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Original Article

Spatially resolved transcriptomics identifies epithelial-specific oncogenic signaling networks in ameloblastoma

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Abstract *Background/purpose:* Ameloblastoma is a benign odontogenic tumor characterized by locally aggressive growth and a high recurrence rate. Although recurrent mutations such as *BRAF V600E* have been identified, the epithelial-specific molecular mechanisms underlying tumor initiation and invasion remain incompletely defined. Previous transcriptomic studies have been limited by stromal contamination and lack of spatial resolution.

Materials and methods: An integrative transcriptomic strategy was applied, combining bulk RNA sequencing of fresh ameloblastoma and dental follicular tissues, RNA sequencing of primary cultured ameloblastoma epithelial cells and dental follicular epithelial cells, and spatial transcriptomic profiling of formalin-fixed, paraffin-embedded ameloblastoma specimens.

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Results: Differential expression and functional enrichment analyses identified 871 upregulated and 1464 downregulated genes in tumor tissues, and 360 upregulated and 306 downregulated genes in primary epithelial cells. Integration of these datasets defined a high-confidence set of 75 consistently dysregulated epithelial genes, including *BEX1*, *NOS1*, and *IRS2*, which were validated by quantitative RT-PCR. Pathway enrichment analyses revealed coordinated activation of Wnt/ β -catenin, PI3K/AKT, MAPK, and Hedgehog signaling pathways. Spatial transcriptomic analysis further demonstrated that *BEX1*, *NOS1*, *IRS2*, Wnt ligands (*WNT3A*, *WNT6*, *WNT7A*), and Hedgehog effectors (*GLI1*, *GLI2*) were predominantly upregulated within ameloblastoma tumor epithelial compartments, confirming pathway activation in situ and minimizing stromal confounding.

Conclusion: Together, these findings provide a refined molecular characterization of ameloblastoma and demonstrate spatially localized activation of multiple oncogenic signaling pathways within tumor epithelium, extending current single-pathway models of ameloblastoma pathogenesis and supporting the potential development of pathway-based or combinatorial therapeutic strategies.

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Introduction

Ameloblastoma is a histologically benign yet locally aggressive odontogenic neoplasm arising in the jaws. According to the World Health Organization (WHO) classification of tumors, ameloblastomas are categorized into three clinicopathological subtypes: solid/multicystic, extraosseous (peripheral), and unicystic forms.¹ Despite their benign histological appearance, ameloblastomas are characterized by a high propensity for local recurrence, particularly following conservative surgical management. Consequently, wide surgical excision with adequate margins remains the current standard of care.¹

Ameloblastoma is widely regarded as originating from odontogenic epithelium, including the epithelial rests of the dental lamina, the developing enamel organ, epithelial rests of Malassez, the lining epithelium of intrabony odontogenic cysts, and, in peripheral cases, the basal cells of the oral mucosa. Among these proposed progenitor cell populations, the epithelial rests of the dental lamina are considered the most likely cell of origin for ameloblastoma.¹ Tooth development, termed odontogenesis, proceeds through a series of well-defined stages, including the bud, cap, and bell stages. During the cap stage, the apical portion of the proliferating dental lamina expands and invaginates to form the enamel organ, which subsequently interacts with the underlying dental papilla to produce enamel during the bell stage. Following tooth bud formation, the residual dental lamina progressively degenerates into epithelial rests dispersed within the fibrous connective tissue of the dental follicle and the marrow spaces of the jaws. Based on this developmental context, it is widely accepted that genetic aberrations arising within the epithelial rests of the dental lamina may represent an early event contributing to the initiation and subsequent development of ameloblastoma.

Currently, the oncogenic *BRAF V600E* mutation has been identified as the most prevalent genetic alteration in

ameloblastoma, with a reported prevalence of up to 87.2% in study cohorts,^{2–6} representing the most common genetic alteration and underpin constitutive ERK signaling that promotes epithelial proliferation and survival. Additional evidence implicates Wnt/ β -catenin,^{6–9} PI3K/AKT,^{10,11} and FGFR^{12–15} signaling in tumor growth, invasion, and resistance to apoptosis, collectively supporting the concept that ameloblastoma arises from aberrant reactivation of odontogenic epithelial programs. However, despite these insights, the molecular pathogenesis of ameloblastoma remains incompletely defined. Most existing transcriptomic analyses rely on bulk tumor tissues, which are intrinsically confounded by stromal, inflammatory, and vascular components, limiting the ability to resolve tumor epithelial-specific molecular events. Moreover, few studies integrate in vivo tumor profiles with purified odontogenic epithelial cell models or validate gene expression within spatial tissue context, resulting in uncertainty regarding the biological relevance and cellular localization of candidate pathways and genes. Consequently, the precise driver genes that link canonical pathway activation to the locally invasive behavior of ameloblastoma and the molecular heterogeneity beyond well-characterized driver mutations remain insufficiently characterized.

In this study, we sought to elucidate the key epithelial drivers and signaling pathways underlying the molecular pathogenesis of ameloblastoma by integrating complementary transcriptomic approaches. We first performed comparative transcriptome analysis between fresh ameloblastoma tissues and dental follicle tissues, representing the presumed odontogenic origin. To further minimize stromal confounding and enrich for tumor epithelial-specific molecular signatures, we next compared gene expression profiles between primary cultured ameloblastoma epithelial cells and cultured dental epithelial cells derived from dental follicles. Differentially expressed genes (DEGs) identified from these in vivo and in vitro comparisons were subsequently overlapped to define a high-confidence gene

set consistently dysregulated in ameloblastoma epithelium. Collectively, this integrative strategy aimed to provide a more refined and biologically relevant understanding of the molecular mechanisms driving ameloblastoma development and local invasiveness.

Materials and methods

Clinical samples

Four multicystic ameloblastoma specimens and three dental follicular tissue samples containing abundant odontogenic epithelial rests were obtained from patients undergoing surgical treatment at the Department of Oral and Maxillofacial Surgery, National Taiwan University Hospital, Taiwan. All specimens were collected immediately after surgical excision and processed for downstream analyses. Demographic characteristics, anatomical sites, histopathological subtypes, and *BRAF* mutation status of all collected samples are summarized in Table 1. This study was approved by the Research Ethics Committee of NTUH (No. 201412058RINA, 201608088RINA, and 201901034RIND).

Cell culture

Primary odontogenic epithelial cells derived from dental follicular tissues and primary ameloblastoma epithelial cells were established from freshly resected ameloblastoma specimens through the explant culture method. Briefly, tissues were processed immediately after surgical excision, and the minced tissue were cultured under defined conditions. The cells were maintained in medium composed of CnT-Prime (CnT-PR, Cellntec Advanced cell systems, Bern, Switzerland) and DMEM+10% FBS in 1:1 ratio with adding 1% pen/strep, 5 μ M Rock inhibitor (Y-27632) (Cat. No. 1254; Tocris Bioscience, Bristol, UK), 1 μ M BMP inhibitor (DMH-1) (Cat. 4126), and 1 μ M TGF- β inhibitor (Cat. 2939) in a humidified incubator at 37 °C with 5% CO₂. All experiments were performed using early-passage cells to minimize

culture-induced phenotypic drift. The demographic and clinicopathological characteristics of samples used for primary cell culture are summarized in Table 1.

Immunohistochemical analysis (IHC)

Cultured epithelial cells derived from dental follicular tissues and ameloblastomas were cultured in chamber slides. After removal of the supernatant, the cells were fixed in 10% neutral-buffered formalin. Immunohistochemical analysis was performed. Briefly, cells were incubated with primary antibodies, Cytokeratin 19 (Clone RCK108; Agilent/Dako, Santa Clara, CA, USA) and PITX2 (Clone AF2330; R&D Systems, Minneapolis, MN, USA), followed by detection using an appropriate secondary antibody system and chromogenic visualization. Cells were counterstained with hematoxylin. Details of the primary antibodies, antigen retrieval conditions, and detection systems are provided by the respective manufacturers.

RNA isolation

Total RNA was extracted from ameloblastoma and dental follicle fresh tissues and primary cultured epithelial cells from ameloblastomas and dental follicles using TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. RNA concentration and quality was quantified by nucleic acid concentrations using a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific). Only RNA samples meeting quality requirements were used for subsequent analyses.

RNA library preparation and next generation sequencing

RNA sequencing libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

Table 1 Epidemiologic data of the collected samples in this study.

	Sample	Age (year)	Gender	Site	Histological type	BRAF V600E mutation
Tissues	DF2	18	Female	Impacted tooth 48	N/A	N/A
	DF3	25	Female	Impacted tooth 48	N/A	N/A
	DF5	19	Female	Impacted tooth 38	N/A	N/A
	AM9	15	Male	Mandible	Plexiform	Mutated
	AM10	24	Female	Mandible	Both follicular and plexiform	Wild-type
	AM13	23	Male	Mandible	Follicular with focal plexiform	Mutated
	AM14	28	Male	Mandible	Both follicular and plexiform	Wild-type
Cultured cells	DF23	22	Male	Impacted tooth 48	N/A	N/A
	DF24	24	Female	Impacted tooth 48	N/A	N/A
	DF25	20	Female	Impacted tooth 38	N/A	N/A
	AMT25	35	Male	Mandible	Follicular and acanthomatous	N/A
	AMT26	26	Female	Mandible	Follicular	N/A

DF: Dental follicle, AM: Ameloblastoma, N/A: Not applicable.

Briefly, 1 µg of total RNA was used as input, and polyadenylated mRNA was purified using oligo(dT)-coupled magnetic beads, followed by fragmentation at elevated temperature. First-strand cDNA synthesis was performed using reverse transcriptase and random primers, followed by second-strand cDNA synthesis to generate double-stranded cDNA. After end repair and adenylation of the 3' ends, sequencing adapters were ligated, and the libraries were purified using the AMPure XP system (Beckman Coulter, Beverly, MA, USA). Library quality and fragment size distribution were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and library quantification was performed using a real-time PCR-based method. Qualified libraries were sequenced on an Illumina NextSeq 500 platform (Illumina) to generate 150-bp paired-end reads. Sequencing was performed by Genomics, BioSci & Tech Co. (New Taipei City, Taiwan).

Transcriptome analysis and identification of differentially expressed genes (DEGs)

Raw sequencing reads were processed and pseudoaligned to the human reference transcriptome (hg38) using the Kallisto algorithm.¹⁶ Transcript-level abundance estimates were imported and summarized at the gene level for downstream analysis. Differential gene expression analysis was performed using the DESeq2 package in R.¹⁷ Genes were considered differentially expressed if they met the following criteria: an absolute $\log_2$ fold change ($|\log_2FC|$) greater than 1 and an adjusted *P*-value < 0.05. More stringent thresholds were applied as indicated in specific analyses and figure legends.

Function annotation of DEGs

Functional enrichment analysis of differentially expressed genes (DEGs) was performed using the Metascape platform (<https://metascape.org>).^{18,19} Gene ontology (GO) enrichment analysis was conducted to evaluate the biological functions of DEGs across three ontology categories: biological processes, cellular components, and molecular functions. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was carried out to identify significantly enriched signaling pathways associated with the DEGs.²⁰ KEGG analyses were performed using both Metascape and KEGG Mapper (<https://www.genome.jp/kegg/>). For enrichment analysis, a minimum gene overlap of three was required, and terms with an adjusted *P*-value < 0.05 were considered statistically significant. The enrichment results were visualized using SRplot.²¹

Reverse transcription (RT) and real-time quantitative PCR (qPCR)

Reverse transcription was performed using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT-PCR was performed using TaqMan Real-Time PCR Assays (Thermo Fisher Scientific) in the CFX Connect Real-Time PCR machine (Bio-Rad Laboratories, Hercules, CA, USA). The primer/probe sets used for this study listed in Additional file 1 (<https://jds.ads.org.tw/>

[cgi/editor.cgi?article=3487&window=additional_files&context=journal](https://jds.ads.org.tw/cgi/editor.cgi?article=3487&window=additional_files&context=journal)). β-actin was used as the internal reference gene for normalization. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method using dental follicle as control. All experiments were performed in duplicate.

Spatial gene expression analysis

Formalin-fixed paraffin-embedded (FFPE) tissue sections were cut at a thickness of 5 µm and mounted onto Visium CytAssist-compatible slides (10x Genomics, Pleasanton, CA, USA). H&E staining was performed according to the manufacturer's protocol, and stained sections were imaged at 20 × magnification using a VS200 slide scanner (Evident Corp., Tokyo, Japan). Spatial transcriptomic analysis was performed using the Visium CytAssist Spatial Gene Expression assay for FFPE tissue (10x Genomics) with the Human Whole Transcriptome Probe Set. Briefly, probes were hybridized to RNA molecules in the tissue sections, followed by ligation and transfer of the probe-derived products onto the Visium slide containing spatially barcoded capture oligonucleotides using the CytAssist instrument (10x Genomics). The resulting ligation products were enzymatically amplified and converted into sequencing-ready libraries according to the manufacturer's instructions. Libraries were sequenced on an Illumina NovaSeq X Plus platform (Illumina, San Diego, CA, USA). Sequencing reads were aligned to the human reference genome (GRCh38) using Space Ranger v3.0.1 (10x Genomics). Spatial gene expression data were visualized and analyzed using Loupe Browser version 8.0.0.

Statistical analysis

Quantitative real-time PCR data were analyzed and visualized using GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). Statistical comparisons between groups were performed using either Student's *t*-test or Welch's *t*-test, as appropriate. A *P*-value < 0.05 was considered statistically significant.

Results

Identification of DEGs and functional analysis between fresh ameloblastoma tumor tissue and dental follicular tissue

To define the molecular landscape of ameloblastoma, tumor tissues from four patients with multicystic ameloblastoma and dental follicular tissues from three patients were subjected to RNA sequencing. As shown in Fig. 1A, principal component analysis (PCA) showed that ameloblastoma and dental follicular tissues segregated in the PC1–PC2 space, with PC1 explaining 39% of the variance and PC2 accounting for an additional 30%. Differential expression analysis identified 871 upregulated and 1464 downregulated genes in ameloblastoma compared with dental follicular tissues ($|\log_2FC| > 1$, adjusted *P* < 0.01; Fig. 1B) (detailed information is provided in Additional file 2 (https://jds.ads.org.tw/cgi/editor.cgi?article=3487&window=additional_files&context=journal)). GO

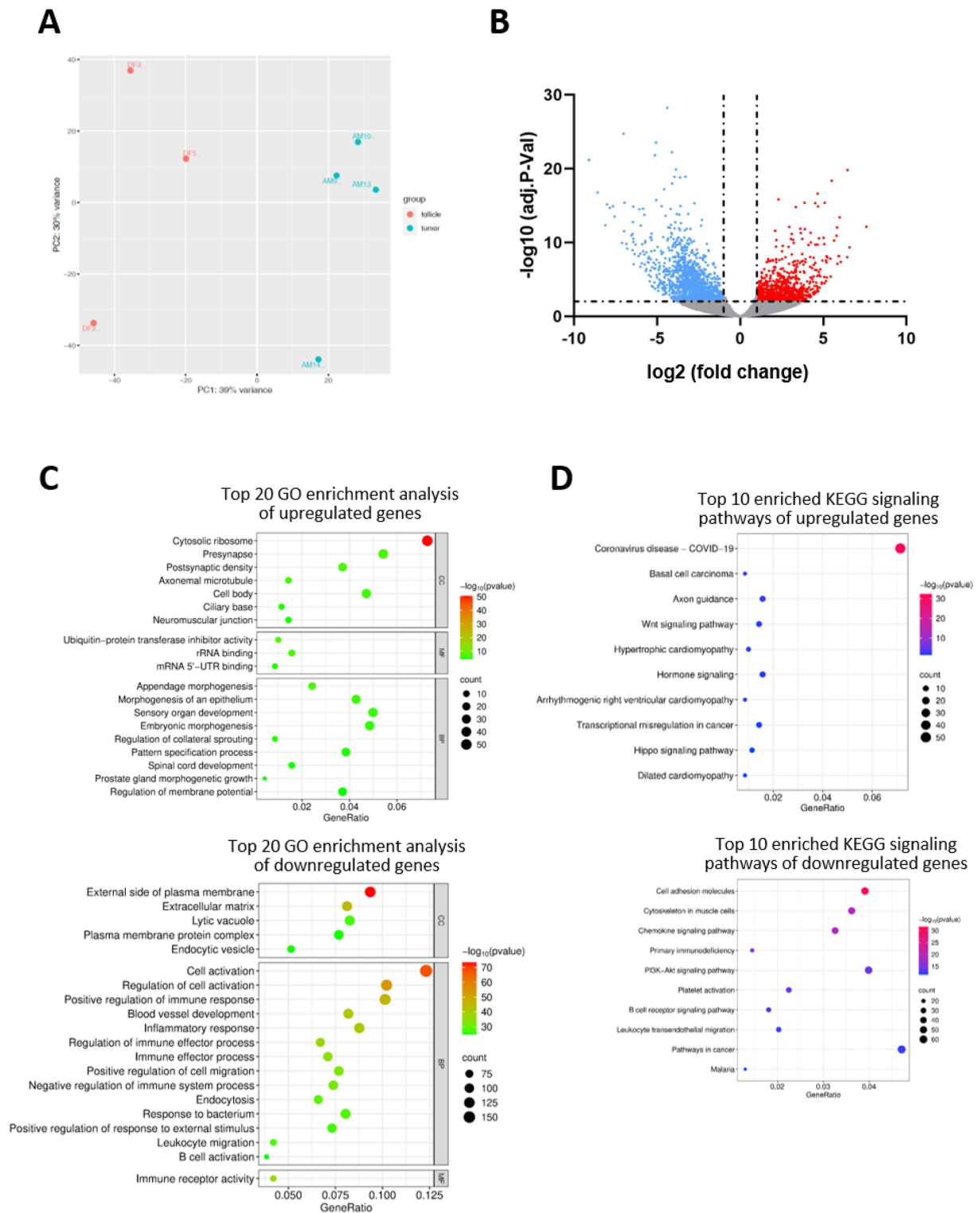


Figure 1 Transcriptomic profiling and functional enrichment analysis of ameloblastoma and dental follicular tissues. (A) Principal component analysis (PCA) showing clear separation between ameloblastoma ($n = 4$) and dental follicular tissues ($n = 3$). (B) Volcano plot of differentially expressed genes (DEGs) between the two groups ($|\log_2\text{FC}| > 1$, adjusted $P < 0.01$). (C) Gene ontology (GO) enrichment analysis of upregulated and downregulated DEGs. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of upregulated and downregulated DEGs. Dot size indicates gene count, and color represents statistical significance ($-\log_{10}$ adjusted P -value).

enrichment analysis revealed that upregulated genes were predominantly associated with ribosome- and translation-related cellular components (e.g., cytosolic ribosome), whereas downregulated genes were enriched in immune- and inflammation-related biological processes (Fig. 1C). KEGG pathway analysis revealed that the significantly upregulated genes in ameloblastoma were enriched in pathways related to Wnt and Hippo signaling, hormone signaling, and transcriptional misregulation, whereas downregulated genes were associated with cell adhesion molecules, chemokine signaling, PI3K–AKT signaling, and other immune- and cytoskeleton-related pathways (Fig. 1D), the majority of which have been implicated in dysregulation of cell proliferation, cell cycle, differentiation, and migration.

Identification of DEGs and functional analysis between ameloblastoma epithelial cells and dental follicular epithelial cells

To verify the DEGs identified from tissue samples and minimize potential stromal and inflammatory influences, primary epithelial cells were cultured separately from two ameloblastoma and three dental follicular specimens, and their

odontogenic epithelial nature was validated by positive immunostaining for odontogenic epithelial markers CK19 and PITX2 (Fig. 2).^{22,23} PCA revealed that dental follicular cells clustered closely and were clearly separated from ameloblastoma cells, indicating preservation of their original phenotypic characteristics during in vitro culture (Fig. 3A). Differential expression analysis identified 360 upregulated and 306 downregulated genes in ameloblastoma cells compared with dental follicular cells ($|\log_2FC| > 1$, adjusted $P < 0.01$; Fig. 3B; Additional file 3(https://jds.ads.org.tw/cgi/editor.cgi?article=3487&window=additional_files&context=journal)). GO analysis revealed that upregulated genes were predominantly associated with extracellular matrix components and organization (including extracellular matrix and basement membrane), tissue and skin development, connective tissue morphogenesis, and epithelial–mesenchymal transition, whereas downregulated genes were mainly enriched in immune- and defense-related biological processes, including inflammatory response, and the regulation of immune and cytokine-mediated signaling pathways (Fig. 3C). KEGG analysis showed enrichment of the Wnt signaling pathway among upregulated genes—consistent with findings from tissue samples—along with immune and metabolic pathways among downregulated genes (Fig. 3D).

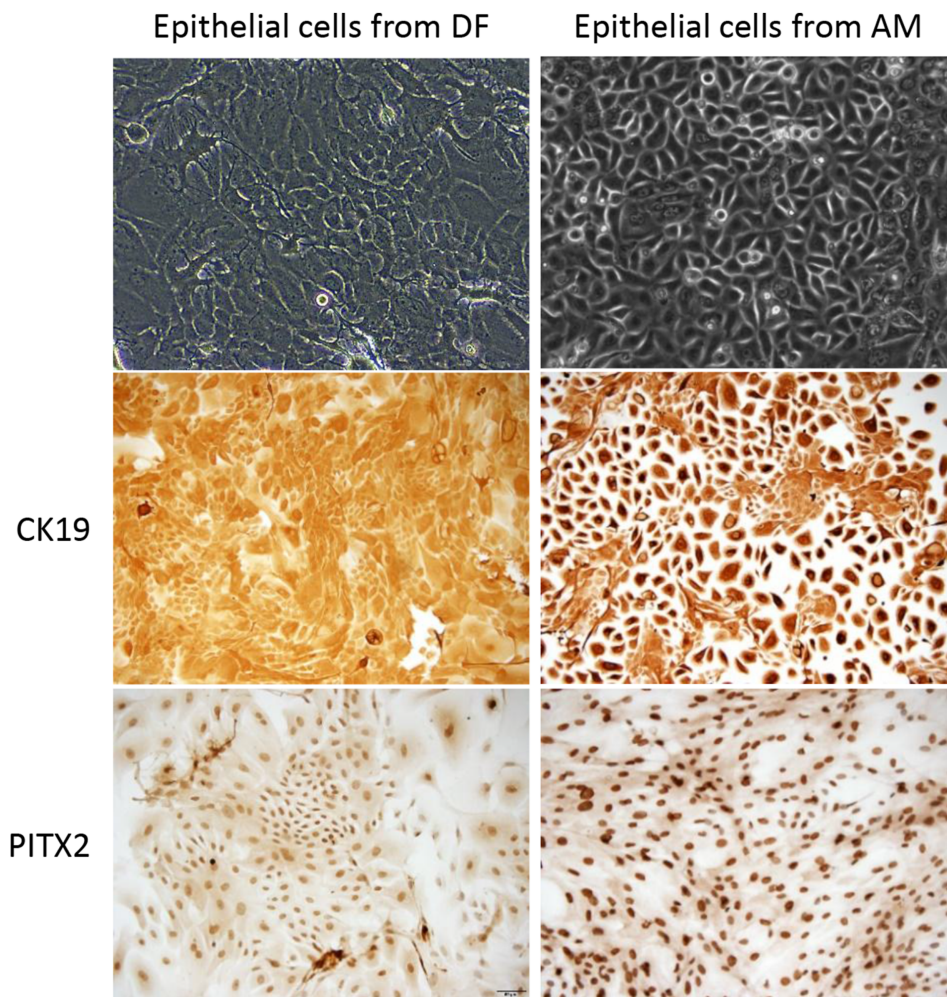


Figure 2 Epithelial cells from dental follicle (DF) and ameloblastoma (AM) demonstrate odontogenic epithelial markers: CK19 and PITX2. (Original magnification; 400 ×).

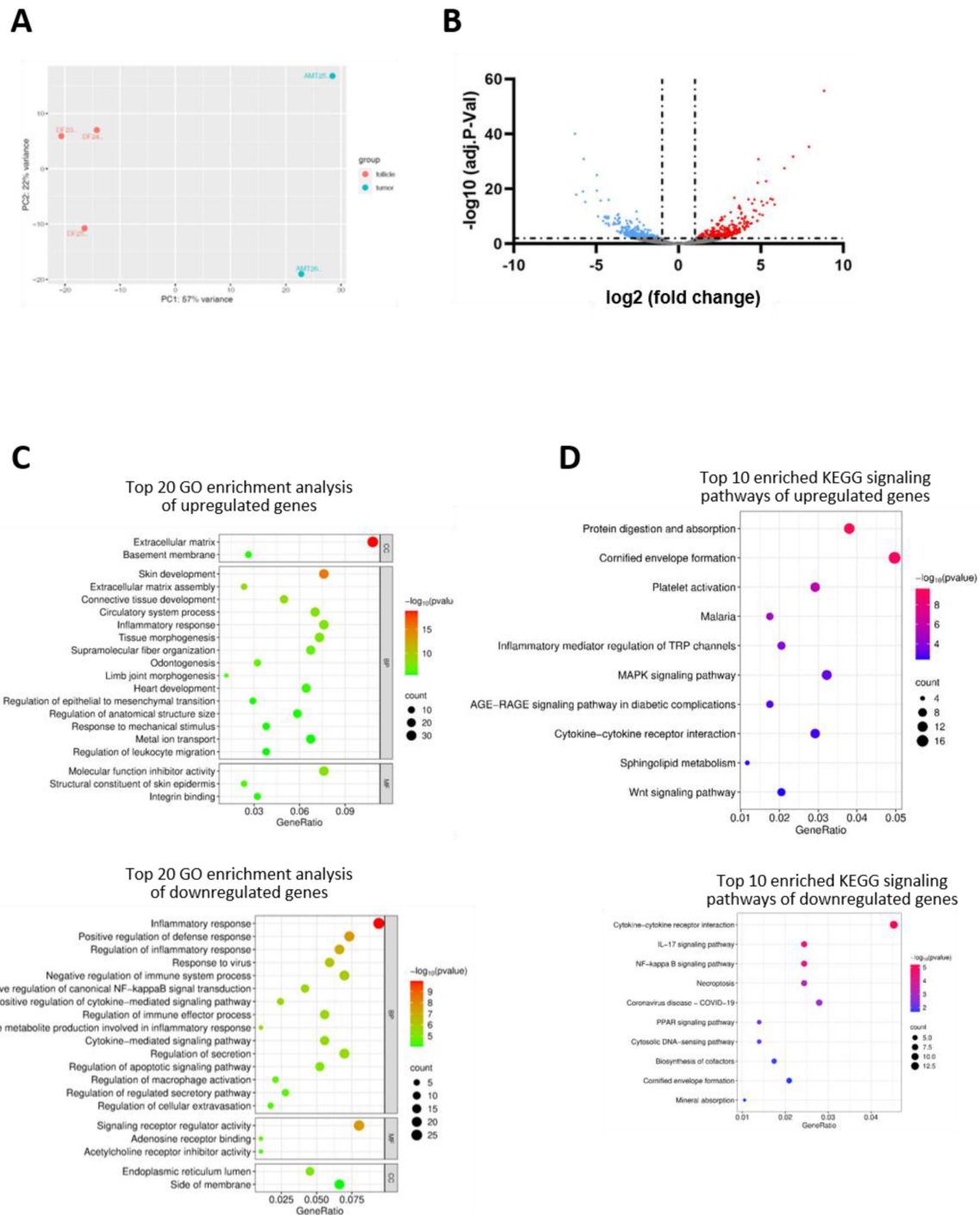


Figure 3 Transcriptomic analysis and functional enrichment of cultured ameloblastoma and dental follicular epithelial cells. (A) Principal component analysis (PCA) showing separation between ameloblastoma cells and dental follicular cells after in vitro culture. (B) Volcano plot of differentially expressed genes (DEGs) between the two cell types ($|\log_2\text{FC}| > 1$, adjusted $P < 0.01$). (C) Gene ontology (GO) enrichment analysis of upregulated and downregulated DEGs. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of upregulated and downregulated DEGs. Dot size represents gene count, and color indicates statistical significance ($-\log_{10}$ adjusted P -value).

Overlap analysis revealed shared DEGs in ameloblastoma tissues and primary cultured ameloblastoma cells

Since our goal was to identify the key pathways involved the genesis of ameloblastoma, overlap analysis was performed

between the 871 and 360 upregulated genes, and between the 1464 and 306 downregulated genes, identified in tissue samples and primary cultured epithelial cells, respectively. We found 26 and 49 genes that were commonly upregulated and downregulated, respectively, in both tissue samples and primary cultured epithelial cells (Fig. 4; Table 2). Of these

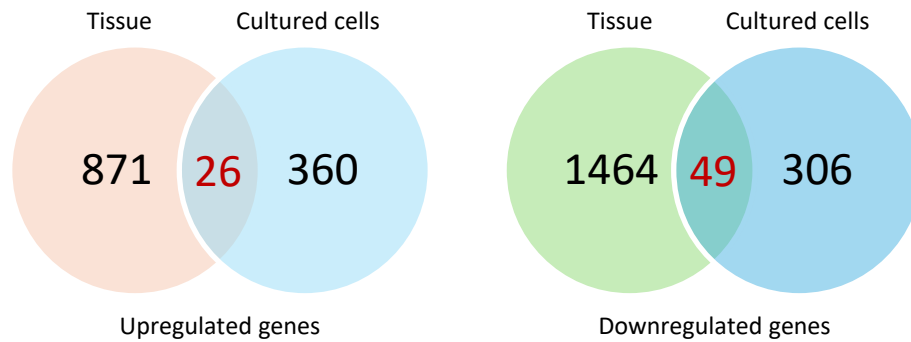


Figure 4 Overlapping differentially expressed genes (DEGs) were identified from two comparisons: ameloblastoma versus dental follicle tissues, and cultured ameloblastoma cells versus dental follicular epithelial cells.

75 DEGs, genes associated with signaling pathways implicated in the pathogenesis of odontogenic lesions, including the PI3K/AKT, MAPK, Hedgehog, and Wnt pathways,^{24,25} were selected for validation by RT-qPCR. Accordingly, the expression levels of brain-expressed X-linked protein 1 (BEX1), nitric oxide synthase 1 (NOS1), and insulin receptor substrate 2 (IRS2) were examined and found to be upregulated in ameloblastoma tissues compared with dental follicle tissues, with *IRS2* showing a statistically significant increase (Fig. 5).

Whole transcriptome spatial in situ data validate selected DEGs, activators of Wnt/ β -catenin and hedgehog signaling pathways in ameloblastoma tissue

To further characterize the spatial organization of tumor and stromal components and to localize pathway-related gene expression within ameloblastoma tissues, spatial transcriptomic analysis was performed. Histopathological annotation, gene expression-based clustering, and

dimensionality reduction were integrated to delineate distinct cellular compartments and signaling activities within the tumor microenvironment. As shown in Fig. 6A and B, gene expression-based clustering showed high concordance with the oral pathologist's histopathological annotations. Dimensionality reduction using uniform manifold approximation and projection (UMAP) further resolved distinct cellular compartments, including peripheral and central tumor epithelial cells, tumor-associated stroma, subepithelial fibrous tissue, and squamous epithelium (Fig. 6C). Spatial expression mapping demonstrated that the selected DEGs, including *IRS2*, *BEX1*, and *NOS1*, were upregulated in ameloblastoma tumor regions (Fig. 6D). Consistent with pathway enrichment analyses, activators of the Wnt signaling pathway, including T cell factor 7 (*TCF7*), *WNT3A*, *WNT6*, and *WNT7A*, were also predominantly upregulated in ameloblastoma tumor cells (Fig. 6E). Additionally, the Hedgehog pathway transcriptional effectors GLI family zinc finger 1 (*GLI1*) and *GLI2* were preferentially upregulated in ameloblastoma tumor epithelial compartments (Fig. 6F). Taken together, these spatial transcriptomic findings corroborate

Table 2 List of overlapping differentially expressed genes (DEGs) from two comparisons: ameloblastoma versus dental follicle tissues, and cultured ameloblastoma cells versus dental follicular epithelial cells.

Upregulated genes (n = 26)				
<i>PNCK</i>	<i>MIRLET7A1HG</i>	<i>HPN</i>	<i>UCN</i>	<i>FXVD6</i>
<i>NOS1</i>	<i>RPL12</i>	<i>EIF4EBP3</i>	<i>TRABD2B</i>	<i>CRLF1</i>
<i>IRS2</i>	<i>ADAMTS17</i>	<i>VIPR2</i>	<i>LOC101928156</i>	<i>LOC101927789</i>
<i>CNNM1</i>	<i>SLC2A12</i>	<i>NFIX</i>	<i>CCNB1IP1</i>	<i>POLR1D</i>
<i>SYT8</i>	<i>BEX1</i>	<i>DHDH</i>	<i>ATP12A</i>	<i>LINC02617</i>
<i>LINC02617</i>				
Downregulated genes (n = 49)				
<i>NFE2</i>	<i>ERG</i>	<i>SP140L</i>	<i>LRMDA</i>	<i>ALOX5</i>
<i>TRIM22</i>	<i>CGAS</i>	<i>CARD11</i>	<i>APOL6</i>	<i>SLFN12</i>
<i>SLC40A1</i>	<i>CIITA</i>	<i>SELENOP</i>	<i>IFI27</i>	<i>TAGAP</i>
<i>PDGFD</i>	<i>SAMD9L</i>	<i>KYNU</i>	<i>ANXA2R</i>	<i>IRAK3</i>
<i>TRANK1</i>	<i>GOS2</i>	<i>QPCT</i>	<i>FMO1</i>	<i>NUP210</i>
<i>CD14</i>	<i>PDZK1IP1</i>	<i>ADA2</i>	<i>ELK2AP</i>	<i>GIPC2</i>
<i>LY75</i>	<i>CD74</i>	<i>ALDH1A1</i>	<i>SLC16A4</i>	<i>SLFN13</i>
<i>MECOM</i>	<i>CNKSR1</i>	<i>TNFAIP2</i>	<i>FLJ32255</i>	<i>ME3</i>
<i>MGAT3</i>	<i>RPP25</i>	<i>CFB</i>	<i>DOC2B</i>	<i>SAMD9</i>
<i>ACKR3</i>	<i>ST6GALNAC1</i>	<i>IFIT3</i>	<i>BIRC3</i>	

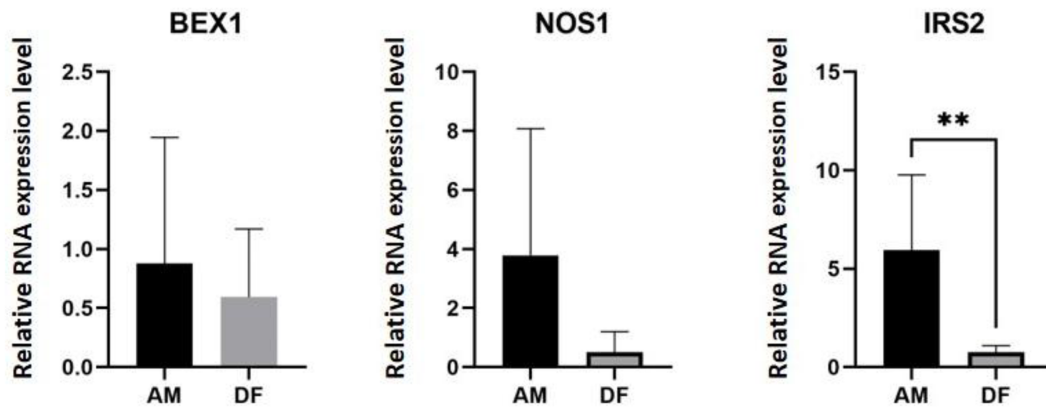


Figure 5 Validation of selected differentially expressed genes by RT-qPCR. Relative mRNA expression levels of brain-expressed X-linked protein 1 (BEX1), nitric oxide synthase 1 (NOS1), and insulin receptor substrate 2 (IRS2) in ameloblastoma (AM) and dental follicle (DF) tissues were measured by RT-qPCR. IRS2 expression was significantly increased in ameloblastoma compared with dental follicle tissues, whereas BEX1 and NOS1 showed an increasing trend. Data are presented as mean $\pm$ SD, $P < 0.01$.

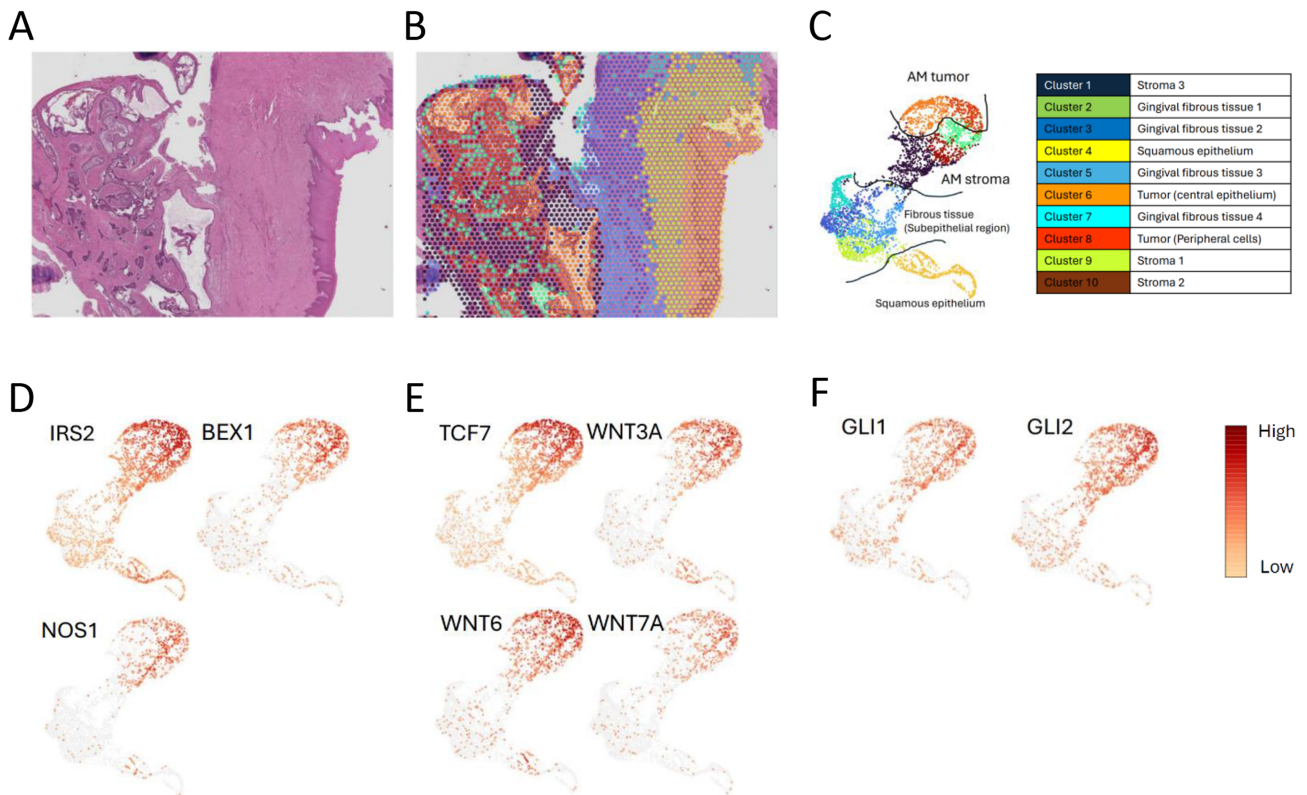


Figure 6 Spatial transcriptomic analysis of ameloblastoma tissue. (A) Representative hematoxylin and eosin (H&E)–stained section of ameloblastoma tissue. (B) Spatial transcriptomic map showing oral pathologist–based annotations and gene expression–based cluster annotations overlaid on the histological section. (C) Uniform manifold approximation and projection (UMAP) visualization of spatial transcriptomic clusters with annotation of major tissue compartments. (D) Spatial expression patterns of selected differentially expressed genes: insulin receptor substrate 2 (IRS2), brain-expressed X-linked protein 1 (BEX1), and nitric oxide synthase 1 (NOS1). (E) Spatial expression of Wnt signaling–related genes: T cell factor 7 (*TCF7*), *WNT3A*, *WNT6*, and *WNT7A*. (F) Spatial expression of Hedgehog pathway transcriptional effectors family zinc finger 1 (*GLI1*) and *GLI2*. Color intensity indicates relative gene expression levels (low to high).

the differential expression and pathway enrichment results obtained from bulk tissue and primary cell analyses, highlighting the spatially localized activation of oncogenic signaling pathways within ameloblastoma tumor epithelium.

Discussion

Ameloblastoma is the most common odontogenic neoplasm of jaws with unknown pathogenesis and is characterized by locally aggressive behavior and a high recurrence rate,

despite being histologically benign. Currently, surgical excision with adequate margins remains the standard treatment,¹ extensive excision often leads to significant functional and esthetic morbidity. The identification of *BRAF V600E* mutation in ameloblastoma has enabled the use of MAPK pathway inhibitors in selected unresectable cases.^{26,27} However, only approximately 60–80% of ameloblastoma harbor this mutation, and residual tumors are often still identified after BRAF/ERK-targeted therapy, likely because MAPK pathway inhibition alone is insufficient to achieve complete tumor eradication, underscoring the need to further elucidate the molecular mechanisms underlying ameloblastoma pathogenesis.^{28,29}

In this study, we integrated bulk tissue RNA sequencing, primary epithelial cell transcriptomics, and spatial transcriptomic profiling to delineate the molecular alterations underlying ameloblastoma. DEG analyses from both fresh tissues and cultured epithelial cells identified upregulation of *BEX1*, *NOS1*, and *IRS2*. Mechanistically, *BEX1* has been reported to inhibit the Wnt/ β -catenin pathway suppressor *RUNX3*,^{30,31} thereby indirectly enhancing β -catenin activity. *NOS1* promotes S-nitrosylation-mediated degradation of PTEN,^{32,33} a key negative regulator of PI3K/AKT signaling, which also facilitates nuclear accumulation of β -catenin.^{34,35} *IRS2* functions as a central adaptor linking receptor tyrosine kinase (RTK) activation to downstream PI3K/AKT and MAPK signaling through PI3K activation and growth factor receptor-bound protein 2 (GRB2) phosphorylation.^{36–39} Importantly, the upregulation of these DEGs was consistent with spatial transcriptomic analysis, which mitigates potential confounding effects from stromal and inflammatory components to further support that these genes were predominantly upregulated in tumor epithelial compartments, thereby providing in situ validation of their tumor-specific expression patterns.

Given that these DEGs converge on signaling networks, particularly Wnt/ β -catenin, PI3K/AKT, and MAPK pathways, it is likely that coordinated pathway dysregulation rather than isolated signaling events in ameloblastoma. Indeed, activation of the Wnt/ β -catenin signaling pathway plays a central role in multiple tumor-related biological processes, including tumorigenesis, proliferation, differentiation, invasion, and tissue homeostasis. Canonical Wnt/ β -catenin signaling is characterized by stabilization and nuclear translocation of β -catenin, resulting in transcriptional activation of downstream target genes essential for early odontogenesis.^{40–43} In fact, aberrant β -catenin expression has been reported in ameloblastoma and other odontogenic carcinomas.^{8,44–46} Notably, mutations in exon 3 of catenin beta 1 (*CTNNB1*), which encodes β -catenin, have been identified in approximately 50% of adenoid variant ameloblastomas, with more than 90% of *CTNNB1*-mutant tumors exhibiting nuclear β -catenin immunoreactivity;^{42,47} importantly, these *CTNNB1*-mutant ameloblastomas often lack concurrent BRAF or KRAS mutations,^{42,47} indicating activation of Wnt/ β -catenin signaling through a MAPK-independent mechanism. In the present study, spatial transcriptomic analysis revealed overexpression of multiple Wnt pathway activators in ameloblastoma tumor cells. Collectively, these findings further substantiate a contributory role of Wnt/ β -catenin signaling in the pathogenesis of a subset of ameloblastomas.

In addition to Wnt/ β -catenin and MAPK signaling, our spatial transcriptomic analysis also indicated activation of the Hedgehog pathway in ameloblastoma, as evidenced by elevated expression of its transcriptional effectors *GLI1* and *GLI2*. Hedgehog signaling plays a critical role in cell growth and differentiation and is aberrantly activated in several malignancies, including medulloblastoma, basal cell carcinoma, and glioblastoma.^{8,48–50} Previous studies have reported activating mutations in *SMO* in a subset of ameloblastomas.⁴ As a key upstream regulator of Hedgehog signaling, activating *SMO* mutations lead to increased *GLI1* expression and sustained pathway activation, providing a plausible mechanism for the elevated *GLI1* and *GLI2* expression observed in this study. Even in the absence of smoothened (*SMO*) mutation, *GLI1* overexpression has been identified in subsets of ameloblastoma, supporting the role of Hedgehog pathway activation in tumorigenesis.⁵¹ Taken together, these findings indicate that ameloblastoma is characterized by coordinated dysregulation of multiple oncogenic signaling pathways, including Wnt/ β -catenin, MAPK, PI3K/AKT, and Hedgehog signaling, which collectively contribute to tumor initiation and progression.

While our study provides integrative transcriptomic insights into the molecular alterations underlying ameloblastoma, a limitation of this study is the use of dental follicular tissue as the control for ameloblastoma. Although dental follicle is commonly adopted in odontogenic tumor studies, the most biologically appropriate controls would be developing tooth germs with normal odontogenic epithelial cells, which more closely represent the putative cell of origin of ameloblastoma. However, these tissues are exceedingly difficult to obtain in sufficient quantity and purity for transcriptomic analyses, particularly in human samples, necessitating the use of dental follicular tissue as a practical and ethically feasible alternative.

In conclusion, this study integrated bulk tissue transcriptomics, primary epithelial cell analyses, and spatial transcriptomic profiling to provide a comprehensive molecular characterization of ameloblastoma. Our findings identified a set of reproducibly dysregulated genes and revealed coordinated perturbation of multiple oncogenic signaling pathways, including Wnt/ β -catenin, PI3K/AKT, MAPK, and Hedgehog signaling, with spatially localized activation within tumor epithelial compartments. These results highlighted the molecular heterogeneity and network-level dysregulation underlying ameloblastoma pathogenesis and extended current understanding beyond single-pathway models. Collectively, this integrative framework may facilitate more refined molecular classification of ameloblastoma and inform future studies aim at developing pathway-based or combinatorial therapeutic strategies.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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