

Secreted phosphoprotein 1 is associated with epithelial cell proliferation and PI3K/AKT signalling in sinonasal inverted papilloma

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Abstract

Objectives: Sinonasal inverted papilloma is a locally aggressive epithelial lesion with a relatively high recurrence rate, yet the molecular basis of its proliferative behaviour remains incompletely understood. This study investigated the clinical relevance of secreted phosphoprotein 1 in sinonasal inverted papilloma and its association with epithelial cell proliferation and related signalling pathways.

Methods: Differentially expressed genes were identified from the GSE193016 dataset, followed by gene set enrichment analysis. The expression of secreted phosphoprotein 1 and the cell cycle-related molecules cell division cycle 25C and cyclin A2 in clinical specimens was assessed using quantitative PCR, Western blotting, and immunohistochemistry. Associations between secreted phosphoprotein 1 expression and postoperative recurrence were assessed based on follow-up data. In vitro experiments using human nasal epithelial cells were conducted to examine the effects of altered secreted phosphoprotein 1 expression on cell proliferation-associated phenotypes, and pathway inhibition assays were performed to investigate the involvement of the PI3K/AKT signalling pathway.

Results: Secreted phosphoprotein 1 expression was significantly upregulated in sinonasal inverted papilloma tissues, with higher levels observed in advanced-stage cases (T3–T4), and was associated with postoperative recurrence. The expression of the cell cycle-related molecules cell division cycle 25C and cyclin A2, positively correlated with secreted phosphoprotein 1 expression and increased with disease progression. In vitro epithelial cell models demonstrated that modulation of secreted phosphoprotein 1 expression was accompanied by corresponding changes in cellular proliferative capacity and cell cycle-related protein expression. Pathway inhibition assays further suggested that PI3K/AKT signalling may be involved in secreted phosphoprotein 1-associated alterations in cell cycle regulation.

Conclusion: These findings indicate that secreted phosphoprotein 1 is highly expressed in sinonasal inverted papilloma and may be associated with epithelial cell proliferation and PI3K/AKT signalling. In addition, secreted phosphoprotein 1 expression correlates with disease recurrence, highlighting its potential research value as a biological marker, while the underlying mechanisms warrant further investigation.

Keywords

sinonasal inverted papilloma, SPPI, epithelial cell proliferation, PI3K/AKT signalling pathway, CDC25C, CCNA2

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Introduction

Sinonasal inverted papilloma (SNIP) is a benign epithelial tumour arising from the Schneiderian mucosa and represents the most common subtype of sinonasal papilloma.^{1,2} Epidemiological studies indicate that SNIP accounts for ~0.5%–4% of primary nasal tumours and predominantly affects middle-aged males.^{3,4} Despite its benign histological classification, SNIP often exhibits aggressive clinical behaviour, including local bone destruction and invasion of adjacent structures,^{5,6} posing substantial challenges for clinical management. Notably, SNIP is associated with a relatively high postoperative recurrence rate of 15%–20%.⁷ Although SNIP is therefore widely recognised as a biologically aggressive epithelial lesion, the molecular basis underlying its development and progression remains incompletely understood, particularly the key molecules and signalling pathways involved in epithelial cell proliferation and recurrence.

The cell cycle consists of a series of tightly regulated processes governing DNA replication and cell division, thereby maintaining cellular proliferative capacity and functional homeostasis.⁸ Aberrant expression of cell cycle-related proteins has been reported in SNIP and may be associated with its aggressive biological behaviour.^{9,10} Cell division cycle 25C (CDC25C) and cyclin A2 (CCNA2) are key regulators of cell cycle progression, playing critical roles in the G2/M transition and mitotic regulation, respectively.^{11,12} Elevated expression of CDC25C and CCNA2 has been observed in various epithelial-derived tumours, including sinonasal, lung, and liver cancers, and is frequently associated with increased tumour aggressiveness and unfavourable clinical outcomes.^{13–15} Together, these findings suggest that dysregulation of specific cell cycle regulators may contribute to abnormal proliferative processes in SNIP; however, their precise roles in this disease context remain to be clarified.

Secreted phosphoprotein 1 (SPP1) is a phosphorylated glycoprotein involved in diverse biological processes, including cell proliferation, cellular invasiveness, and tissue remodelling in a range of disease contexts, such as malignant tumours, metabolic disorders, and fibrotic conditions.^{16–18} Previous studies have reported elevated SPP1 expression in SNIP tissues and its association with clinical disease severity.¹⁹ However, the cytological functions of SPP1 in SNIP and its related molecular regulatory networks remain poorly characterised. The present exploratory study integrates analyses of clinical specimens with *in vitro* epithelial cell models to examine the association between SPP1 and cell cycle-related molecules, thereby providing further insight into the biological behaviour of SNIP.

Methods

Public database data acquisition and bioinformatics analysis

The GSE193016 dataset was obtained from the Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo/query/>

[acc.cgi?acc=GSE193016](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193016)) and generated using the GPL21047 platform. This dataset comprises four normal nasal mucosa samples obtained from patients undergoing septoplasty and four SNIP tissue samples. Raw expression data were preprocessed and analysed in the R version 4.4.1. After data normalisation, differentially expressed genes (DEGs) between SNIP and control tissues were identified using the limma package. Gene set enrichment analysis (GSEA) was subsequently performed on the ranked gene list using the clusterProfiler package, with the KEGG pathway gene set (c2.cp.kegg.v7.5.1.symbols.gmt) and 10,000 permutations.^{20,21} Genes were ranked based on $-\log_{10}$ adjusted *p* values. Following the identification of significantly enriched cell cycle-related pathways, core enrichment genes were extracted and visualised using heatmap analysis. The association between SPP1 expression and core cell cycle-related genes was assessed using Pearson correlation analysis. To further evaluate the relationship between SPP1 expression and cell cycle activation, genes were ranked according to their Pearson correlation coefficients with SPP1 expression and subjected to GSEA analysis. In addition, samples were stratified into SPP1-high and SPP1-low groups according to the median expression level of SPP1, and ssGSEA implemented in the GSVA package was used to quantify pathway activity and immune cell infiltration based on predefined gene signatures, with differences assessed using the Wilcoxon rank-sum test. DEGs were additionally ranked according to log₂ fold change and adjusted *p* values. The complete list of genes is provided in Supplementary Table S3, while significantly DEGs are summarised in Supplementary Table S4, and the ranked list of DEGs according to log₂ fold change is presented in Supplementary Table S5. Among the significantly upregulated genes identified in the dataset, SPP1 showed a relatively high fold change and statistical significance and was therefore selected for further functional investigation based on its differential expression and previously reported association with SNIP.¹⁹

Human tissue samples

This was a retrospective observational study. This study enrolled 54 patients with SNIP who underwent surgical treatment at Qingdao University Affiliated Hospital between January 2020 and March 2022. Patients were included if they had histopathologically confirmed SNIP and complete clinical and follow-up data. Patients were excluded if they had a history of sinonasal malignancy, prior radiotherapy or chemotherapy to the sinonasal region, or incomplete clinical records. All patients received preoperative paranasal sinus computed tomography examinations and were clinically staged according to the Krouse classification system.²² The distribution of disease stages was as follows: T1 (*n* = 13), T2 (*n* = 15), T3 (*n* = 15), and T4 (*n* = 11). All patients underwent endoscopic sinus surgery. During surgery, efforts were made to identify and completely remove the tumour attachment site (pedicle) together with the surrounding mucosa and, when necessary, the underlying bone to achieve complete resection. Recurrence was defined as the detection of newly

Table 1. Characteristics of clinical subjects.

Characteristic	Control (n = 18)	SNIP (n = 54)	p value
Age (years)	46.11 ± 5.71	49.74 ± 8.03	0.081
Male	8 (44.44%)	31 (57.41%)	0.339
Recurrence	NA	9 (16.68)	NA
Krouse stage			
T1 (%)	NA	13 (24.07%)	NA
T2 (%)	NA	15 (27.78%)	NA
T3 (%)	NA	15 (27.78%)	NA
T4 (%)	NA	11 (20.37%)	NA

Note. Continuous variables are presented as mean ± SD and compared using an unpaired Student's *t*-test. Categorical variables are presented as *n* (%) and compared using the chi-square test. Recurrence and Krouse stage were assessed only in SNIP patients.

NA: not applicable; SD: standard deviation; SNIP: sinonasal inverted papilloma.

developed SNIP lesions at the original surgical site or adjacent sinonasal regions during follow-up based on endoscopic examination and/or radiological imaging. All suspected recurrent lesions were subsequently surgically resected and confirmed by histopathological examination. All patients were followed for 36 months through outpatient visits and telephone consultations. Based on postoperative recurrence status, patients were classified into a recurrence group (*n*=9) and a non-recurrence group (*n*=45). The control group comprised 18 patients who underwent endoscopic septoplasty during the same period at the same institution. These individuals had no history of nasal or paranasal sinus tumours or inflammatory diseases, and normal nasal mucosal tissues were collected as control samples. Clinical and pathological features of the enrolled subjects are presented in Table 1. The study received approval from the Ethics Committee of Qingdao University Affiliated Hospital (no. QYFYWZLL28986), and all participants provided written informed consent before enrolment. The sample size was determined based on the number of eligible patients treated during the study period and was not predefined by statistical power calculation.

Immunohistochemical staining

Paraffin-embedded tissue sections were routinely deparaffinised and rehydrated through graded ethanol solutions, followed by antigen retrieval in sodium citrate buffer (pH 6.0) using heat-induced epitope retrieval for 10 min. After cooling to room temperature, sections were blocked with 10% sheep serum for 30 min and incubated overnight at 4 °C with the appropriate primary antibodies. After washing with phosphate-buffered saline (PBS), sections were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactivity was visualised using a diaminobenzidine chromogen, followed by haematoxylin counterstaining. For each sample, three independent tissue sections were analysed. For each section, representative regions were selected for analysis in a blinded manner. The

interpretation and quantification of staining results were independently performed by two investigators who were blinded to the clinical information. Semi-quantitative analysis was conducted with ImageJ software (v 1.54f) by calculating the proportion of positively stained area relative to the selected tissue area. The results were reviewed for consistency, and discrepancies were resolved by consensus. The mean value of the three sections was used for subsequent statistical analysis. Details of the antibodies used are provided in Supplementary Table S1.

Nasal secretion collection and enzyme-linked immunosorbent assay

To quantify SPP1 protein levels in nasal secretions, samples were collected using the expanded sponge method. Briefly, ~150 mg of sterile expanded sponge was weighed and placed into an eppendorf tube for each sample, then inserted into the nasal cavity to absorb nasal secretions. After removal, the sponge was reweighed, and the difference in weight before and after collection was used to estimate the volume of absorbed secretions. The secretion-soaked sponge was subsequently transferred into a 5 mL disposable syringe, and 1 mL of sterile physiological saline was added. The syringe was placed vertically in a centrifuge tube and incubated at room temperature for 2 h to allow elution of nasal secretions. The eluate was then centrifuged at 4 °C and 1500g for 10 min, and the supernatant was collected and stored at –80 °C until analysis. SPP1 concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, E-EL-H1347, China) according to the manufacturer's instructions. All samples were analysed in triplicate.

Cell culture and lentiviral transduction

The human nasal epithelial cell (HNEpC; SHTPBio, Shanghai, China) was maintained in RPMI-1640 medium (Procell, PM150110, China) containing 10% foetal bovine serum at 37 °C in a humidified incubator with 5% CO₂.²³ To generate SPP1 overexpression or knockdown models, HNEpC cells were infected with lentiviral vectors (Genechem, China) carrying an SPP1 overexpression construct or an SPP1-targeting interference sequence. The multiplicity of infection was set at 10, and cells were exposed to lentivirus for 48 h. Following infection, puromycin selection was applied for an additional 48 h to enrich transduced cells. The efficiency of SPP1 overexpression or knockdown was confirmed by assessing SPP1 mRNA and protein expression levels using real-time quantitative PCR (qPCR) and Western blot analysis.

Western blot analysis

Tissue samples were lysed in radioimmunoprecipitation assay (RIPA) buffer, while cell samples were lysed using a buffer containing 2% SDS to extract total protein. Lysates were centrifuged to remove insoluble debris, and protein

concentrations were determined using the Lowry method. Equal protein amounts were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under constant voltage (80 V for stacking and 110 V for separating gels) and then transferred to pre-activated polyvinylidene difluoride (PVDF) membranes (Millipore, IPFL00010, USA). Membranes were blocked in 5% skimmed milk for 1.5 h, followed by overnight incubation with the appropriate primary antibodies at 4 °C. Following tris-buffered saline with tween 20 (TBST) washes, membranes were incubated with HRP-conjugated secondary antibodies (1:10,000; Elabscience) for 1 h at room temperature. Protein bands were visualised using enhanced chemiluminescence and detected with a chemiluminescent imaging system. All experiments were independently repeated three times. Antibody information is provided in Supplementary Table S1.

Real-time qPCR

Total RNA was extracted from tissue samples and cultured cells using the TRIzol method. Briefly, samples were lysed with TRIzol reagent (Vazyme, R401-01, China), followed by phase separation with chloroform. After centrifugation, the aqueous phase was collected, and RNA was precipitated with isopropanol. The RNA pellet was washed with 75% ethanol, air-dried, and dissolved in RNase-free water. Complementary DNA (cDNA) was synthesised from 1 µg of total RNA using the HiScript IV First Strand cDNA Synthesis Kit (+gDNA wiper; Vazyme). The resulting cDNA was diluted fivefold and used as the template for qPCR with SYBR Green Premix Pro Taq HS reagent. Relative mRNA levels were determined by the $2^{-\Delta\Delta C_t}$ method.²⁴ Primer sequences are listed in Supplementary Table S2.

Immunofluorescence staining

Cell smears were placed in 24-well plates and cultured for 48 h. Cells were then washed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. After fixation, cells were permeabilised with 0.1% Triton X-100 for 10 min, followed by blocking with blocking solution at room temperature for 1 h. Cells were subsequently incubated overnight at 4 °C with the appropriate primary antibodies. After washing with PBS, cells were incubated with species-specific fluorescently labelled secondary antibodies at room temperature for 1 h. Nuclei were counterstained with an anti-fade mounting medium containing DAPI. Immunofluorescence images were acquired using a fluorescence microscope. Details of the antibodies used are provided in Supplementary Table S1.

Cell proliferation assay (Cell Counting Kit-8)

Cells were enzymatically dissociated to obtain single-cell suspensions and plated in 96-well plates at 2000 cells/well.

To minimise edge effects, peripheral wells were filled with 100 µl of PBS. Cells were cultured at 37 °C in a humidified incubator with 5% CO₂, and cell proliferation was assessed at 24, 48, and 72 h. At each time point, 10 µl of Cell Counting Kit-8 (CCK-8) reagent (Solarbio, CA1210, China) was added to the wells and incubated for 3 h. Absorbance at 450 nm was recorded using a microplate reader. Blank control wells containing culture medium and CCK-8 reagent only were included. All assays were performed in triplicate, and cell proliferation was expressed as optical density values after subtraction of the blank control.

Statistical analysis

Fifty-four SNIP specimens were stratified into high- and low-expression groups ($n=27/\text{group}$) based on the median SPP1 mRNA expression level. Recurrence rates between groups were compared using contingency tables, with the chi-square test applied where appropriate and Fisher's exact test used when expected cell counts were <5 . Because the number of recurrence events was limited in this cohort, multivariate regression analysis was not performed to avoid potential model overfitting. Continuous variables were compared between two groups using independent-samples t -tests, whereas comparisons involving more than two groups were performed by analysis of variance, when appropriate. Normality of the data distribution was assessed prior to applying parametric tests. Correlations between variables were assessed using Pearson correlation analysis. For the GSE193016 dataset, correlation analysis was performed using all available samples (SNIP and control combined) due to the limited sample size ($n=4/\text{group}$). In contrast, for the clinical cohort, correlation analysis was conducted using SNIP samples only ($n=54$). Results are expressed as the mean $\pm$ standard deviation. All in vitro experiments were independently repeated at three times. A $p < 0.05$ was considered statistically significant, and statistical analyses were conducted using GraphPad Prism software (version 9.5.0, San Diego, CA, USA).

Results

Elevated SPP1 expression in SNIP is associated with disease recurrence

Differential expression analysis of the GSE193016 dataset identified multiple dysregulated genes between SNIP and control tissues, as illustrated by the volcano plot (Supplementary Figure S1(A)) and detailed in Supplementary Tables S3 to S5. Among these, SPP1 was significantly upregulated ($\log_2\text{FC}=2.42$, adjusted $p=0.0159$) and was selected for further investigation. Consistently, analysis of the GSE193016 dataset showed significantly higher SPP1 mRNA expression in SNIP tissues compared with controls (Figure 1(a)), which was further validated by qPCR in clinical SNIP specimens (Figure 1(b)). Stratification by Krouse

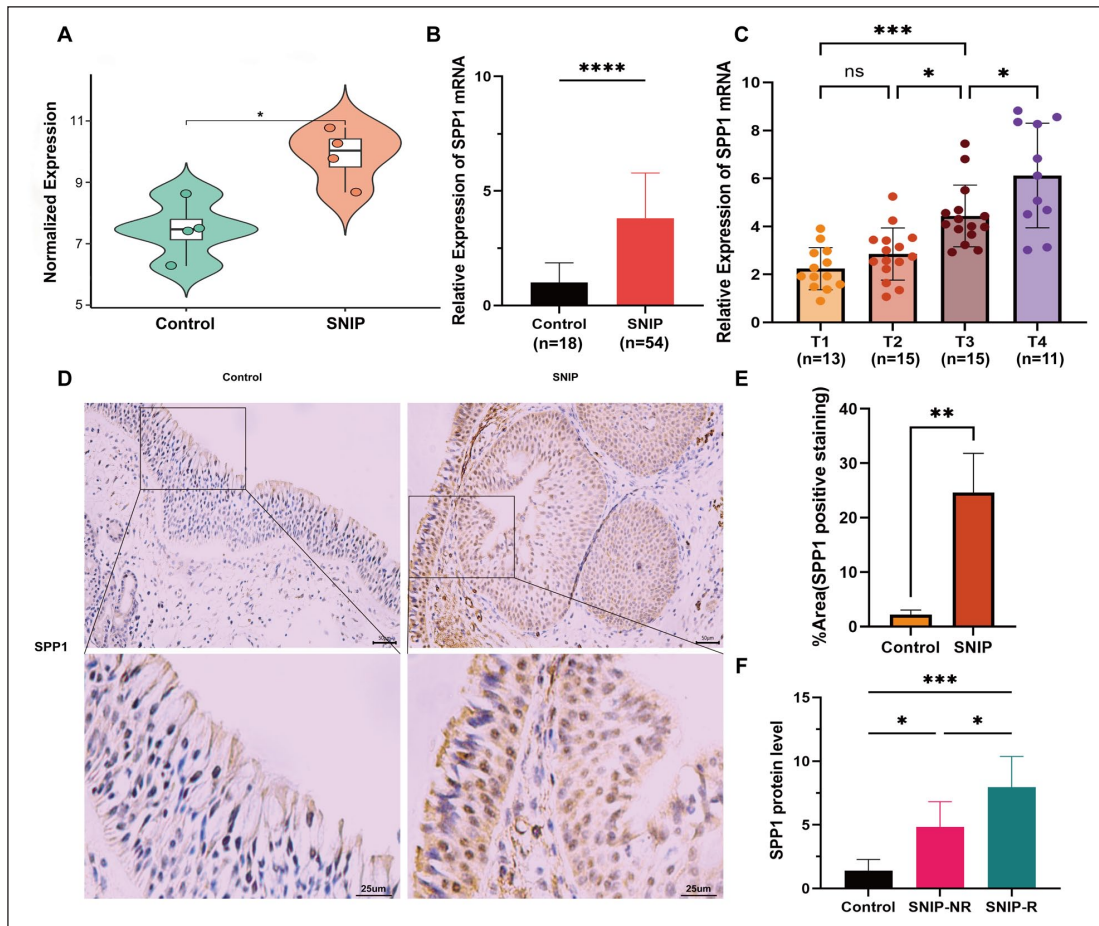


Figure 1. Elevated SPP1 expression in SNIP and its association with disease recurrence: (a) Differential expression analysis of SPP1 between control tissues and SNIP tissues in the GSE193016 dataset, (b) quantitative PCR analysis of SPP1 mRNA expression levels in control and SNIP tissues, (c) SPP1 mRNA expression levels in SNIP tissues stratified by Krouse stage (T1–T4), (d) representative immunohistochemical staining images showing SPP1 expression in control and SNIP tissues, (e) semi-quantitative analysis of SPP1-positive staining, expressed as the %Area, and (f) SPP1 protein levels in nasal secretions from control subjects, SNIP-NR patients, and SNIP-R patients.

ns: not statistically significant; SNIP: sinonasal inverted papilloma; SNIP-NR: non-recurrent SNIP; SNIP-R: recurrent SNIP; SPP1: secreted phosphoprotein 1; %Area: percentage of positive area.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

stage demonstrated a progressive increase in SPP1 expression in advanced-stage SNIP (T3–T4) compared with early-stage disease (T1–T2; Figure 1(c)). Immunohistochemical staining further demonstrated increased SPP1 protein expression in SNIP tissues compared with control tissues (Figure 1(d) and (e)). SPP1 staining was predominantly localised in the cytoplasm of epithelial tumour cells in SNIP tissues, whereas minimal staining was observed in stromal components.

According to the median level of SPP1 mRNA expression, 54 patients with SNIP were divided into high- and low-expression groups ($n=27$ in each group). The high-expression group showed a higher recurrence rate (29.6%, 8/27) than the low-expression group (3.7%, 1/27; Fisher's exact test, $p=0.0243$; Table 2).

In addition, SPP1 protein levels in nasal secretions were significantly higher in SNIP patients than in control subjects, and were further elevated in patients who experienced

postoperative recurrence compared with those without recurrence (Figure 1(f)). Together, these results suggest that SPP1 is highly expressed in SNIP and correlates with a higher risk of disease recurrence.

SPP1 expression in SNIP correlates with activation of cell cycle-related pathways

To explore the biological processes potentially involved in SNIP, GSEA was performed on the GSE193016 dataset using KEGG pathway annotations. Several pathways were significantly enriched in SNIP tissues, including cornified envelope formation, oxidative phosphorylation, and the cell cycle (Figure 2(a)). Given the prominent epithelial proliferative features of SNIP, subsequent analyses focussed on cell cycle-related pathways. The GSEA enrichment plot demonstrated significant positive enrichment of the cell

Table 2. Association between SPPI expression and recurrence.

Expression	Recurrence, n (%)	No recurrence, n (%)	Total
Low	1 (3.7%)	26 (96.3%)	27
High	8 (29.6%)	19 (70.4%)	27

Note. Statistical significance was assessed using Fisher’s exact test ($p=0.0243$).
SPPI: secreted phosphoprotein 1.

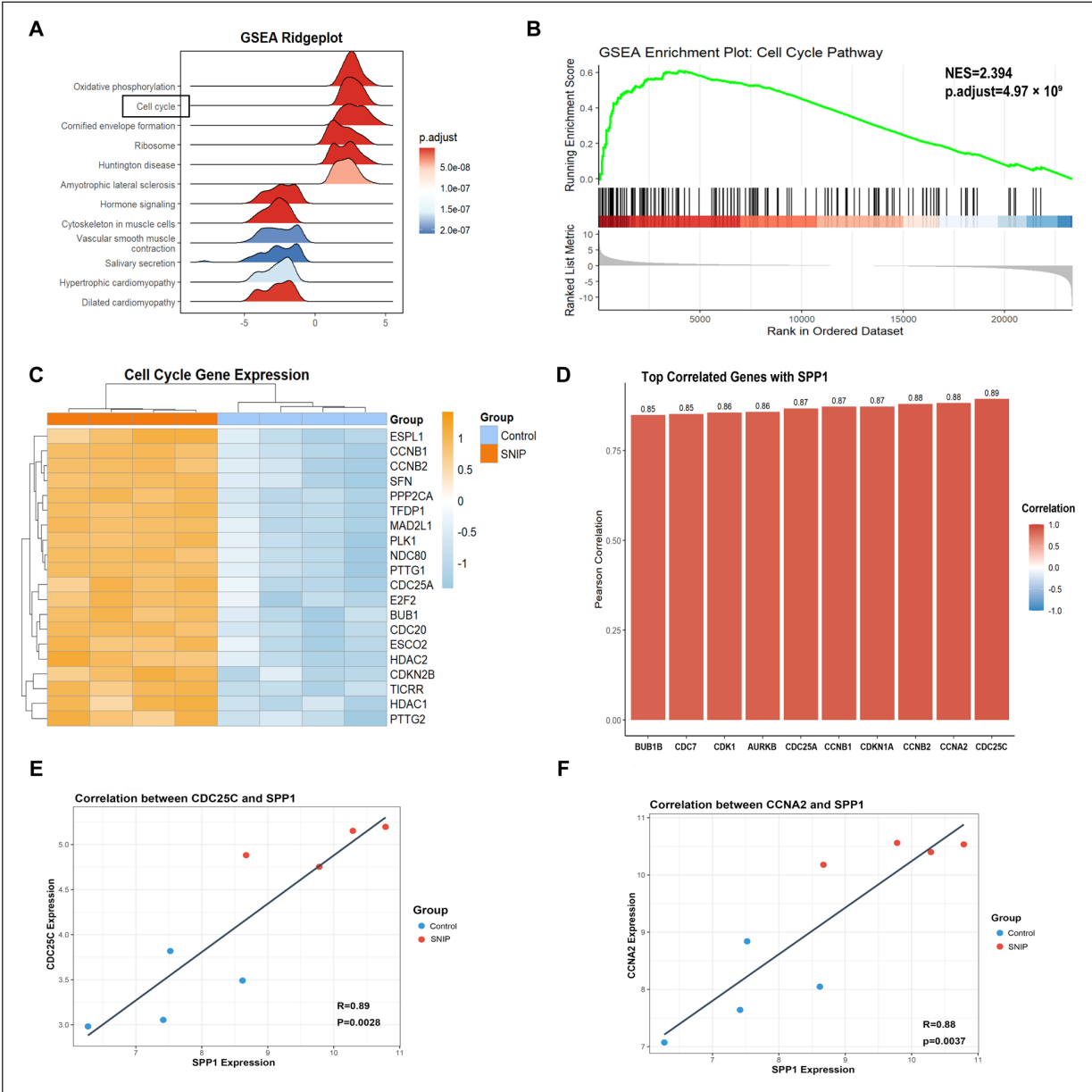


Figure 2. Bioinformatics analysis shows enrichment of cell cycle and association with SPPI expression: (a) ridge plot showing the top 12 KEGG pathways significantly enriched in SNIP, as identified by GSEA, (b) GSEA enrichment plot for the KEGG cell cycle pathway, showing significant positive enrichment in SNIP tissues, (c) heatmap illustrating the top 20 core genes enriched in the cell cycle pathway based on GSEA results in SNIP tissues, (d) Pearson correlation analysis between SPPI expression levels and the expression levels of cell cycle-related genes, and (e, f) scatter plots depicting correlations between SPPI expression and CDC25C (e) and CCNA2 (f) expression levels. CCNA2: cyclin A2; CDC25C: cell division cycle 25C; GSEA: gene set enrichment analysis; ns: not statistically significant; SNIP: sinonasal inverted papilloma; SPPI: secreted phosphoprotein 1; NES: normalized enrichment score.

cycle pathway in SNIP, with cell cycle-related genes preferentially distributed at the leading edge of the ranked gene list (NES=2.394, adjusted $p=4.97 \times 10^{-9}$; Figure 2(b)). To further investigate whether cell cycle activation was associated with SPP1 expression, additional analyses centred on SPP1 were performed. GSEA based on genes ranked according to their correlation with SPP1 expression revealed significant enrichment of the KEGG cell cycle pathway (NES=3.18, adjusted $p=1 \times 10^{-10}$; Supplementary Figure S1(B)). In addition, ssGSEA analysis showed significantly higher cell cycle pathway activity in the SPP1-high group compared with the SPP1-low group (Supplementary Figure S1(C)).

The top 20 core genes contributing to cell cycle pathway enrichment were subsequently visualised using heatmap analysis, revealing distinct expression patterns between SNIP and control tissues (Figure 2(c)). To further examine the association between SPP1 and cell cycle-related molecules, correlation analyses were performed based on mRNA expression levels using all samples in the GSE193016 dataset. SPP1 expression showed positive associations with multiple cell cycle-related genes (Figure 2(d)). Among the SPP1-correlated cell cycle genes, CDC25C and CCNA2 were selected as representative regulators of cell cycle progression for further analysis. Additional correlation analyses confirmed significant positive associations between SPP1 expression and CDC25C and CCNA2 expression levels (Figure 2(e) and (f)). Given that SPP1 is a secreted mediator implicated in inflammatory regulation, we further explored whether SPP1 expression was associated with differences in the immune microenvironment of SNIP tissues. ssGSEA-based immune infiltration analysis suggested that neutrophil scores were higher in the SPP1-high group, whereas NK cell infiltration tended to be lower compared with the SPP1-low group (Supplementary Figure S1(D)).

Altered SPP1 expression is associated with cell cycle-related molecular changes and proliferative phenotypes

To examine the association between SPP1 expression levels and cell cycle regulation as well as cellular proliferation, HNEpC cells with SPP1 overexpression (SPP1_OE) or SPP1 knockdown (SPP1_KD) were established. qPCR analysis showed that, compared with control cells, SPP1 overexpression was accompanied by increased mRNA levels of the cell cycle-related genes CDC25C and CCNA2, whereas reduced expression of these genes was observed following SPP1 knockdown (Figure 3(a)). Western blotting further showed that the protein expression patterns of CDC25C and CCNA2 were consistent with the observed mRNA alterations (Figure 3(b)).

Immunofluorescence staining was conducted to evaluate the expression of the proliferation marker Ki-67. The proportion of Ki-67-positive cells increased in the SPP1 overexpression group and decreased in the SPP1 knockdown group (Figure 3(c) and (d)). In addition, CCK-8 assays

demonstrated enhanced proliferative activity in SPP1-overexpressing cells compared with control cells at 24, 48, and 72 h, whereas reduced proliferation was observed following SPP1 knockdown (Figure 3(e)). Collectively, these findings indicate that altered SPP1 expression is closely associated with changes in cell cycle-related gene expression and cellular proliferative capacity.

SPP1 expression is associated with PI3K/AKT pathway activation and cell cycle-related proliferative changes

Given the established role of the PI3K/AKT signalling pathway in cell cycle regulation, we next examined whether alterations in SPP1 expression were associated with changes in PI3K/AKT pathway activity. Western blot analysis revealed that SPP1 knockdown markedly reduced PI3K and AKT phosphorylation, whereas total PI3K and AKT levels remained unchanged (Figure 4(a)), suggesting an association between SPP1 expression and PI3K/AKT pathway activation.

Consistent with these findings, qPCR analysis demonstrated that SPP1 overexpression was accompanied by increased mRNA expression of the cell cycle-related genes CDC25C and CCNA2. Importantly, treatment with the PI3K inhibitor Pilaralisib attenuated the upregulation of these genes induced by SPP1 overexpression (Figure 4(b)). Similar patterns were observed at the protein level, as Western blot analysis confirmed that Pilaralisib partially reversed SPP1-associated increases in CDC25C and CCNA2 expression (Figure 4(c)).

To further evaluate the functional relevance of PI3K/AKT pathway activation, cellular proliferation was assessed using Ki-67 immunofluorescence staining. SPP1 overexpression was associated with an increased proportion of Ki-67-positive cells, whereas Pilaralisib treatment significantly diminished this effect (Figure 4(d) and (e)). In line with these observations, CCK-8 assays revealed enhanced proliferative capacity in SPP1-overexpressing cells over time, which was partially suppressed following PI3K inhibition (Figure 4(f)).

Collectively, these results demonstrate that SPP1-associated activation of the PI3K/AKT signalling pathway is accompanied by increased expression of key cell cycle regulators and enhanced cellular proliferative capacity.

CDC25C and CCNA2 are highly expressed in SNIP and are associated with disease severity and SPP1 expression

To further support the in vitro findings, the expression of the cell cycle-related molecules CDC25C and CCNA2 was examined in clinical SNIP specimens. qPCR analysis demonstrated significantly increased mRNA expression levels of CDC25C and CCNA2 in SNIP tissues compared with control tissues (Figure 5(a) and (b)). Stratified analysis according to the Krouse staging system revealed higher expression levels of CDC25C and CCNA2 in advanced-stage SNIP tissues (T3–T4) relative to early-stage cases

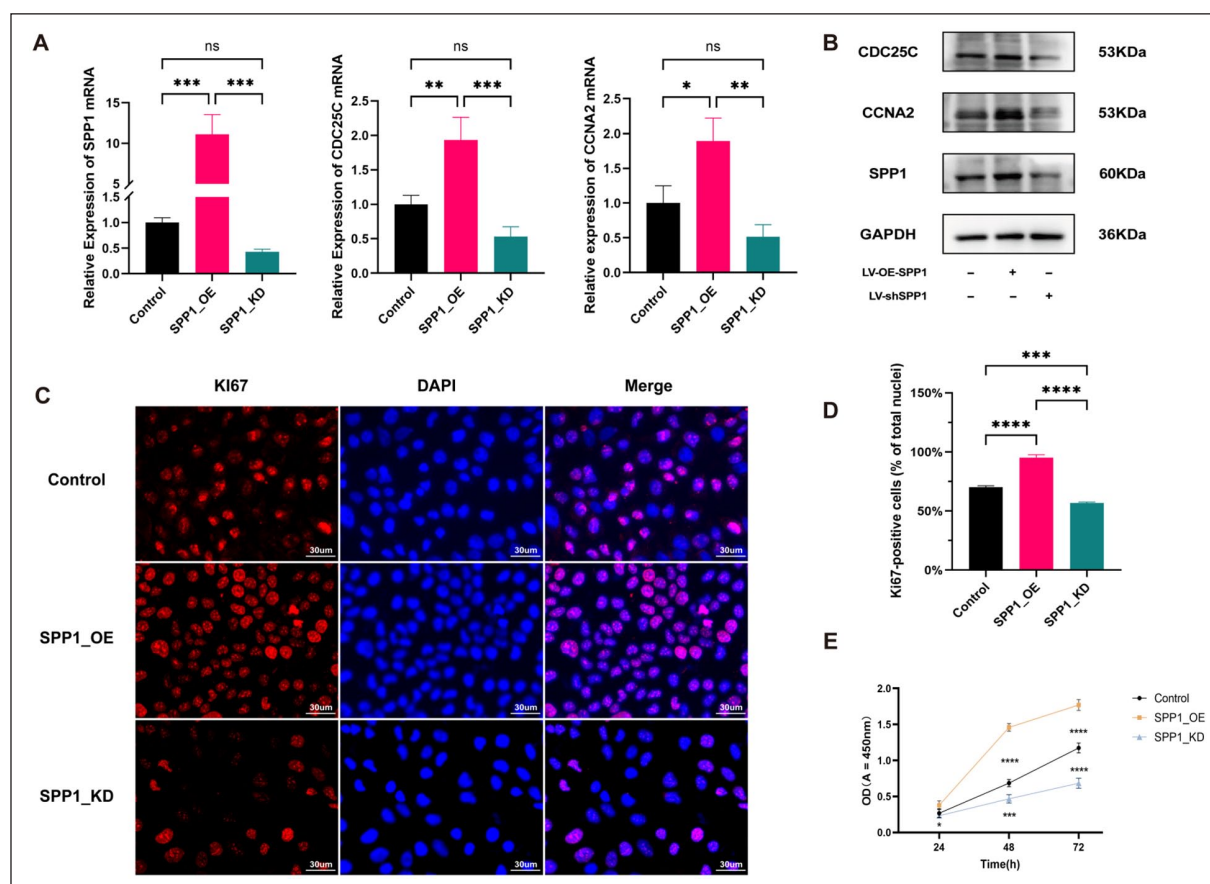


Figure 3. SPP1 expression is associated with CDC25C/CCNA2 expression and proliferative capacity: (a) quantitative PCR analysis of CDC25C and CCNA2 mRNA expression levels in control, SPP1-overexpressing, and SPP1-knockdown cells, (b) Western blot analysis of CDC25C and CCNA2 protein expression under different SPP1 expression conditions, (c) representative immunofluorescence images of Ki-67 staining showing proliferative status in each group, (d) quantitative analysis of the proportion of Ki-67-positive cells based on immunofluorescence staining, and (e) CCK-8 assay assessing cell proliferation in control, SPP1-overexpressing, and SPP1-knockdown groups.

Data are presented as mean $\pm$ SD and analysed by ANOVA. All experiments were independently repeated three times.

ANOVA: analysis of variance; CCK-8: Cell Counting Kit-8; ns: not statistically significant; SD: standard deviation; SPP1: secreted phosphoprotein 1.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

(T1–T2; Figure 5(c) and (d)). Correlation analyses based on mRNA expression in SNIP samples further revealed positive associations between SPP1 and CDC25C and CCNA2 expression levels (Figure 5(h) and (i)).

At the protein level, immunohistochemical staining confirmed higher expression of CDC25C and CCNA2 in SNIP tissues compared with control tissues (Figure 5(e)–(g)). Collectively, these clinical findings indicate that CDC25C and CCNA2 are highly expressed in SNIP and exhibit expression patterns that are associated with disease stage and SPP1 expression levels.

Discussion

This study systematically characterised the expression profile of SPP1 in SNIP and explored its potential association with signalling pathways related to epithelial cell proliferation. By integrating public database analyses, bioinformatics

approaches, in vitro functional assays, and validation using clinical tissue specimens, we provide multi-level evidence demonstrating that SPP1 is highly expressed in SNIP tissues, and that its expression shows a close association with disease stage and postoperative recurrence. In addition, alterations in SPP1 expression were accompanied by corresponding changes in cell cycle-related molecules and epithelial proliferative phenotypes, suggesting that SPP1 may participate in the regulation of SNIP biological behaviour.

SPP1 is recognised as a regulator of inflammatory and immune responses and has been reported to contribute to tumour growth, invasion, and microenvironmental remodelling across multiple malignancies.^{25,26} In SNIP, previous studies have also observed elevated SPP1 expression and a positive correlation with clinical stage.¹⁹ Liu et al. previously reported increased SPP1 expression in SNIP tissues and its association with clinical stage. However, that study primarily focussed on describing the expression pattern of SPP1. In

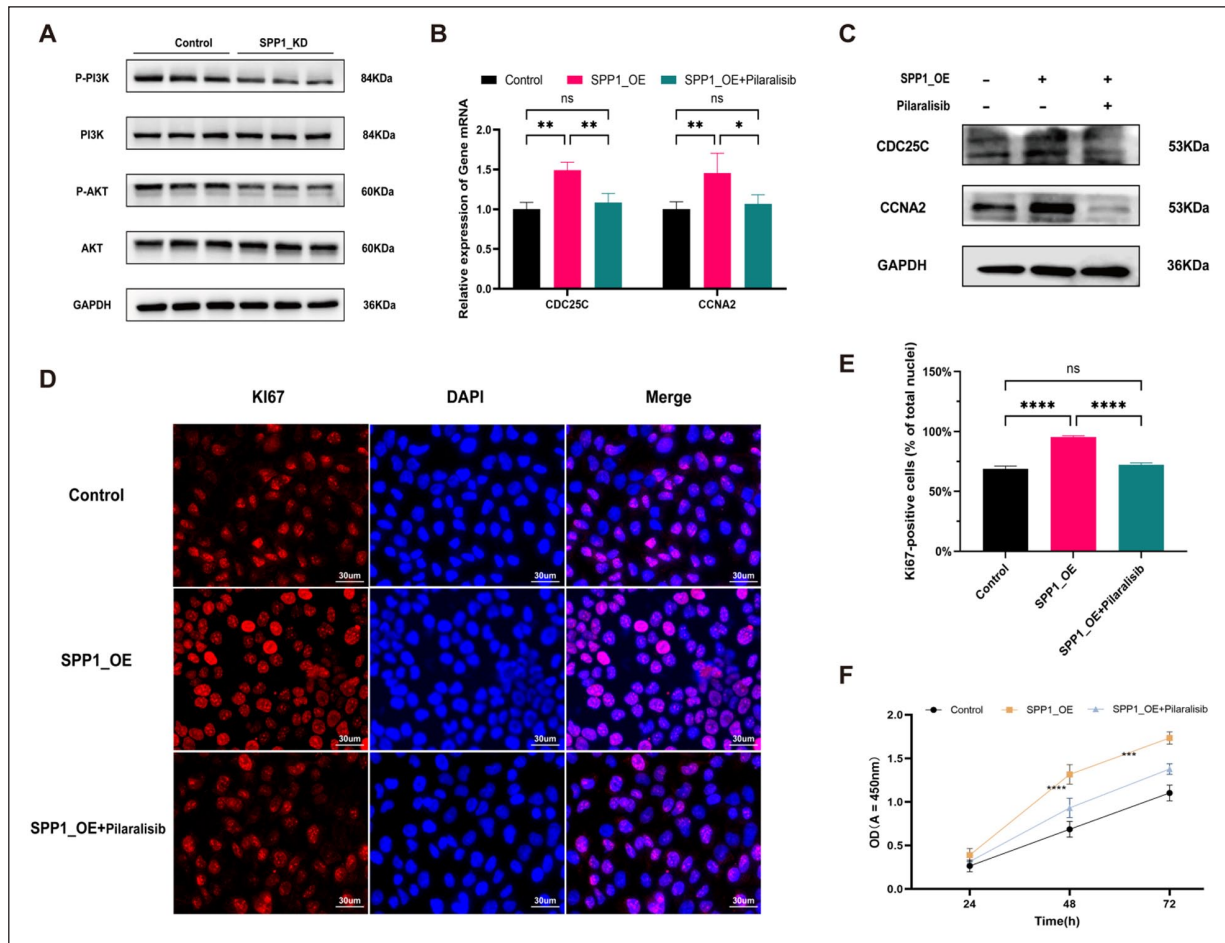


Figure 4. PI3K/AKT activation associates with CDC25C/CCNA2 expression and proliferation: (a) Western blot analysis of phosphorylated and total PI3K and AKT protein levels in control cells and SPP1-knockdown cells, (b) quantitative PCR analysis of mRNA expression levels of the cell cycle-related genes CDC25C and CCNA2 in control cells, SPP1-overexpressing cells, and SPP1-overexpressing cells treated with the PI3K inhibitor Piliaralisib, (c) Western blot analysis of CDC25C and CCNA2 protein expression under the indicated experimental conditions, (d) representative immunofluorescence images of Ki-67 staining in control cells, SPP1-overexpressing cells, and SPP1-overexpressing cells treated with Piliaralisib, (e) quantitative analysis of the proportion of Ki-67-positive cells based on immunofluorescence staining, and (f) CCK-8 assay assessing cell proliferation in the indicated cell groups at different time points. Data are presented as mean $\pm$ SD and analysed by ANOVA. All experiments were independently repeated three times. ANOVA: analysis of variance; CCK-8: Cell Counting Kit-8; CCNA2: cyclin A2; CDC25C: cell division cycle 25C; ns: not statistically significant; SD: standard deviation; SPP1: secreted phosphoprotein 1. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.0001$.

contrast, the present study extends these findings by integrating bioinformatics analysis, in vitro functional experiments, and clinical validation to explore the potential role of SPP1 in epithelial proliferation and its association with postoperative recurrence. Building upon these observations, the present study further confirms sustained overexpression of SPP1 in SNIP tissues, with progressively higher levels detected in advanced disease stages. Notably, high SPP1 expression was associated with an increased risk of postoperative recurrence, and elevated SPP1 protein levels were detected in nasal secretions from patients with recurrent SNIP. These findings suggest that SPP1 expression may reflect disease activity during clinical progression and recurrence, providing new molecular insight into the aggressive biological behaviour and recurrence propensity of SNIP.

At the mechanistic level, cell cycle-related pathways were significantly enriched in SNIP tissues, with key regulatory molecules such as CDC25C and CCNA2 exhibiting higher expression in advanced-stage lesions. Given the limited sample size of currently available SNIP transcriptomic datasets, the bioinformatics analyses in this study were primarily intended to provide exploratory, hypothesis-generating insights rather than definitive conclusions. Accordingly, the enrichment results were interpreted with caution and were further supported by validation in an independent clinical cohort and complementary in vitro experiments, thereby strengthening the overall biological relevance of the findings. Dysregulation of cell cycle control represents a fundamental mechanism underlying abnormal cellular proliferation,²⁷ and therapeutic strategies targeting cell cycle processes have

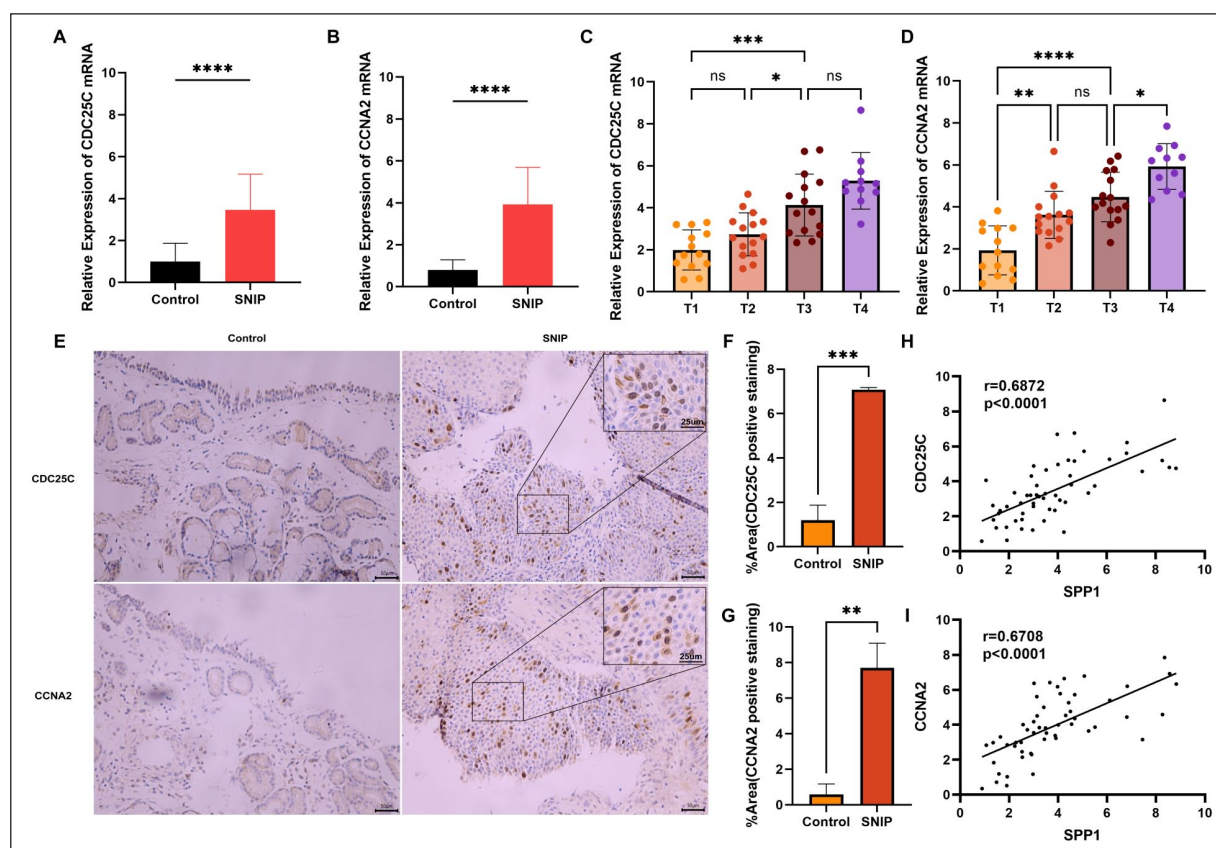


Figure 5. Elevated CDC25C/CCNA2 in SNIP associate with disease stage and SPP1 expression: (a, b) quantitative PCR analysis of CDC25C (a) and CCNA2 (b) mRNA expression levels in control and SNIP tissues, (c, d) CDC25C (c) and CCNA2 (d) mRNA expression levels in SNIP tissues stratified by Krouse stage (T1–T4), (e) representative immunohistochemical staining images showing CDC25C and CCNA2 expression in control and SNIP tissues, (f, g) semi-quantitative analysis of CDC25C (f) and CCNA2 (g) immunopositive area, expressed as the %Area, and (h, i) Pearson correlation analysis between SPP1 expression levels and CDC25C (h) and CCNA2 (i) mRNA expression levels in clinical SNIP samples. For panels (a, b) and (f, g), statistical analyses were performed using independent-samples *t*-tests; for panels (c, d), ANOVA was applied. Data are presented as mean $\pm$ SD. ANOVA: analysis of variance; CCNA2: cyclin A2; CDC25C: cell division cycle 25C; ns: not statistically significant; SD: standard deviation; SNIP: sinonasal inverted papilloma; SPP1: secreted phosphoprotein 1; %Area: percentage of positive area. **p* < 0.05. ***p* < 0.01. ****p* < 0.001. *****p* < 0.0001.

demonstrated potential clinical value across multiple tumour types.^{28,29} Consistent with these concepts, our *in vitro* findings showed that modulation of SPP1 expression was accompanied by corresponding changes in CDC25C and CCNA2 expression, together with altered proliferative capacity in nasal epithelial cells. These results support a close association between SPP1 expression and aberrant regulation of cell cycle-related molecules, suggesting that SPP1 may contribute to abnormal epithelial proliferation in SNIP through modulation of the cell cycle regulatory network.

In granulosa cell models, elevated SPP1 expression activates PI3K/AKT signalling, promoting cell proliferation while inhibiting apoptosis.³⁰ In addition, SPP1 can interact with cell surface receptors such as integrins or CD44, triggering downstream signalling cascades including PI3K/AKT that are involved in proliferation-related molecular events.^{31,32} In the present study, changes in SPP1 expression were accompanied by corresponding alterations in PI3K and AKT phosphorylation status, and pharmacological inhibition

of the PI3K pathway using Pilaralisib partially reversed SPP1-associated upregulation of cell cycle regulators (CDC25C and CCNA2) as well as proliferative phenotypes. From a functional perspective, these findings support an important role for PI3K/AKT signalling in SPP1-associated proliferative regulation. Taken together, PI3K/AKT signalling may serve as a key signal integration node in SPP1-mediated cell cycle regulation, providing a molecular basis for the abnormal activation of cell cycle-related molecules observed in SNIP. However, it should be emphasised that PI3K/AKT signalling is unlikely to represent the sole downstream mechanism of SPP1 action. As a multifunctional secreted protein, SPP1 may simultaneously influence epithelial proliferation through multiple signalling networks. Our findings indicate that PI3K/AKT signalling plays an important supportive role in SPP1-associated epithelial proliferation in SNIP, while the precise downstream molecular events and their coordination with other signalling axes remain to be further elucidated. In addition, exploratory immune

infiltration analysis suggested that SPP1-high SNIP tissues might exhibit altered immune cell composition, including increased neutrophil scores and reduced NK cell infiltration. These observations are consistent with the known role of SPP1 as a secreted mediator involved in inflammatory regulation and may indicate a potential link between SPP1-associated epithelial proliferation and the inflammatory microenvironment in SNIP.

Despite the relatively comprehensive analytical strategy employed in this study, several limitations should be acknowledged. First, the *in vivo* effects of SPP1 on epithelial proliferation and cell cycle regulation were not evaluated using animal models. Second, although exploratory immune infiltration analysis suggested potential differences in immune cell composition between SPP1-high and SPP1-low tissues, SNIP is characterised by a complex inflammatory microenvironment, and further validation using larger cohorts and tissue-based analyses will be required to clarify the immunological role of SPP1 in SNIP. Furthermore, the transcriptomic analysis was based on the GSE193016 dataset, which contains a relatively small number of samples (four SNIP tissues and four control tissues). Therefore, the bioinformatics analysis in this study should be considered exploratory and hypothesis-generating. In addition, because this study was retrospective and based on available clinical specimens collected during the study period, a formal sample size calculation was not performed prior to data collection. Future studies incorporating larger clinical cohorts, primary cell models, and tissue-based or *in vivo* approaches will be valuable for further elucidating the molecular mechanisms and clinical significance of SPP1 in SNIP.

In conclusion, this study provides integrated clinical, molecular, and functional evidence linking SPP1 expression with epithelial proliferative features in SNIP. By placing SPP1 within the broader framework of cell cycle regulation and multi-pathway signalling networks, our findings offer new insight into the biological behaviour of SNIP and establish an experimental foundation for future mechanistic investigations and potential translational exploration.

Conclusion

This study demonstrates that SPP1 is highly expressed in SNIP, with elevated levels observed in advanced-stage disease. Increased SPP1 expression was associated with a higher rate of postoperative recurrence. In addition, aberrant activation of cell cycle-related pathways was identified in SNIP, accompanied by consistent associations between disease severity and the expression of key regulators, including CDC25C and CCNA2. Functional analyses further showed that modulation of SPP1 expression was accompanied by corresponding alterations in PI3K/AKT signalling activity and in CDC25C and CCNA2 expression *in vitro*, together with changes consistent with a proliferative cellular phenotype. Overall, these findings suggest that SPP1 is closely linked to cell cycle dysregulation and epithelial proliferation

in SNIP and provide a rationale for further investigation of its role in disease development and progression.

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Ethical considerations

This study was approved by the Ethics Committee of Qingdao University Affiliated Hospital (no. QYFYWZLL28986). This study was conducted in accordance with the Declaration of Helsinki and did not involve any interventional clinical trial.

Consent to participate

Written informed consent was obtained from all participants prior to enrolment.

Author contributions

W.T. and L.W. conceived and designed the study. W.T., L.W., and X.Y. performed the experiments. J.Z., L.Y., and H.C. collected clinical samples and acquired the data. C.Z., Z.D., and Q.Z. performed data analysis and interpretation. L.H. and Y.J. provided critical intellectual input and supervised the study. W.T. and L.W. drafted the article. L.H. and Y.J. revised the article critically for important intellectual content. All authors read and approved the final article.

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Data availability statement

The GSE193016 dataset was downloaded from the Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193016>). All data generated during this study are available from the corresponding author on reasonable request.

Supplemental material

Supplemental material for this article is available online.

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