuefu Road, Nan'gang District, Harbin, Heilongjiang Province, 150000, China.

Tel: 13633631706; E-mail: syn2767@126.com

Abstract

Background: Laryngeal squamous cell carcinoma (LSCC) remains a clinically challenging subtype of head and neck squamous cell carcinoma, with limited prognostic markers and

1 actionable therapeutic targets. Glycosylated small RNAs (GlycoRNAs) are small RNAs
2 modified with N-glycans and displayed on the cell surface, yet the GlycoRNA-associated
3 miRNA landscape and its biological relevance in LSCC remain poorly defined.

4 **Methods:** Technical validation of GlycoRNA detection was performed using metabolic labeling
5 and nuclease-sensitivity controls. GlycoRNA-associated small RNAs from paired LSCC tumors
6 and adjacent mucosa were enriched by wheat germ agglutinin (WGA) and profiled utilizing the
7 Arraystar Human GlycoRNA Microarray. Candidate miRNAs were prioritized through the
8 integration of GSE132222 data, RT-qPCR, TargetScan/miRDB predictions, SOX17-centered
9 network and enrichment analyses, a supportive GSEA panel, and CLEAR-CLIP-derived
10 orthogonal evidence. The functional relevance of GlycoRNA-associated miRNAs was assessed
11 via in vitro RNase A/RNasin perturbation and an in vivo nude mouse xenograft model, followed
12 by Transwell and Ki67/TUNEL assays. Direct interaction between miR-1228-3p and SOX17
13 was further interrogated utilizing a dual-luciferase reporter assay.

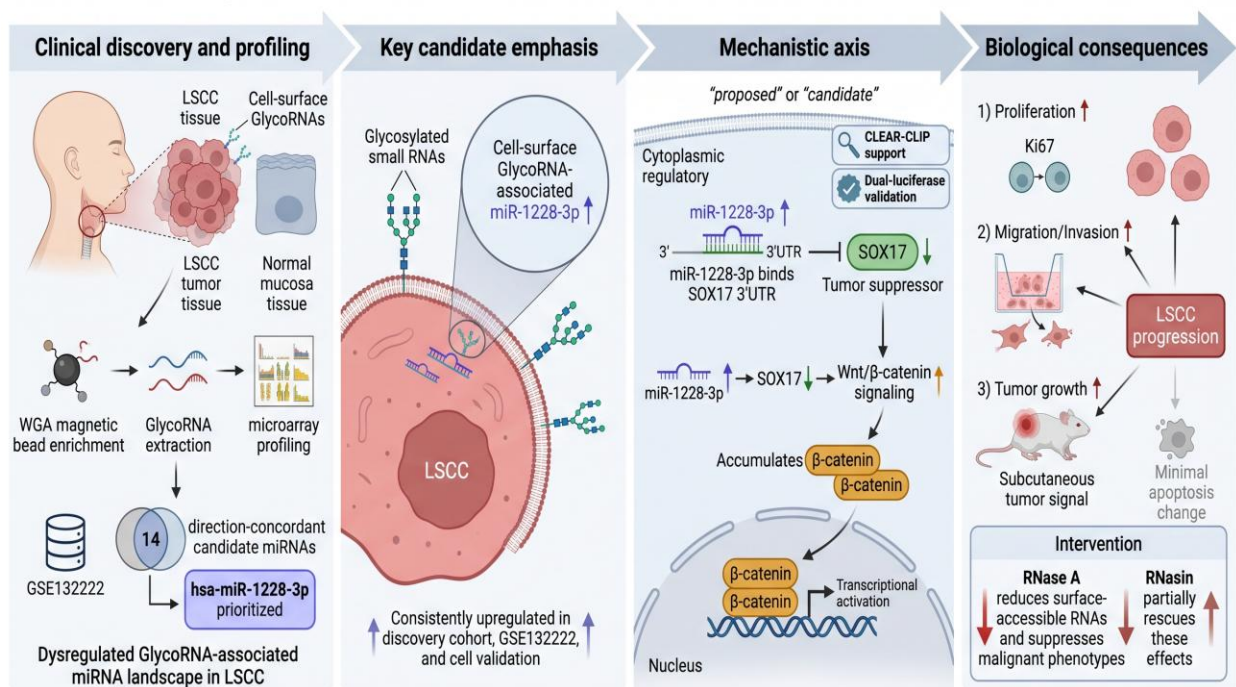
14 **Results:** Technical controls validated RNase-sensitive GlycoRNA-associated signals, and
15 sample-quality metrics supported the downstream microarray analysis. Discovery profiling
16 revealed a distinct LSCC-associated GlycoRNA small-RNA pattern, and integration with the
17 GSE132222 dataset identified 14 directionally concordant candidate miRNAs. Among five
18 prioritized candidates, hsa-miR-1228-3p exhibited the most consistent upregulation across
19 external cohorts and cell-based validation. In vitro RNase A treatment attenuated invasion and
20 reduced Ki67 staining without notably inducing apoptosis, whereas in vivo administration
21 markedly attenuated tumor-associated signals; RNasin co-treatment partially rescued these
22 alterations. Integrative target analyses prioritized SOX17; furthermore, enrichment analyses,
23 target-prioritization plots, TCGA-HNSC cohort validation, a supportive SOX17-stratified GSEA

panel, and CLEAR-CLIP-derived orthogonal evidence collectively confirmed a compatible hsa-miR-1228-3p binding site within the SOX17 3'UTR, an interaction directly supported by the dual-luciferase reporter assay.

Conclusions: LSCC harbors a significantly dysregulated GlycoRNA-associated miRNA signature. GlycoRNA-associated hsa-miR-1228-3p emerges as a reproducible candidate mechanistically linked to Glycosylated miRNA biology in LSCC. The proposed hsa-miR-1228-3p/SOX17/Wnt/ β -catenin axis is substantiated by robust bioinformatic screening, CLEAR-CLIP evidence, and dual-luciferase reporter validation, whereas future gain- and loss-of-function and rescue experiments remain requisite to definitively establish pathway causality.

Keywords: laryngeal squamous cell carcinoma; GlycoRNA; miR-1228-3p; SOX17;

Proposed GlycoRNA-associated miR-1228-3p/SOX17/Wnt/ β -catenin axis in LSCC



Background

Laryngeal squamous cell carcinoma (LSCC) is a major anatomical subtype of head and neck squamous cell carcinoma (HNSCC) and remains an important cause of cancer-related morbidity and mortality worldwide^[1]. Global cancer statistics for 2022 estimated nearly 20 million new cancer cases and 9.7 million cancer deaths overall, with larynx cancer alone accounting for 189,191 new cases and 103,359 deaths worldwide^[1,2]. Despite advances in surgery, radiotherapy, systemic therapy, and organ-preservation strategies, recurrent disease, treatment resistance, and the lack of robust biomarkers or actionable therapeutic targets continue to limit outcome improvement in LSCC and broader HNSCC^[3,4].

Glycosylated small RNAs (GlycoRNAs) represent a recently described class of RNAs modified with N-glycans and displayed on the cell surface^[5,6]. Their surface accessibility and capacity to interact with glycan-binding proteins suggest potential roles in extracellular communication, immune modulation, and receptor-proximal signaling that are not captured by conventional intracellular transcriptomic profiling. In parallel, microRNAs (miRNAs) are well-established post-transcriptional regulators of gene expression, and dysregulated miRNA networks have been widely implicated in HNSCC pathogenesis as candidate diagnostic, prognostic, and therapeutic biomarkers, including in LSCC^[7-9]. Moreover, emerging cancer studies suggest that perturbation of cell-surface glycoRNAs can influence malignant cell proliferation, further supporting the biological and translational relevance of this newly recognized RNA class^[10].

Among oncogenic signaling programs relevant to HNSCC, canonical Wnt/ β -catenin signaling is strongly linked to proliferation, migration, invasion, stem-like behavior, and treatment resistance^[11]. Prior molecular and clinical studies have implicated Wnt-pathway dysregulation in head and neck tumors, including laryngeal carcinomas, where Wnt activity has shown prognostic

1 relevance^[12]. SOX17 is recognized as a tumor-suppressive antagonist of β -catenin-driven
2 transcriptional output in epithelial cancers^[13], and a miR-1228-3p/SOX17/ β -catenin regulatory
3 axis has been reported in triple-negative breast cancer^[14], providing a plausible mechanistic
4 framework for interrogation in LSCC.

5 Herein, WGA-based enrichment and microarray profiling were performed to define GlycoRNA-
6 associated miRNAs in paired LSCC and adjacent tissues. Subsequently, the discovery results
7 were integrated with the independent LSCC dataset GSE132222, prioritizing five specific
8 candidates for cellular validation. Both in vitro and in vivo RNase-based perturbation models
9 were utilized to probe the phenotypic relevance of GlycoRNA-associated miRNAs. Finally,
10 consensus target prediction, clinical cohort integration (TCGA-HNSC), pathway analysis,
11 CLEAR-CLIP-supported evidence, and dual-luciferase reporter validation were systematically
12 combined to prioritize a candidate hsa-miR-1228-3p/SOX17/Wnt/ β -catenin axis in LSCC.

13 **Methods**

14 **Study design and public datasets**

15 Discovery profiling of GlycoRNA-associated miRNAs in paired LSCC tumor and adjacent
16 normal tissues was performed utilizing WGA enrichment followed by miRNA microarray
17 analysis. Candidate miRNAs were subsequently validated against the public LSCC cohort
18 GSE132222. Mechanistic nomination integrated consensus target prediction, enrichment
19 analysis, Sankey-network visualization, a SOX17-stratified supportive GSEA plot, and CLEAR-
20 CLIP-supported interaction evidence, with contextual interpretation informed by established
21 HNSCC molecular paradigms.

Clinical specimens

Paired LSCC tumor tissues and adjacent non-tumor mucosal tissues were collected from patients undergoing surgical resection at the Second Affiliated Hospital of Harbin Medical University between May 2023 and December 2025. Fresh specimens were snap-frozen in liquid nitrogen immediately following resection and maintained at -80°C until downstream processing. The collection and utilization of human specimens received ethical approval from the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (GZRDWSYYS-016) and were conducted in strict adherence to institutional guidelines. Written informed consent was obtained from all participants prior to sample acquisition.

Cell culture

Human LSCC cell lines AMC-HN-8, TU177, TU212, and TU686 were utilized for candidate-expression validation and functional assays. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin, and maintained at 37°C in a humidified atmosphere containing 5% CO_2 .

Enrichment of GlycoRNAs and miRNA microarray

Total RNA was subjected to affinity capture utilizing lectin wheat germ agglutinin (WGA)-conjugated magnetic beads to specifically enrich GlycoRNAs. The bead-bound fraction was subsequently extracted using standard TRIzol reagent. Recovered GlycoRNAs were treated with T4 polynucleotide kinase to eliminate residual 3'-monophosphate and cyclic phosphate groups, generating uniform 3'-OH termini. Cy3 fluorescent dye was ligated to the 3'-OH ends utilizing

T4 RNA ligase. The Cy3-labeled GlycoRNAs were hybridized onto the Arraystar Human GlycoRNA Microarray to profile small GlycoRNAs, encompassing miRNAs, tsRNAs (tRFs and tiRNAs), pre-miRNAs, tRNAs, snoRNAs, and other small RNA species. Microarray slides were scanned utilizing an Agilent G2505C scanner, and raw images were processed via Agilent Feature Extraction Software (v11.0.1.1). Quantile normalization and subsequent bioinformatic analyses were executed in GeneSpring GX v12.1. Following signal quality control (QC) flag filtering, probe signals targeting identical GlycoRNAs were averaged to yield a consensus expression value. Differentially expressed GlycoRNAs were identified based on strict thresholds of $P < 0.05$ and an absolute fold change ≥ 1.3 .

RT-qPCR validation and enrichment controls

Total RNA was isolated utilizing either a phenol-chloroform protocol or a commercial extraction kit. Reverse transcription was performed employing miRNA-specific stem-loop primers, followed by qPCR quantification using SYBR Green or probe-based chemistries. The U6 snRNA served as the endogenous internal control, and relative expression levels were quantified via the $2^{(-\Delta\Delta Ct)}$ method. For prioritized candidates, RT-qPCR was additionally employed to assess expression dynamics between the 0 h and 24 h conditions.

Differential expression and intersection analysis in GSE132222

Normalized miRNA expression profiles for the GSE132222 cohort were retrieved from the GEO database to facilitate paired comparisons between LSCC tumors and adjacent normal tissues. For each miRNA, the log2 fold change (tumor versus adjacent) and adjusted P values (utilizing the Benjamini–Hochberg procedure) were computed. Directionally concordant miRNAs consistently

dysregulated across both the discovery GlycoRNA microarray and the external GSE132222 cohort were prioritized for downstream functional validation.

Cell-surface RNA depletion

To delineate the functional relevance of surface-accessible RNAs, LSCC cells were subjected to RNase A treatment to enzymatically deplete extracellular and membrane-exposed RNA species. For phenotypic rescue conditions, an RNase inhibitor (RNasin) was co-administered with RNase A. Following targeted depletion, cells were washed and immediately subjected to functional assays. Depletion efficacy was rigorously validated via RT-qPCR and/or Northern blot analysis of GlycoRNA-associated signals.

Blot-based validation of GlycoRNA-associated signals

Blot-based confirmation of GlycoRNA signals was executed utilizing standard periodate oxidation and aldehyde-reactive probe labeling, followed by denaturing gel electrophoresis and membrane transfer. Briefly, total RNA was chemically functionalized, purified, resolved via denaturing PAGE or agarose gel electrophoresis, and transferred onto a positively charged nylon membrane. Following UV crosslinking and blocking, the membranes were incubated with streptavidin-HRP and visualized utilizing a chemiluminescent substrate in accordance with standard protocols.

Transwell assays

Cellular invasive capacity was evaluated utilizing Matrigel-precoated Transwell chambers. Treated cells were seeded into the upper chamber suspended in serum-free medium, whereas the lower chamber was supplemented with medium containing 10% FBS to serve as a

chemoattractant. Following a predetermined incubation period, non-invading cells on the apical surface were mechanically removed; cells that had successfully invaded the basal surface were fixed, stained, and quantified via light microscopy.

Immunofluorescence assay

For immunofluorescence evaluation, cultured cells were fixed in 4% paraformaldehyde, permeabilized utilizing Triton X-100, and blocked with bovine serum albumin (BSA). Samples were subsequently incubated with an anti-Ki67 primary antibody and processed for TUNEL staining strictly according to the manufacturer's protocols. Nuclei were counterstained with DAPI. Fluorescent images were acquired under identical exposure parameters and analyzed in parallel across all experimental cohorts.

Nude-mouse subcutaneous xenograft imaging

To extrapolate the depletion-and-rescue framework to an in vivo setting, luciferase-expressing TU177 cells were established, subjected to the indicated in vitro treatments (Control, RNase A, or RNase A + RNasin), and subsequently inoculated subcutaneously into nude mice to establish xenograft models. Upon successful tumor formation, representative whole-body fluorescence images were captured utilizing strictly matched imaging parameters.

Bioinformatic target prediction, CLEAR-CLIP, and enrichment analysis

Putative targets of hsa-miR-1228-3p were retrieved from TargetScan and miRDB; the intersection of these predictive datasets was defined as the consensus target list for downstream functional enrichment and visualization. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were executed on this consensus set utilizing

standard bioinformatic pipelines. To acquire orthogonal validation of direct miRNA-mRNA interactions, CLEAR-CLIP sequencing was performed using an AGO-associated chimeric RNA capture workflow, and the resultant data were processed via standardized pipelines—encompassing quality control, adapter trimming, alignment against the hg38 and small-RNA reference genomes, and the precise annotation of chimeric reads. Validated interactions supported by chimeric reads were collapsed into representative miRNA-target pairs to facilitate downstream mechanistic interpretation.

Dual-luciferase reporter assay

Wild-type (WT) and mutant (MUT) SOX17 3'UTR reporter constructs, harboring the predicted hsa-miR-1228-3p-binding motif, were synthesized for dual-luciferase validation. TU177 cells were co-transfected with either the WT-SOX17 or MUT-SOX17 3'UTR reporter plasmids, alongside an hsa-miR-1228-3p mimic or a scrambled negative control, utilizing a standard transient-transfection protocol. Relative luciferase activity was quantified 24–48 h post-transfection utilizing a dual-luciferase reporter system, with signals normalized as the firefly/Renilla luminescence ratio. The design and analytical interpretation of this 3'UTR mutagenesis strategy adhered to established rigorous principles for validating direct miRNA-target interactions .

Statistical analysis

Quantitative data are presented as the mean $\pm$ standard deviation (SD) unless otherwise specified. Two-group comparisons were evaluated utilizing either Student's t-test or the Wilcoxon rank-sum test, contingent upon data distribution normality. Paired continuous variables were assessed via paired t-tests. Multiple-group comparisons were conducted using one-way analysis of

variance (ANOVA) followed by appropriate post hoc testing. A P value < 0.05 was predefined as the threshold for statistical significance.

Results

Identification and profiling of GlycoRNAs and GlycoRNA-associated miRNAs in LSCC

To validate the detection workflow, Ac4ManNAz metabolic labeling followed by blot-based analysis was conducted in reference cell lines. After 24 hours of labeling and subsequent proteinase K treatment, robust streptavidin-detectable signals were observed in AMC-HN-8 and TU177 cells, whereas the 0-hour controls exhibited minimal signal (Figure 1A). Enzymatic perturbation assays confirmed the RNA-derived nature of the signal: treatment with DNase failed to abolish the signal, whereas RNase treatment markedly reduced it, and co-treatment with the RNasin preserved it to a substantial extent (Figure 1B). Collectively, these data establish a technically robust and RNase-sensitive GlycoRNA-associated signal, enabling subsequent downstream profiling in paired LSCC tumor and adjacent normal tissues. Spectrophotometric analysis confirmed that RNA purity metrics (A260/280 and A260/230 ratios) were within acceptable ranges for both groups (Figure 1C). Furthermore, representative gel electrophoresis demonstrated high RNA integrity prior to downstream processing (Figure 1D). Analysis of the enriched fractions revealed a heterogeneous composition of multiple small-RNA classes, supported by their respective sequence counts and abundance distributions (Figure 1E). To comprehensively profile these modifications, the discovery workflow incorporated wheat germ agglutinin (WGA) capture-based enrichment of GlycoRNAs, followed by RNA extraction, fluorescent labeling, and hybridization onto the Arraystar microarray platform (Figure 1F). Specifically, among all RNA species captured within this system, the diversity of glycosylated

1 miRNAs (n = 24) ranked third, following pre-miRNAs (n = 39) and tsRNAs (n = 28), indicating
2 that mature miRNAs constitute a predominant class of glycosylated non-coding RNAs. Given
3 that pre-miRNAs are precursor molecules requiring Dicer-mediated processing for direct
4 regulatory function, and tsRNAs are tRNA-derived fragments primarily implicated in stress
5 responses and translational regulation—mechanisms fundamentally distinct from the core post-
6 transcriptional regulation targeted in this study—subsequent in-depth analyses were exclusively
7 directed toward mature miRNAs possessing direct regulatory activity. Application of statistical
8 thresholds of $P < 0.05$ and absolute fold change ≥ 1.3 identified a distinct profile of dysregulated
9 glycosylated miRNAs between tumor and adjacent tissues. Unsupervised hierarchical clustering
10 robustly separated the paired sample groups based on their miRNA expression profiles (Figure
11 1G), while volcano plot analysis highlighted a specific subset of differentially modified
12 miRNAs, providing high-confidence candidates for downstream prioritization (Figure 1H).

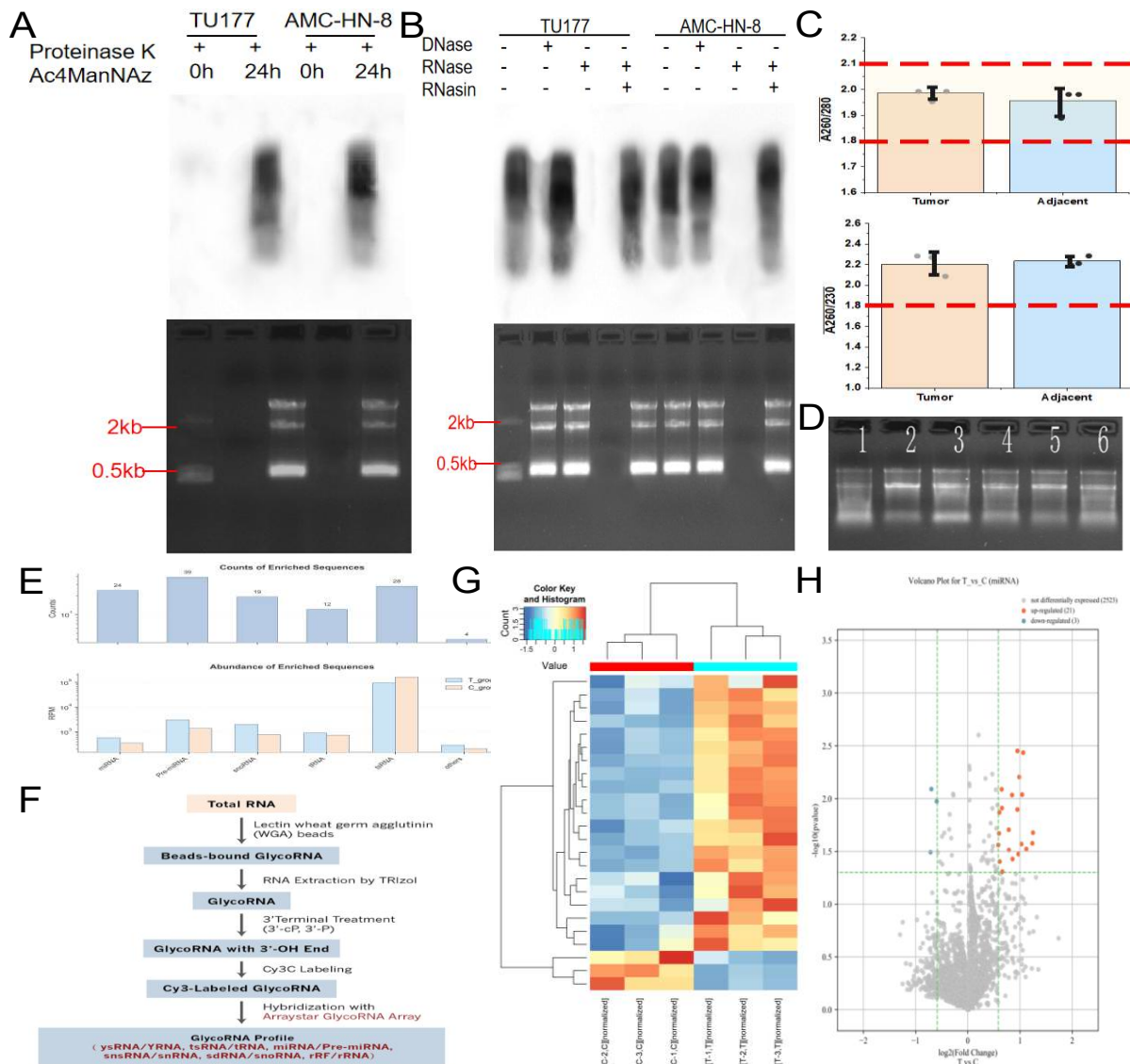


Figure 1. Technical validation and profiling of GlycoRNAs in LSCC.

(A) Streptavidin-based detection of Ac4ManNAz-labeled GlycoRNAs in LSCC cell lines. (B) Sensitivity of GlycoRNA signals to DNase, RNase, and RNasin treatments. (C) RNA purity metrics (A260/280 and A260/230 ratios) for paired tumor and adjacent tissues. (D) Representative agarose gel showing RNA integrity. (E) Distribution of counts and abundance across enriched small-RNA classes. (F) Schematic of the WGA-based GlycoRNA enrichment and microarray discovery workflow. (G) Hierarchical clustering of differentially expressed glycosylated miRNAs in paired samples. (H) Volcano plot identifying significantly dysregulated glycosylated miRNAs.

External validation of dysregulated GlycoRNA-associated miRNAs

To enhance the clinical relevance and robustness of the findings, an independent, publicly available LSCC miRNA dataset (GSE132222) was analyzed. Unsupervised hierarchical clustering and volcano plot analysis revealed a distinct expression profile distinguishing tumor from adjacent normal tissues in this public cohort (Figure 2A-B), serving as an independent benchmark to validate the GlycoRNA-associated miRNA candidates. Subsequent intersection of the differentially expressed miRNAs from GSE132222 with the dysregulated GlycoRNA-associated miRNAs identified in the discovery cohort yielded 14 overlapping candidate miRNAs (Figure 2C; Table 1). To bridge candidate prioritization with downstream mechanistic exploration, a Sankey diagram integrating the candidate miRNAs, their shared predicted targets, and inversely expressed downstream genes was constructed (Figure 2D). This network analysis highlighted SOX17 as a prominent convergence node, providing a strong rationale for its selection in subsequent functional assays. To further assess cross-platform reproducibility, a fold-change concordance analysis of these candidates was performed (Figure 2E). This analysis validated the consistent dysregulation across cohorts and facilitated the specific prioritization of five representative miRNAs (hsa-miR-210-5p, hsa-miR-1228-3p, hsa-miR-6889-3p, hsa-miR-129-2-3p, and hsa-miR-6824-3p) for validation. Validation within the GSE132222 cohort confirmed that all five selected miRNAs were significantly upregulated in LSCC compared to non-tumor tissues, with hsa-miR-1228-3p exhibiting the most robust and consistent separation (Figure 2F). Subsequent RT-qPCR analysis in TU177 and AMC-HN-8 cell lines corroborated these findings, demonstrating significantly elevated expression levels and reinforcing hsa-miR-1228-3p as the optimal priority miRNA for downstream functional studies (Figure 2G).

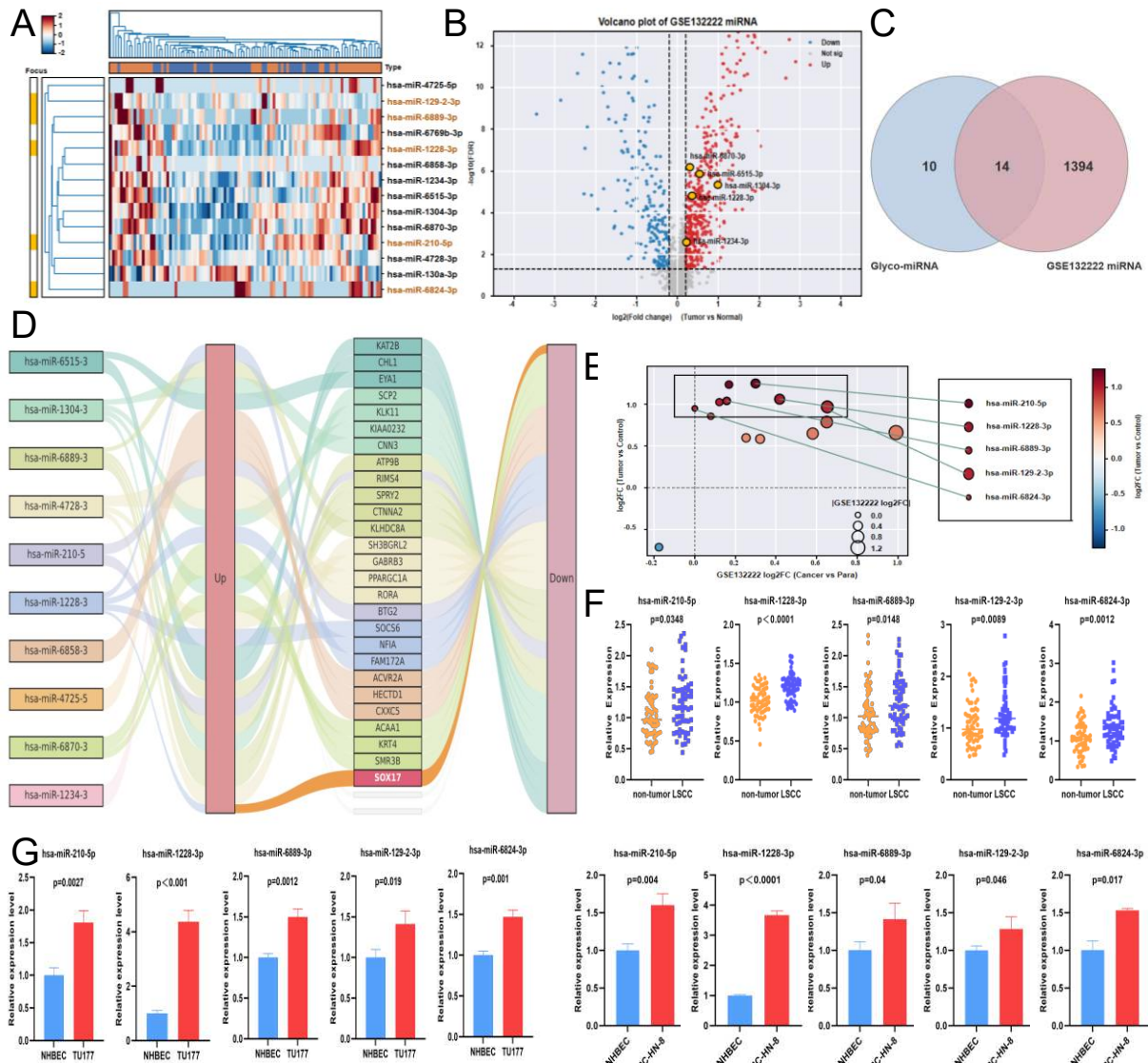


Figure 2. Cross-cohort validation and candidate prioritization.

(A) Hierarchical clustering heatmap of differentially expressed miRNAs in the GSE132222 cohort.(B) Volcano plot identifying dysregulated miRNAs in the GSE132222 cohort.(C) Venn diagram showing the overlap of dysregulated miRNAs between the discovery and validation cohorts.(D) Sankey diagram integrating candidate miRNAs, their shared targets, and downstream genes.(E) Bubble plot illustrating fold-change concordance of prioritized miRNAs across cohorts.(F) Expression levels of five prioritized miRNAs in tumor versus non-tumor tissues (GSE132222).(G) RT-qPCR validation of prioritized candidate miRNAs in LSCC cell lines.

Rank	miRNA	Discovery trend	Discovery FC	GSE132222 log2FC	GSE132222 (BH) q
1	hsa-miR-210-5p	up	2.380	0.348	0.108253
2	hsa-miR-6858-3p	up	2.358	1.011	0.001956
3	hsa-miR-1228-3p	up	2.088	1.139	0.000022
4	hsa-miR-6889-3p	up	2.058	0.851	0.002034
5	hsa-miR-6769b-3p	up	2.039	0.470	0.122866
6	hsa-miR-129-2-3p	up	1.959	1.005	1.000000
7	hsa-miR-6824-3p	up	1.934	0.007	1.000000
8	hsa-miR-4725-5p	up	1.813	0.563	0.026209
9	hsa-miR-4728-3p	up	1.727	0.986	0.787298
10	hsa-miR-1304-3p	up	1.582	1.148	0.000004
11	hsa-miR-6515-3p	up	1.569	1.063	0.000006
12	hsa-miR-1234-3p	up	1.514	0.678	0.008765
13	hsa-miR-6870-3p	up	1.500	1.198	0.000000
14	hsa-miR-130a-3p	down	0.610	-0.177	0.090824

Table 1. Direction-concordant intersecting miRNAs between the discovery cohort and GSE132222.

Abbreviations: FC, fold change; BH, Benjamini-Hochberg adjusted q value. Discovery FC indicates paired tumor-versus-adjacent fold change in the GlycoRNA microarray cohort; positive GSE132222 log2FC values indicate higher expression in tumor.

Depletion of total GlycoRNAs inhibits LSCC cell proliferation and invasion.

To investigate the temporal dynamics of candidate miRNA expression, Ac4ManNAz labeling was applied to laryngeal squamous cell carcinoma (LSCC) cell lines, including AMC-HN-8, TU177, TU212, and TU686. Subsequent RT-qPCR analysis revealed that hsa-miR-1228-3p was significantly enriched across all evaluated cell lines at 24 h compared to the 0-h controls (Figure

3A). Integration of GlycoRNA sequencing data with public RNA-seq datasets further pinpointed hsa-miR-1228-3p as a critical LSCC-associated candidate, providing a strong rationale for its functional validation. Due to the lack of highly specific inhibitors for cell-surface GlycoRNAs, an enzymatic depletion strategy using RNase A was employed to target surface-accessible RNAs. Independent Northern blot analyses in AMC-HN-8 and TU177 cells demonstrated a near-complete ablation of GlycoRNA-associated signals following RNase A treatment relative to the control group (Figure 3B). Conversely, RNasin supplementation in the RNase A + RNasin co-treatment group substantially rescued these signals (Figure 3B), validating the efficacy of the enzymatic depletion-and-rescue framework at the macromolecular level. To extrapolate this depletion-and-rescue framework to an in vivo setting, a nude mouse subcutaneous xenograft model was established and subsequently subjected to Control, RNase A, or RNase A + RNasin treatments. Representative in vivo fluorescence imaging revealed a marked attenuation of tumor-associated signals in the RNase A-treated group compared to the control, whereas the RNase A + RNasin group exhibited partial signal recovery (Figure 3C). These qualitative in vivo observations align seamlessly with the in vitro depletion-and-rescue findings, which were further corroborated by subsequent functional assays. Transwell migration and invasion assays demonstrated a significant impairment in cellular migration and invasion capacities within the RNase A-treated group compared to the control (Figure 3D). Importantly, this inhibitory effect was partially reversed by RNasin co-treatment, exhibiting a statistically significant phenotypic rescue ($P < 0.05$). Furthermore, Ki67/TUNEL dual immunofluorescence staining was utilized to evaluate cellular dynamics. The proportion of Ki67-positive cells was profoundly decreased in RNase A-treated AMC-HN-8 and TU177 cells compared to controls (Figure 3E). Conversely, Ki67 positivity in the RNase A + RNasin co-treatment group was largely restored, showing no

statistically significant difference relative to the control. Concurrently, TUNEL staining revealed no notable differences in the proportion of apoptotic cells among the experimental groups (Figure 3E). Collectively, these data suggest that the depletion of surface-accessible RNAs suppresses LSCC progression primarily by impairing proliferative, migratory, and invasive capacities rather than inducing overt apoptosis.

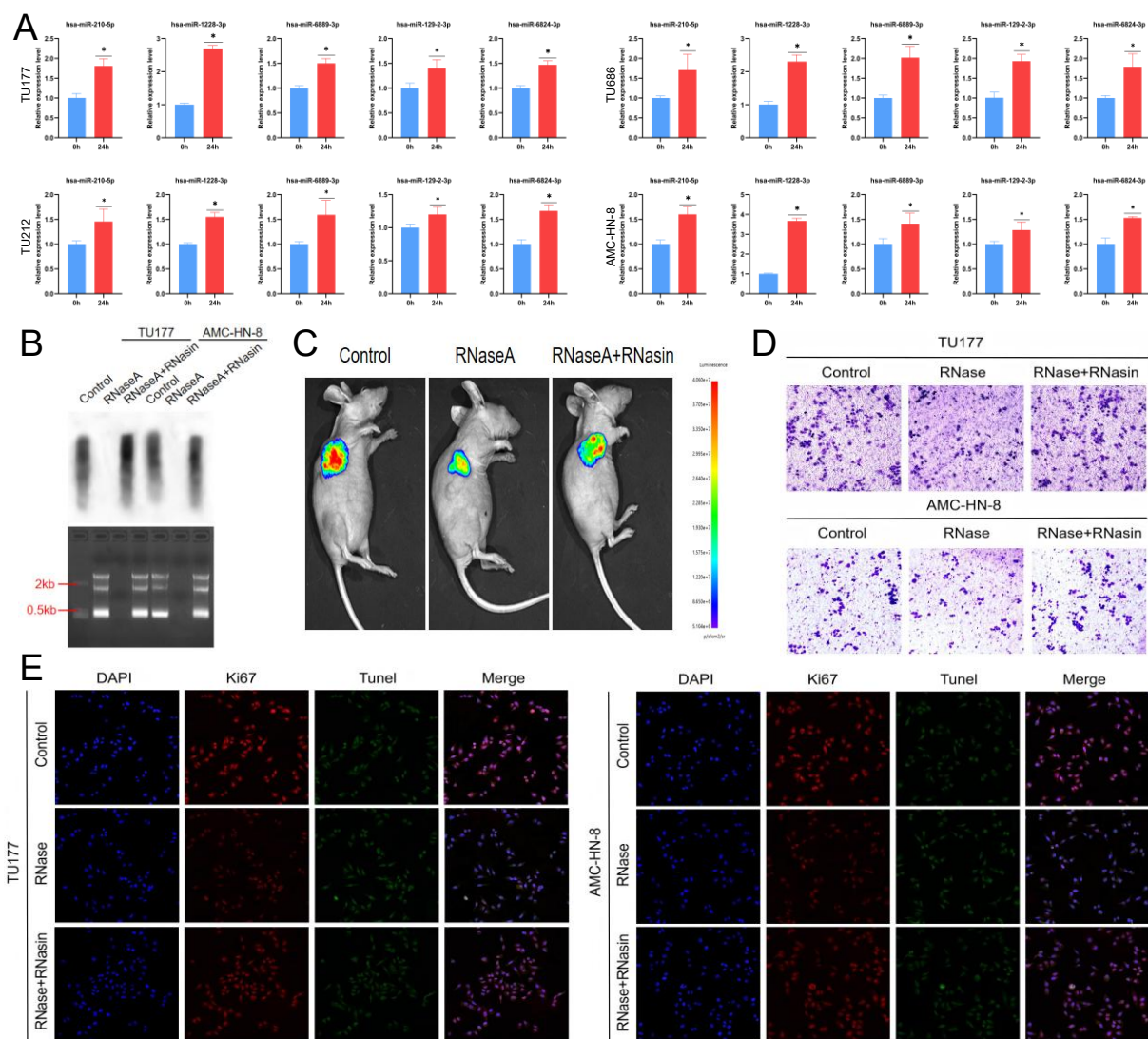


Figure 3. Depletion of total GlycoRNAs inhibits LSCC cell proliferation and invasion.

(A) RT-qPCR analysis of the five prioritized candidate miRNAs at 0 h and 24 h across four LSCC cell lines (TU177, TU212, TU686, and AMC-HN-8). (B) Northern blot analysis demonstrating RNase A sensitivity and subsequent RNasin rescue of GlycoRNA-associated signals in TU177 and AMC-HN-8 cells. (C) Representative in vivo fluorescence images of nude mouse subcutaneous xenografts subjected to Control, RNase A, and RNase A + RNasin treatments. (D) Representative images of Transwell migration and invasion assays for TU177 and AMC-HN-8 cells under the indicated depletion-and-rescue treatment conditions. (E) Double immunofluorescence staining for Ki67 (red, proliferation) and TUNEL (green, apoptosis), demonstrating impaired proliferative capacity following RNase A treatment and partial phenotypic rescue by RNasin. DAPI (blue) was used for nuclear staining. Data in (A) are presented as mean $\pm$ SD. * $p < 0.05$, $p < 0.01$, * $p < 0.001$.

Identification and validation of the hsa-miR-1228-3p/SOX17/Wnt/ β -catenin axis

By intersecting TargetScan and miRDB prediction algorithms, we identified a consensus target set for hsa-miR-1228-3p. Subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of these putative targets revealed significant enrichment in biological processes and signaling pathways pertinent to tumor biology (Figure 4A-B). Target-prioritization visualizations highlighted SOX17 as a core node within the miRNA-target interaction network (Figure 4C-D). Following integration with expression-direction filtering, SOX17 emerged as a biologically plausible downstream effector. Notably, Analysis of the TCGA-HNSC cohort revealed that SOX17 expression was found to be significantly downregulated in tumor tissues compared to normal controls (Figure 4E), exhibiting a tumor-relevant inverse-expression profile. This finding aligns with the recognized tumor-suppressive role of SOX17 and its well-established antagonistic relationship with canonical Wnt/ β -catenin signaling. To obtain orthogonal evidence of direct miRNA-mRNA physical interactions, CLEAR-CLIP was performed on human samples, followed by comprehensive bioinformatic

1 analysis of the resulting sequencing dataset. The sequencing output demonstrated an expected
2 read-length distribution and typical genomic-feature composition (Figure 4F-G). For the primary
3 analysis pipeline, raw reads were trimmed using fastp, aligned to the hg38 and small RNA
4 reference genomes via STAR, and chimeric interactions were subsequently annotated using
5 HybriDetector. Sequence-level inspection of the chimeric interaction output revealed a highly
6 compatible hsa-miR-1228-3p-binding motif within the 3' untranslated region (UTR) of SOX17
7 (Figure 4H). To functionally validate this binding, a dual-luciferase reporter assay was conducted
8 in TU177 cells co-transfected with either wild-type (WT) or binding-site mutant (MUT) SOX17
9 3'UTR reporter constructs. Consistent with the in silico predictions and CLEAR-CLIP data, hsa-
10 miR-1228-3p mimics significantly repressed the luciferase activity of the WT-SOX17 3'UTR
11 reporter. Crucially, mutation of the predicted binding site entirely abrogated this suppressive
12 effect (Figure 4I). These results provide direct, reporter-level evidence for the physical
13 interaction and negative regulation of SOX17 by hsa-miR-1228-3p, a mechanism consistent with
14 established regulatory paradigms in other epithelial malignancies. Finally, pre-ranked Gene Set
15 Enrichment Analysis (GSEA) based on a SOX17-stratified transcriptional profile revealed a
16 significant enrichment of the KEGG_WNT_SIGNALING_PATHWAY, linking the SOX17-
17 associated transcriptional state to broader Wnt-pathway activity (Figure 4J). Although the false
18 discovery rate (FDR) indicates that these correlations provide supportive rather than definitive
19 causal evidence, the integrated findings robustly reinforce the proposed mechanistic framework.

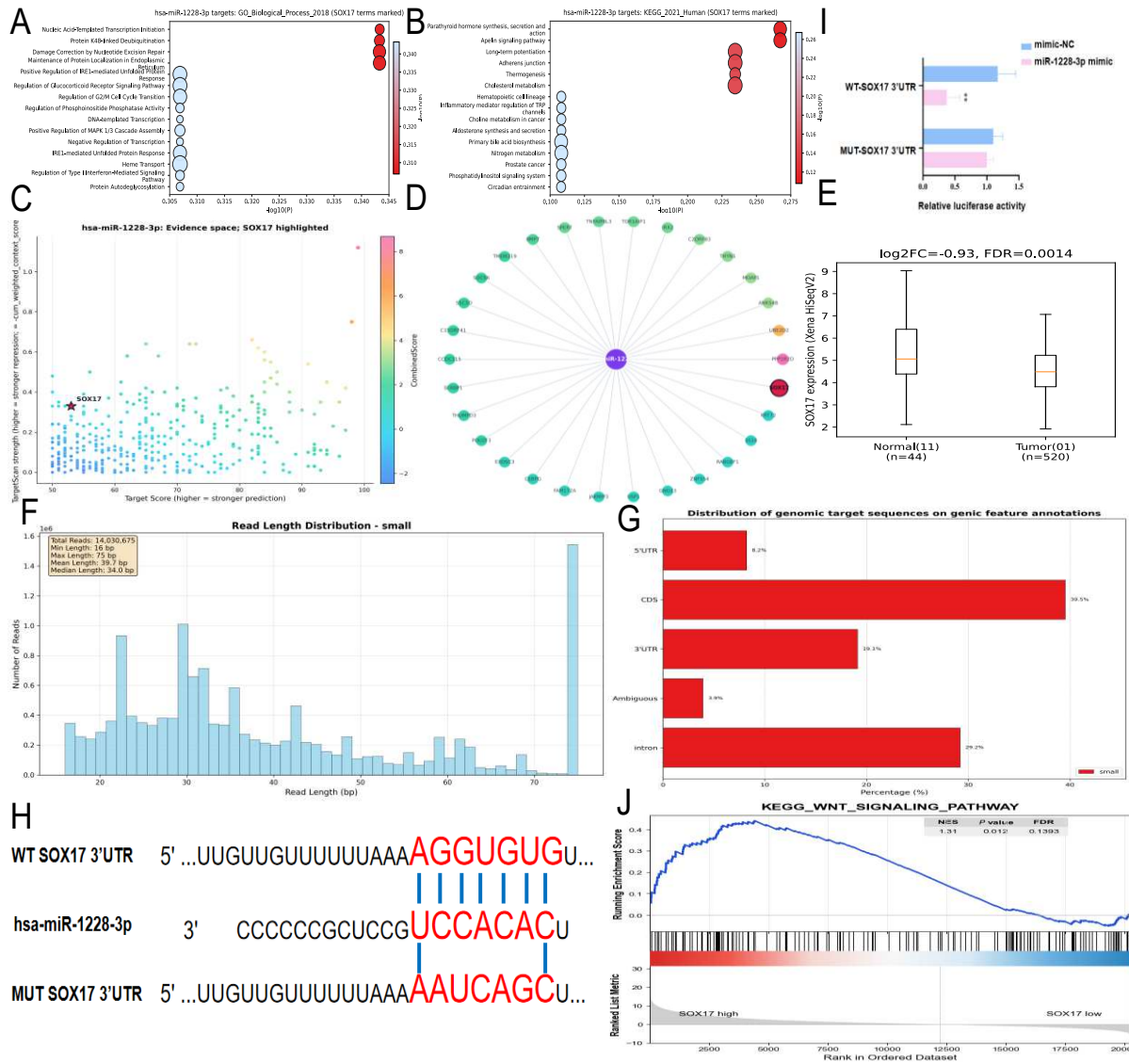


Figure 4. Validation of the hsa-miR-1228-3p/SOX17/Wnt/β-catenin axis.

(A-B) GO and KEGG enrichment analyses identifying biological processes and pathways associated with hsa-miR-1228-3p targets. (C-D) Target-prioritization visualizations, including evidence-space distribution and the miRNA-centered interaction network. (E) Boxplot illustrating the significant downregulation of SOX17 expression in tumor tissues compared to normal tissues ($\log_2FC = -0.93$, $FDR = 0.0014$). (F-G) CLEAR-CLIP sequencing output showing read-length distribution and genomic-feature composition. (H) Chimeric interaction analysis from CLEAR-CLIP data identifying the hsa-miR-1228-3p binding site in the SOX17 3'UTR. (I) Dual-luciferase reporter assay results in TU177 cells confirming direct targeting

1 and suppression of SOX17 by hsa-miR-1228-3p.(J) Pre-ranked GSEA plot for the
2 KEGG_WNT_SIGNALING_PATHWAY demonstrating the association between the SOX17-stratified
3 transcriptional state and Wnt signaling.

4 **Discussion**

5 In the present study, GlycoRNA enrichment, discovery profiling, external validation, in vitro and
6 in vivo RNase-based perturbations, and direct reporter validation were integrated to characterize
7 GlycoRNA-associated miRNAs in LSCC. The revised data presentation supports four central
8 conclusions: first, paired LSCC tissues harbor a distinct GlycoRNA-associated small-RNA
9 pattern; second, cross-cohort integration prioritizes hsa-miR-1228-3p as a highly reproducible
10 candidate; third, both in vitro and in vivo evidence corroborates that Glycosylated miRNA
11 significantly contribute to invasion-associated behaviors and proliferative activity in LSCC cells;
12 and fourth, direct luciferase validation strengthens the candidacy of an hsa-miR-1228-
13 3p/SOX17/Wnt/ β -catenin regulatory axis.

14 A notable methodological strength of the study lies in the separation of technical workflow
15 validation from disease-specific discovery. The metabolic-labeling and nuclease-sensitivity
16 controls successfully established that the detected signal is RNA-derived and RNase sensitive,
17 thereby providing robust support for the utilized GlycoRNA-detection strategy, which is
18 consistent with the current experimental framework used in the GlycoRNA field^[5,6]. Importantly,
19 however, these panels were generated in reference cell systems rather than specifically in
20 primary LSCC cells; accordingly, they should be interpreted as proof-of-principle technical
21 validation.

1 The integration with the external GSE132222 dataset substantially strengthens candidate
2 prioritization. The identified 14-miRNA overlap significantly reduces the likelihood that the
3 discovery signal is driven solely by cohort size limitations or enrichment artifacts. Among the
4 overlapping candidates, fold-change concordance analysis prioritized a subset of five miRNAs.
5 Within this group, hsa-miR-1228-3p emerged most prominently due to its consistent elevation
6 across both the public dataset and RT-qPCR validation, thoroughly justifying its selection for
7 downstream mechanistic analysis.

8 The RNase A/RNasin perturbation experiments demonstrate that GlycoRNA-associated miRNAs
9 harbor functional relevance in LSCC. Based on the present findings, the functional evidence is
10 supported across distinct experimental models: in vitro RNase A treatment diminished Ki67
11 staining and Transwell invasion without altering TUNEL apoptosis profiles, while in vivo
12 xenograft imaging revealed distinct tumor-associated signal attenuation. The introduction of
13 RNasin partially restored these malignant phenotypes across both settings. Because RNase A
14 exerts broad perturbation effects on extracellular and membrane-exposed RNAs, these findings
15 support the broader conceptual framework that GlycoRNA-associated miRNAs can contribute to
16 malignant cellular behavior, primarily through proliferative and invasion-associated phenotypes
17 rather than overt apoptosis, in line with emerging functional observations in cancer systems^[10].

18 The target-nomination analyses, reinforced by the significant downregulation of SOX17
19 identified in the TCGA-HNSC cohort, converge on a biologically plausible, SOX17-centered
20 mechanism. The incorporation of the luciferase assay substantially strengthens the mechanistic
21 chain by advancing the hsa-miR-1228-3p/SOX17 relationship from in silico prediction and
22 orthogonal chimera support to direct, reporter-level validation. Given that the GSEA FDR
23 remained above conventional significance thresholds, the pathway-level results are most

1 accurately interpreted as supportive contextual evidence rather than standalone proof of Wnt-
2 pathway activation. Nevertheless, the overall mechanistic model remains biologically coherent in
3 light of prior evidence linking Wnt/ β -catenin activity to HNSCC progression, identifying SOX17
4 as a negative regulator of this pathway, and describing a miR-1228-3p/SOX17/ β -catenin axis in
5 other epithelial cancers^[11-14].

6 Recent literature indicates that GlycoRNA biology is increasingly connected to immune
7 regulation, cell-surface signaling, and cancer-related translational opportunities^[6]. At the same
8 time, LSCC-oriented non-coding RNA studies continue to highlight the promise of miRNA-
9 centered biomarker development, including minimally invasive strategies, although clinical
10 standardization remains incomplete^[8,9]. Together with emerging functional cancer evidence
11 showing that cell-surface glycoRNA perturbation can alter tumor-cell proliferation^[10], the
12 present work provides a rationale for evaluating GlycoRNA-associated miRNAs as accessible
13 biomarker candidates in larger, independent LSCC cohorts.

14 Several limitations within the current study warrant consideration. The discovery platform
15 utilized a microarray-based approach, which does not fully resolve the complete structural
16 diversity of GlycoRNA species. Additionally, although the current investigation encompasses
17 direct reporter validation of the SOX17 3'UTR, the functional evidence remains predominantly
18 derived from enzymatic depletion models, lacking specific gain- and loss-of-function
19 experiments for hsa-miR-1228-3p itself. The precise modulation of endogenous SOX17 and
20 downstream Wnt/ β -catenin readouts following the direct manipulation of this miRNA also
21 requires definitive establishment. Lastly, although processed through a rigorous bioinformatic
22 pipeline (incorporating fastp, STAR, and HybriDetector), the CLEAR-CLIP evidence was
23 procured from a single human sample and served primarily as orthogonal interaction support.

These constraints justify the interpretation of the proposed relationship as a highly plausible candidate axis rather than a definitively established mechanism.

Future investigations should strategically prioritize executing comprehensive hsa-miR-1228-3p gain- and loss-of-function studies, conducting SOX17 rescue experiments to map epistasis, and performing expanded clinical validation across larger, independent LSCC cohorts.

Conclusions

LSCC exhibits a significantly dysregulated GlycoRNA-associated miRNA signature. Through rigorous integration of discovery profiling, public-dataset validation, and comprehensive cell-based assays, hsa-miR-1228-3p is consistently prioritized as a crucial molecular entity. Supported by combined in vitro and in vivo xenograft evidence, GlycoRNA-associated miRNAs are implicated in sustaining invasion-associated behaviors and driving proliferative activity within LSCC cells. Comprehensive target analyses, clinical cohort integration, orthogonal CLEAR-CLIP evidence, and direct dual-luciferase assays collectively substantiate a candidate hsa-miR-1228-3p/SOX17/Wnt/ β -catenin axis, which now definitively warrants subsequent direct rescue validation to establish functional causality.

List of abbreviations

CLEAR-CLIP: covalent ligation of endogenous Argonaute-bound RNAs-crosslinking immunoprecipitation

FC: fold change

GO: Gene Ontology

GSE: Gene Expression Omnibus Series

- 1 **HNSCC:** head and neck squamous cell carcinoma
- 2 **KEGG:** Kyoto Encyclopedia of Genes and Genomes
- 3 **LSCC:** laryngeal squamous cell carcinoma
- 4 **miRNA:** microRNA
- 5 **RNase:** ribonuclease
- 6 **RT-qPCR:** reverse transcription quantitative polymerase chain reaction
- 7 **WGA:** wheat germ agglutinin

8 **Declarations**

9 **Ethics approval and consent to participate**

10 This study was reviewed and approved by the Ethics Committee of the Second Affiliated
11 Hospital of Harbin Medical University . All the participants were aware of and provided written
12 informed consent to participate in this study. All animal experimental protocols were reviewed
13 and approved by the Animal Ethics Committee of the Second Affiliated Hospital of Harbin
14 Medical University and complied with government and international guidelines for animal
15 experiments.

16 **Consent for publication**

17 Not applicable.

1 **Availability of data and materials**

2 The public dataset analyzed in this study (GSE132222) is available in the Gene Expression
3 Omnibus. Other data supporting the findings of this study are available from the corresponding
4 author on reasonable request.

5 **Competing interests**

6 The authors declare that they have no competing interests.

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11 **Authors' contributions**

12 Peng Wang: Conceived and designed the study, analyzed data, drafted the manuscript,
13 Supervised the entire study, obtained funding, critically revised the manuscript, approved the
14 final version for publication.

15 Zuer Liu: Conceived and designed the study, performed animal experiments and molecular tests,
16 analyzed data, drafted the manuscript.

17 Shuo Liu: RT-qPCR and Immunofluorescence, assisted with animal modeling, analyzed data.

18 Baiying Liu: Assisted with animal modeling, analyzed data.

19 Xiang Li: Analyzed data.

Yanan Sun: Supervised the entire study, obtained funding, critically revised the manuscript, approved the final version for publication.

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