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Original Article

Evaluation of the prognostic and therapeutic potential of inhibin beta B for oral squamous cell carcinoma

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KEYWORDS
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Abstract *Background/purpose:* Oral squamous cell carcinoma (OSCC) is a common cancer worldwide, and its metastasis is difficult to predict and prevent. Inhibin beta B (INHBB) protein has been linked to cancer prognosis and epithelial-mesenchymal transition (EMT). However, previous study about INHBB expression focused on patients in a single region while the risk factors vary among regions. This study aimed to provide a broader perspective on INHBB expression in OSCC.
Materials and methods: Tissue micro-arrays comprising 118 specimens were subjected to immunohistochemistry, and all slides were quantified using StrataQuest software.
Results: The ratio of INHBB-positive cells to total cells was significantly higher in OSCC samples than in normal samples, and the intensity of INHBB expression was significantly greater in the late-stage OSCC. After classifying specimens into high and low INHBB expression groups, a significant association with clinical staging was found. Though a previous study suggested that menin regulates INHBB, menin expression was not detected in specimens.
Conclusion: The ratio of INHBB-positive cells in OSCC may be druggable for targeting tumor cells or assisting in diagnosis, and the intensity of INHBB expression may provide prognostic

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information for predicting potential metastasis. Moreover, the regulatory mechanism of INHBB in OSCC remains unclear and requires further investigation.

Introduction

Oral squamous cell carcinoma (OSCC) is a prevalent cancer worldwide, which affects chewing and swallowing functions of human beings. OSCC can be life-threatening with tumor invasion and metastasis. According to the latest research, approximately 377,000 new cases of OSCC were diagnosed in 2020, causing 145,000 deaths.[1] Though the five-year overall survival rate of OSCC is approximately 50—60%,[2] it is essential to note that the survival rates vary widely between different stages. Tsai et al. reported a drop in the survival rate of OSCC from 82.3% in the first stage to 39.3% in the fourth stage in Taiwan.[3] In addition to primary tumor size, depth of invasion,[4] the rate of lymph node metastasis,[5] and perineural invasion[6] are also prognostic factors of OSCC. Therefore, the discovery of a tool to aid in early diagnosis is crucial.

The quality of life of patients after anticancer treatments poses a significant challenge in current medical practice. For OSCC patients, the primary anticancer strategy is surgery, which may leave a postsurgical scar on the face.[7,8] A combination of surgery and chemotherapy or radiotherapy is also utilized to enhance the effectiveness for advanced stages.[9] However, these treatments often result in various side effects and negative impacts, such as hair loss, diarrhea, fatigue, marrow suppression, and other severe symptoms, which can significantly affect patients' well-being.[10,11] The impacts may contribute to that the suicide mortality rate of head and neck cancer patients was highest compared to other types of cancers.[12] Hence, a novel intervention modality for OSCC treatment is essential. These days, targeted therapies have become a rising star against cancer due to high selectivity and specificity.[13] To control early progression and aid in early detection of OSCC, the identification of a druggable target is necessary for the development of effective anticancer drugs.

Inhibin beta B (INHBB) gene encodes for the protein subunits of activin B and inhibin B. Both belong to the transforming growth factor-β (TGF-β) superfamily. Activin is a cytokine that was first discovered for its activity to stimulate the secretion of follicle-stimulating hormone.[14] Inhibin, on the other hand, has the opposite biological effect.[15] In addition to its significance in reproductive medicine, activin involves in fibrosis, inflammation, immune regulation, and tumorigenesis.[16] Researchers have observed that $INHBB^{-/-}$ mice show a correlation with oligodendrocyte apoptosis and a decrease in oligodendrocyte progenitor cells.[17] Moreover, accumulating studies have linked INHBB to cancers. Overexpression of INHBB in colorectal cancer was found to be negatively correlated with survival and positively related to liver metastasis.[18,19] A similar result has been found in gastric cancer, where INHBB was correlated with immune infiltration.[20] Furthermore, INHBB provided an environment suitable for metastasis of hepatocellular carcinoma.[21] Conversely, other studies in the epigenetic field held a contrary opinion. CpG island hypermethylation was found to be associated with the promoter of INHBB in colorectal cancer.[22,23] Also, expression of INHBB decreased during the epithelial-mesenchymal transition (EMT) in non-small cell lung carcinoma.[24] These information suggest a broad potential for INHBB-targeted interventions in cancer, which however, requires further investigation regarding to tissue specific scenario and detailed mechanistic information.

Though there are currently no drugs specifically targeting INHBB, several antagonists have been developed and tested in clinical trials for their effectiveness in TGF-β pathway.[25] Medical interventions have been reported to modulate INHBB expression. INHBB was negatively regulated by menin via Akt/Ezh2-mediated histone modification in mouse embryonic fibroblasts.[26] Wang et al. have found that *Camellia. leave. saponins* of golden-flowered tea could downregulate INHBB *in vivo* with a concomitant suppression of non-small cell lung cancer xenograft growth.[27] Li et al. suggested that INHBB served as a potential marker for OSCC treatment in immunosuppressed patients using machine learning.[28] These studies provided evidence that regulation INHBB expression can be therapeutic intervention target. Moreover, in a study using OSCC specimens in Japan, Kita et al. reported that high INHBB expression may enhance proliferation and EMT.[29] However, due to the inconsistent findings on INHBB across different cancer and even within a same cancer,[18,19,22,23] further investigation of INHBB expression in OSCC is necessary to provide direction to explore prognostic value. Furthermore, the development of medical interventions targeting INHBB could be valuable for anticancer therapy, as there is currently no research focusing on INHBB intervention in OSCC. Since INHBB is primarily expressed in reproductive organs,[30] it may represent a suitable target for localized intervention through onsite injection in OSCC tumor cells to prevent systemic effects.

The aim of this study was to evaluate and understand the implications of INHBB expression in OSCC. StrataQuest software was implemented to analyze clinical head and neck cancer specimen for comparing the differential expression of INHBB in normal tissues and different OSCC stages. We also evaluated whether INHBB might serve as a diagnostic biomarker for OSCC and used as a target for treatment of OSCC.

Materials and methods

Tissue micro-arrays

OR481a (Biomax, Derwood, MD, USA) and ORC1021 (Pantomics, Fairfield, CA, USA) tissue micro-arrays (TMAs) slides were used in this study. These slides were constructed using formalin-fixed paraffin-embedded tissues obtained from patients. The characters on each core of slides include age, sex, anatomic site, pathology diagnosis, TNM status, and stage. Additionally, the experiments on these TMAs were approved by National Taiwan University Hospital Research Ethics Committee (Approval No. 202201055RIND).

Immunohistochemistry

The slides were subjected to deparaffinization by immersing them in xylene for 30 min, followed by immersion in 95%, 85%, 75% ethanol, and ddH_2O for 5 min each. Then, antigen retrieval was performed by citrate acid buffer (pH 6.0) for 19 min in a microwave oven. Endogenous peroxidase activity was quenched using 3% H_2O_2. Non-specific IgG binding was blocked using normal goat serum (BioGenex, Fremont, CA, USA) for 30 min in phosphate-buffered saline (PBS), prior to incubating the slides with INHBB primary antibody (1:200, LS-C165242, LifeSpan BioSciences, Seattle, WA, USA) or menin primary antibody (1:200, PA5-115569, Invitrogen, Waltham, MA, USA) at room temperature for 2 h.

REAL EnVision detection system (Dako, Glostrup, Denmark) was then used to incubate the slides with the secondary antibody and chromogen 3,3′-diaminobenzidine (DAB) staining, following the manufacturer's instructions. During the process, slides were washed three times using PBS and phosphate-buffered saline with tween-20 (PBST) for 5 min each. Finally, hematoxylin was briefly used to counterstain the nuclei.

Image acquisition and quantification

TMA slides after processing immunohistochemical staining were scanned by TissueFAXS system (TissueGnostics, Vienna, Austria) at 20 × magnification. StrataQuest version 6 software (TissueGnostics) was used to record and quantify INHBB expression. Briefly, hematoxylin and DAB-staining are unmixed into separate channels for analysis. Segmentation of nuclei, exclusion of poorly stained nuclei, and exclusion of nuclear debris were individually screened to ensure the detective results were plausible in all cores. For each core of TMAs, three random regions with tumor cells (0.1 × 0.1 mm, 0.01 mm^2 each) were chosen to calculate the percentage of INHBB(+) cells and the intensity of INHBB expression.

Statistical analysis

The study utilized JASP 0.16.4 (JASP Team, Amsterdam, Netherlands) and R 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria) to analyze the raw data. Comparison of the average INHBB(+) ratio and INHBB(+) cell intensity among clinical stages were examined by one-way ANOVA with Tukey post hoc test. Furthermore, the correlation between the characteristics of each core and the difference of INHBB expression were performed through chi-square test. Logistic regression was employed to assess the relationship between binomial clinical stage status (early and late stage), T status (T1+T2 and T3+T4), N status (N0 and N1+N2), and INHBB(+) cell intensity. The significant level was set at $\alpha = 0.05$.

Results

INHBB protein expression between normal and OSCC samples

There was a total of 150 cores on the TMA slides. Prior to analyzing the data obtained from StrataQuest software, 22 cores were excluded because they were neither diagnosed as normal nor OSCC. Additionally, 10 cores were removed because they did not contain enough tumor or epithelial regions to include the required three 0.10 × 0.10 mm^2 regions for evaluation. Finally, two OSCC cores lacking staging information were included in the analysis comparing normal and OSCC but excluded from the staging analysis (Table 1).

INHBB(+) cells were found in all samples (n = 118) in both normal epithelium and OSCC tumor cells, with a mean INHBB(+) cell ratio of 59.74 ± 23.21%. Immunohistochemical staining images showed that INHBB expression was predominantly located in the cytoplasm of cells and extracellular region (Fig. 1A). The INHBB(+) cell ratio was found to be significantly greater in OSCC samples compared to normal samples ($P = 0.046$) (Fig. 1B), while intensity was not (Fig. 1C).

INHBB protein expression among OSCC clinical staging

Then, we examined the ratio and intensity of INHBB(+) cells in various stages. INHBB(+) cells ratio is non-

Table 1 Diagnosis distribution of the samples used in the study.

Diagnosis	Applicable cores	Inapplicable cores[a]	Total
Normal	**6**	**5**	**11**
OSCC	**112**	**5**	**117**
Stage 1	22	1	23
Stage 2	33	2	35
Stage 3	41	0	41
Stage 4	14	2	16
No Staging Information	2	0	2
Non-OSCC Tumors	**0**	**22**	**22**
Total	**118**	**32**	**150**

Note: OSCC, oral squamous cell carcinoma.
The bolded words in the table represent major categories or totals, while the non-bolded words represent subcategories within those major categories.
[a] Samples that were not diagnosed as normal or with OSCC, as well as samples lacking sufficient epithelial or OSCC tumor regions for analysis, were not applicable and were therefore excluded from the study.

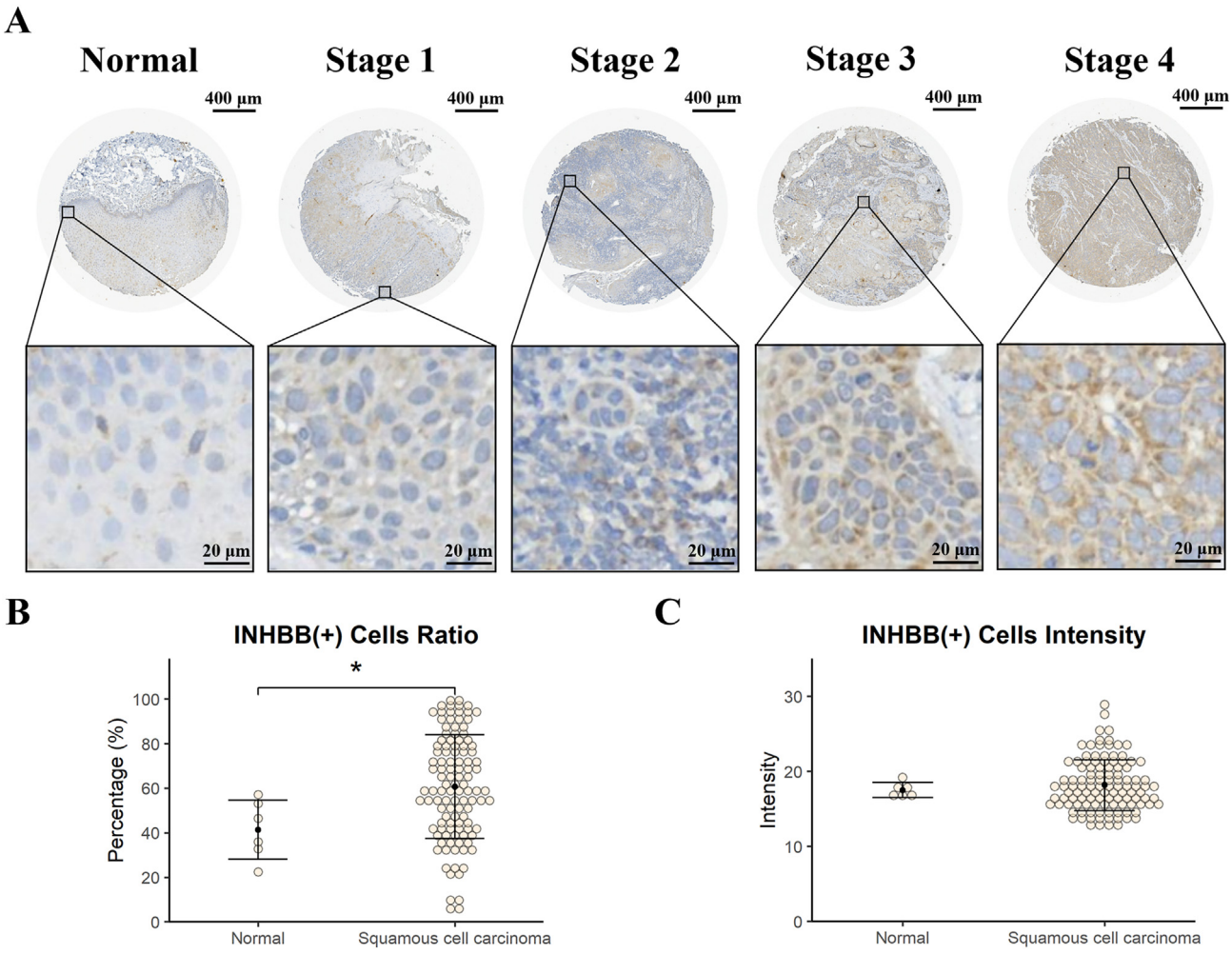

Fig. 1 Immunohistochemical staining of INHBB in normal and OSCC tissues. (A) Immunohistochemistry images from each stage. (origin magnification 20 ×) (B) Ratio of INHBB-positive cells to total cells. (C) Average immunostaining intensity of INHBB-positive cells. Data are presented as mean and standard deviation. * denotes statistical significance with $P < 0.05$ after Student's t-test.

significant between stages ($P = 0.089$), though the average INHBB(+) cells ratio is gradually higher in different stages (Fig. 2A). The result showed that the intensity of INHBB(+) in normal tissue, early stage, and late stages was significantly different. Specifically, the average intensity of INHBB(+) cells was higher in the late stage (Stage 3 + Stage 4) compared to the early stage (Stage 1 + Stage 2) ($P = 0.033$) (Fig. 2B).

Association of INHBB expression with OSCC clinical characteristics

Following the previous observation that intensity may be a potential predictor, we proceeded to divide the tissue blocks into two groups based on the intensity of INHBB expression levels, namely high and low INHBB expression groups, using a cut-off point of 18.495 (mean of intensity + standard deviation of intensity in the normal group). In the correlation study, we found that the late stage (Stage 3 + Stage 4) exhibited higher INHBB(+) cell intensity compared to early-stage OSCC (Stage 1 + Stage 2) ($P = 0.011$) (Table 2). Nevertheless, other characteristics, including diagnosis, age, gender, T status, and N status, did not yield significant results in statistical analysis.

Student's t-test showed a significant difference between staging and intensity of INHBB expression ($P = 0.012$), and so did logistic regression ($P = 0.011$). The results presented a nearly medium power[31] of difference (Cohen's $d = 0.487$) between clinical staging (Table 3).

Menin may not be expressed in OSCC

A previous study showed that INHBB was regulated by menin via H3K27 histone modification,[26] providing a potential therapeutic link in OSCC. To confirm the relationship, we performed immunohistochemistry with menin primary antibody on slides from the same patients as INHBB protein. However, menin wasn't expressed in OSCC cells. It has only been expressed in the duct and duct-like tissues in samples (Fig. 3).

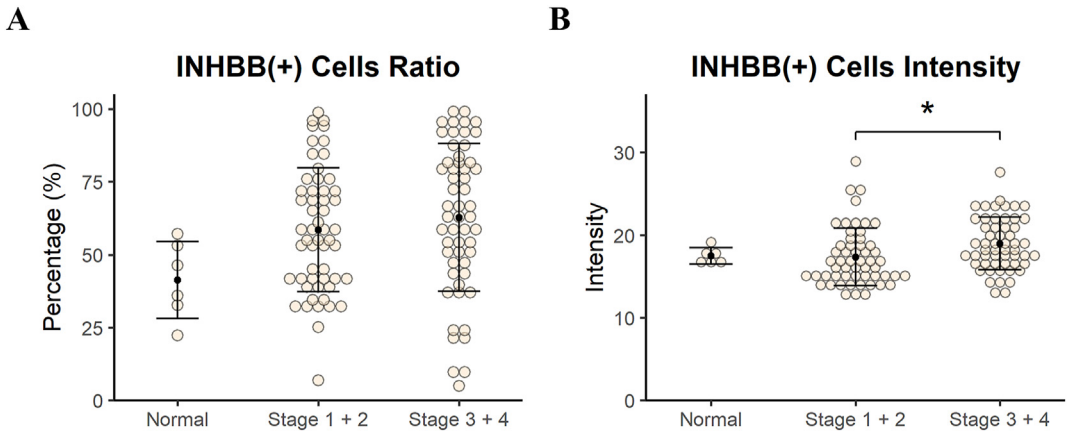

Fig. 2 Immunohistochemical staining of INHBB in OSCC classified by clinical staging. (A) Ratio of INHBB-positive cells to total cells in different stages. (B) Average immunostaining intensity of INHBB-positive cells in different stages. Data are presented as mean and standard deviation. * denotes statistical significance with $P < 0.05$ after one-way ANOVA and Tukey post hoc test.

Discussion

Drawing on recent advances in oral cancer, the present research focused on the difference and correlation of INHBB expression in different processes of OSCC. INHBB is a glycoprotein in TGF-β family. Our findings revealed significant differences in the INHBB(+) cell ratio between normal and OSCC tissues. Moreover, the intensity of INHBB expression varied among clinical stages, with higher expression observed in late-stage tumors. Correlation analysis indicated that clinical staging was significantly associated with INHBB expression, suggesting that increased INHBB expression may serve as a potential predictor of OSCC staging. These results may contribute to the development of novel therapeutic strategies for the treatment of OSCC.

Table 2 Correlations of INHBB(+) intensity with clinical characteristics.

Groups		INHBB(+) intensity		*P*-value
		High	Low	
Age				0.347
≧65	n = 23	11	12	
<65	n = 89	33	56	
Gender				0.731
Female	n = 30	11	19	
Male	n = 82	33	49	
Clinical staging				0.011*
Stage 1 + 2	n = 55	15	40	
Stage 3 + 4	n = 55	28	27	
T status				0.470
T1+T2	n = 76	28	48	
T3+T4	n = 34	15	19	
N status				0.053
N0	n = 78	26	52	
N1+N2	n = 32	17	15	

* denotes statistical significance with $P < 0.05$.
Note: INHBB, inhibin beta B.

There are two explanations based on our findings. Firstly, the ratio of INHBB(+) cells to total cells may be used as a diagnostic tool to distinguish between normal tissue and tumor cells by establishing an appropriate threshold. Additionally, the result also suggested that INHBB showed potential druggability for cancer cell targeting, while further research is indispensable to develop precise and effective inhibitors or regulating mechanisms.

Next, the intensity of INHBB expression may serve as a marker for prognosis prediction. Patients with high INHBB expression in early stages may require aggressive treatment, such as neck dissection, to prevent undetected lymph node metastasis though tumor cells were not sufficient to be detected on X-rays. In other words, the amount of INHBB expression of each tumor cell in OSCC is an essential indicator, implying more invasive cancerous characteristics of tumor cells. Coupled with the study from Kita et al.,[29] INHBB presented a metastatic phenotype. Notwithstanding, we found that clinical staging is correlated with INHBB expression, rather than N status alone. Though the late stage is also highly dependent on N status, it indicated that both primary tumor size and regional metastasis play a role in INHBB expression.

It is widely accepted that epigenetic markers are important for oral cancer diagnosis[32] and regulation via epidrug may have therapeutic potential.[33] In the case of INHBB, possible regulatory methods included promoter and upstream modulation, as well as medicine-food homology food. First, CpG island hypermethylation has been found to regulate INHBB in colorectal cancer.[22,23] Gherardi et al. discovered that menin could negatively regulate INHBB.[26] However, in our examination, menin expression was scarce in OSCC tumor tissue (Fig. 3), indicating that INHBB may not be regulated by menin in OSCC. In addition, a recent study showed that active fractions from golden-flowered tea dropped INHBB expression in tumor cells of non-small cell lung cancer.[27] Combined with the finding from immune infiltration result in gastric cancer[20] and immunocompromised patients via deep learning in OSCC,[28] it is suggested that tumor immune microenvironment may play a role in INHBB modulation. After unraveling the mystery of the regulatory mechanism of INHBB in oral

Table 3 Association of INHBB(+) intensity level with clinical staging, T status, and N status.

	P-value	Effect size
Mean intensity comparison		
Clinical Staging	0.012*	0.487
T status	0.256	0.236
N status	0.229	0.254
Logistic regression		
Clinical Staging	0.011*	0.077
T status	0.471	0.007
N status	0.055	0.047

* denotes statistical significance with $P < 0.05$.
Note: INHBB, inhibin beta B.

cancer, it would help develop possibly specific anticancer drugs.

Taken together, our findings and previous research point out that increased INHBB expression is a potential marker for predicting the prognosis of OSCC. We are the first who identify the correlation between INHBB expression and clinical staging in OSCC.

In the study, there are three main limitations. Firstly, the sample size of normal tissues was relatively low. It can be difficult and ethically controversial to collect normal or healthy tissue from patients for biopsy. However, the results of our study are significant and suggest a clear difference in INHBB(+) cell ratio between normal and tumor tissues. Another limitation is that a few extreme samples were found. As mentioned previously, it may provide a predictor of whether the cancerous characteristic of this patient is more invasive or not. If the patient in the early stage shows high INHBB expression, it may imply that the patient may be advanced to the late stage sooner. Further research has to be done to confirm this finding. Finally, evaluation for risk factors such as betel quid was limited. Betel quid and areca nut use are major risk factors for oral cancer for people living in East Asia, South Asia, and the Pacific islands.[34] Both are also related to poor prognosis.[35] The study could not distinguish the association between INHBB expression and betel quid use.

In the present article, we identified that INHBB is a potential biomarker and predictor in OSCC. OSCC patients showed a higher INHBB(+) cells ratio than normal tissues and both mean intensity expression comparison and correlational study had significant results in clinical staging. Our findings shed light on the role of INHBB in OSCC. However, our data suggested that regulation of INHBB through menin via epigenetic mechanisms is unlikely found in OSCC. Future research could build upon our work by exploring the molecular mechanism of INHBB, particularly whether the active component of golden-flowered tea could regulate INHBB in oral cancer. This could lead to the development of INHBB as a therapeutic target for preventing regional metastasis and finally elevating survival rates in OSCC patients.

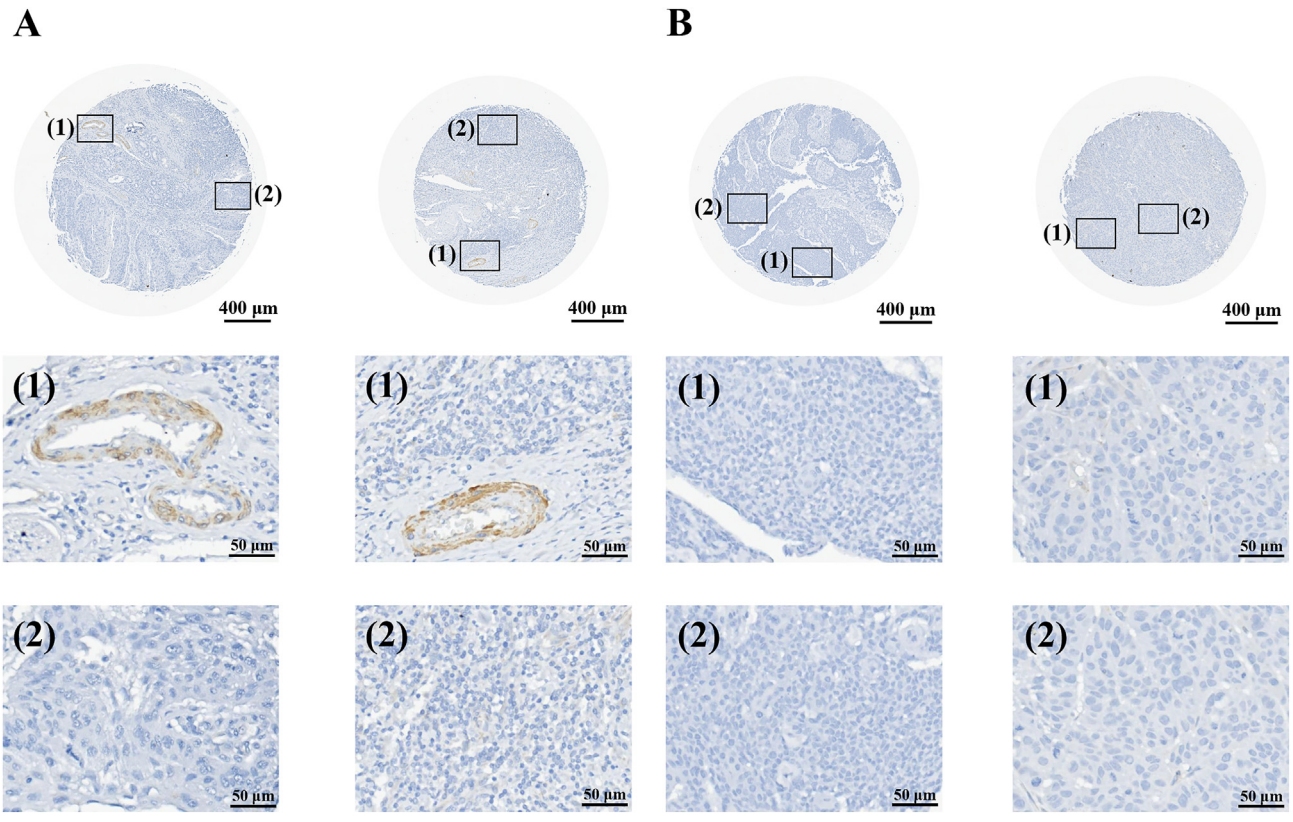

Fig. 3 Absence of menin expression in OSCC tumor tissues, with expression detected in nearby duct or duct-like tissues. (A) Immunohistochemical staining showing menin expression in duct or duct-like tissues but not in tumor tissues. (B) No menin expression detected in tumor tissues.

Declaration of competing interest

The authors report no conflicts of interest related to this article.

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