


# Inhibin B suppresses anoikis resistance and migration through the transforming growth factor- $\beta$ signaling pathway in nasopharyngeal carcinoma

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Inhibin B (INHBB), a heterodimer of a common  $\alpha$ -subunit and a  $\beta$ B-subunit, is a glycoprotein belonging to the transforming growth factor- $\beta$  (TGF- $\beta$ ) family. In this study, we observed INHBB expression was reduced in nasopharyngeal carcinoma (NPC) tissues compared to non-tumor nasopharyngeal epithelium tissues, and INHBB was associated with lymph node metastasis, stage of disease, and clinical progress. Positive expression of INHBB in NPC predicted a better prognosis (overall survival,  $P = 0.038$ ). However, the molecular mechanisms of INHBB have not been addressed in NPC. We induced anoikis-resistant cells in NPC cell lines under anchorage-independent conditions, then found epithelial-mesenchymal transition markers changed, cell apoptosis decreased, cell cycle was modified, and invasion strengthened in anoikis-resistant NPC cells. These anoikis-resistant NPC cells showed decreased expression of INHBB compared with adhesion cells. Furthermore, INHBB was found to influence the above-mentioned changes. In the anoikis-resistant NPC cells with INHBB overexpression, apoptotic cells increased, S phase cells weakened, vimentin, matrix metalloproteinase-9, and vascular endothelial growth factor A expression were downregulated, and E-cadherin expression was upregulated, and vice versa in knockdown of INHBB (INHBB shRNA) anoikis-resistant NPC cells. Diminished INHBB expression could activate the TGF- $\beta$  pathway to phosphorylate Smad2/3 and form complexes in the nucleus, which resulted in the above changes. Thus, our results revealed for the first time that INHBB could suppress anoikis resistance and migration of NPC cells by the TGF- $\beta$  signaling pathway, decrease p53 overexpression, and could serve as a potential biomarker for NPC metastasis and prognosis as well as a therapeutic application.

## KEYWORDS

anoikis, inhibin B, metastasis, nasopharyngeal carcinoma, TGF- $\beta$

**Abbreviations:** ActRII, type II activin receptor; EBV, Epstein-Barr virus; EMT, epithelial-mesenchymal transition; IHC, immunohistochemistry; INHB, inhibin; INHBB, inhibin B; MMP, matrix metalloproteinase; NPC, nasopharyngeal carcinoma; rAd-p53, human adenovirus p53; TGF- $\beta$ , transforming growth factor- $\beta$ ; TMA, tissue microarray; VEGF-A, vascular endothelial growth factor-A.

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## 1 | INTRODUCTION

Nasopharyngeal carcinoma is a head and neck cancer derived from the epithelium of the nasopharynx, which is associated with EBV infection and is highly invasive and metastatic. The NPC population is mainly distributed in Southeast Asia, especially in southern China. Although treatment outcomes have improved with technological developments, metastasis and recurrence are still the main causes of death. Therefore, it is crucial to clarify the mechanisms of NPC metastasis. It has been shown that anoikis resistance was involved in NPC metastasis, and reducing the anoikis-resistant capacity of NPC cells could reverse their invasive properties.<sup>1</sup>

Anoikis is a specific programmed cell death from the ECM, which is conducive to physical development and balance. It is the primary condition of invasion and metastasis for the characteristics of anoikis resistance in tumor cells, attributed to changes in cell polarity<sup>2</sup> and the induction of EMT.<sup>3</sup> Anoikis inhibition confers a re-adhesion ability to tumor cells in heterogeneous tissues and organs, therefore, anoikis is considered to be a prerequisite for survival of metastatic tumor cells in the circulation system.<sup>4,5</sup> As one of the highly metastatic tumors, NPC reportedly has anoikis resistance.<sup>6</sup> Manganese superoxide dismutase could regulate the anoikis resistance and tumor metastasis in NPC,<sup>7</sup> however, the related mechanisms have rarely been addressed.

Inhibin, a heterodimer formed by a disulfide linkage of an  $\alpha$ -subunit and a  $\beta$ -subunit, is a kind of glycoprotein hormone that is mainly secreted by ovarian granulosa cells and testicular Sertoli cells. Inhibin belongs to member of the TGF- $\beta$  family. Inhibin B, which is mainly composed of  $\alpha$ -subunits and  $\beta$ B-subunits and has a  $32 \times 10^3$  relative molecular weight, suppresses the secretion of the pituitary follicle-stimulating hormone by the negative feedback mechanism, and regulates the generation of follicle. The knockdown INHBB granule cells upregulated cell cycle protein D1, E, and Bcl2 expression and downregulated Bax expression, which could promote cell apoptosis and block the cell cycle in the G<sub>1</sub> phase.<sup>8</sup> In recent studies, it has been reported to affect the development and prognosis of ovarian cancer,<sup>9</sup> colorectal cancer,<sup>10</sup> and breast cancer<sup>11</sup> because INHBB influences DNA synthesis.

Our pilot studies of preliminary experiments showed that INHBB mRNA presented the negative signal in major NPC tissues, INHBB expression was downregulated in NPC tissues. In addition, INHBB was negatively correlated with lymph node metastasis, histologic type, clinical progression, tumor size, and invasive range. Studies showed that the loss of inhibin responsiveness generated stronger aggressive tumorigenicity of ovarian cancer cells<sup>12</sup> and inhibins were tumor suppressors in peripheral tissues.<sup>13</sup>

As far as we are aware, there has been no extensive research involving the association between INHBB and the clinical outcomes of NPC patients, and the correlative mechanisms. Therefore, we used NPC TMAs to examine the correlation of INHBB expression and clinical prognosis of NPC. In this study, we induced the NPC cell lines HNE1 and 5-8F into anoikis-resistant cells, further influenced

HNE1 anoikis-resistant cells with INHBB overexpression and INHBB knockdown in 5-8F anoikis-resistant cells. We found that INHBB could inhibit anti-anoikis and metastasis in NPC.

## 2 | MATERIALS AND METHODS

### 2.1 | Samples for TMA

All the samples were obtained from January 2002 to October 2004 from the Department of Otolaryngology, Xiangya Hospital of Central South University (Changsha, China). The patients' mean age was 48 years (range, 13-78 years). All subjects provided their informed consent. The specimens were fixed with 10% polyformaldehyde, which were made into paraffin samples. After pathological diagnosis, the selected tissue samples were prepared for the examination of the TMA of NPC. The TMA of different stages of NPC was constructed according to our previous study.<sup>14,15</sup> Immunohistochemistry was carried out using a standard procedure as previously described.<sup>15</sup> Inhibin B was detected using rabbit polyclonal antibody at a 1:100 dilution. The results were observed by optical microscope (E600; Nikon, Tokyo, Japan). The tissues included 141 cases of normal and chronic inflammation of nasopharyngeal epithelium, 98 cases of dysplastic nasopharyngeal epithelium, 88 cases of adjacent epithelium of NPC, and 434 cases of NPC. The research methods conformed to the standards stipulated in the Declaration of Helsinki and were approved by the Xiangya Hospital Ethics Committee of Central South University.

### 2.2 | Cell culture and reagents

Two highly metastatic NPC cell lines, HNE1 and 5-8F, were supplied by the Central South University Advanced Research Center. The NPC cell lines were cultured in medium containing 15% FBS (Gibco, Rio De Janeiro, Brazil), RPMI-1640 medium (Gibco, Beijing, China), and 100 U/mL penicillin-streptomycin (Gibco, Carlsbad, CA, USA), in 5% CO<sub>2</sub> and 95% O<sub>2</sub> in a 37°C culture incubator (Thermo Fisher Scientific, Marietta, Ohio, USA). Cell growth covered 70%-80% of the area, digested by 0.25% trypsin-EDTA (Gibco, Carlsbad, CA, USA), and the culture was transferred every 24 hours. The RIPA lysis buffer and BCA protein quantitation assay kit were purchased from KeyGEN BioTECH (Nanjing, China).

### 2.3 | Preparation for anoikis-resistant culture plates

Ten grams of poly-(2-hydroxyethyl methacrylate) HEMA powder (25249-16-5; Sigma, St. Louis, MO, USA) was dissolved in 100 mL of 95% alcohol (100 mg/mL), vibrated until completely dissolved, then diluted four times into 25 mg/mL with 95% alcohol. One milliliter per well of liquid was coated for a 6-well plate, 0.5 mL/well was coated for a 24-well plate, and 100  $\mu$ L/well was coated for a 96-well plate. Package plates were placed at room temperature in a bio-safety cabinet, dried for 24 hours, and irradiated by UV radiation before use.

## 2.4 | Choosing anoikis-resistant NPC cells

The logarithmic phase HNE1 and 5-8F NPC cells (adhesion culture, abbreviated as HNE1<sup>ad</sup> and 5-8F<sup>ad</sup>) were added ( $5 \times 10^5$ /well) to a 96-well anoikis-resistant culture plate, and suspended to culture with 2% FBS for 0, 24, 48, or 72 hours. For MTT assay (analysis 15 wells for every point): 20  $\mu$ L MTT reagent was added per well, 1.5 mL of Eppendorf tube of culture liquid was collected after 4 hours, centrifuged for 10 minutes (62 g), supernatant was discarded, 100  $\mu$ L DMSO reagent was added to every well, shaken at low speed for 10 minutes (30°C, 2.1 g), and the absorbance value of every well was measured by a microplate reader at 490 nm. The percentage of cells was defined as the ratio of the mean absorbance value at every time point and the value at 0 hour. We chose the time of non-shift percentage of cells as the induction time of anoikis-resistant cells. These cells were anoikis-resistant (suspension culture, abbreviated as HNE1<sup>sus</sup> and 5-8F<sup>sus</sup>). The anoikis-resistant cells were recultured in adhesion conditions for 24 hours, and re-adhesion of anoikis-resistant cells was obtained (re-adhesion culture, abbreviated as HNE1<sup>re-ad</sup> and 5-8F<sup>re-ad</sup>). Morphological changes were observed under an inverted microscope.

## 2.5 | Cell transfection

The transfection plasmids, INHBB overexpression and its control vector, INHBB shRNA and its negative control, were obtained from Shanghai Genechem (Shanghai, China). HNE1 and 5-8F adhesion cells, anoikis-resistant cells and re-adhesion anoikis-resistant cells were seeded in a 6-well plate, and when cells reached 80% density, the recombinant plasmid with Lipofectamine 2000 was transfected. The experimental cells were divided into three groups: normal control group (no intervention treatment), overexpression and negative control group transfection (INHBB/Vector), and interference and negative control group (INHBB shRNA/NC shRNA).

## 2.6 | Detection of cell apoptosis and cell cycle by flow cytometry

The experimental cells were cultured with free-serum culture medium for 12 hours, and the cell growth cycle was synchronized. We adjusted the cell number of single-cell suspension to  $1-5 \times 10^6$ /mL. After centrifugation, the supernatant liquid was discarded, and cells were stained with propidium iodide (PI). Cell apoptosis and the cell cycle were measured by flow cytometry (NovoCyte, San Diego, CA, USA). All measurements were carried out in triplicate. The results were analyzed with NovoExpress 1.1.0 software (NovoCyte, San Diego, CA, USA).

## 2.7 | Migration and invasion assay

Wound-healing assay was used to assess migration. HNE1 and 5-8F cells were treated with INHBB as described above. The cells were seeded in 6-well plates and cultured under permissive conditions until 90% confluence, were removed by trypsinization, and counted at  $4 \times 10^5$  cells/well. The wells were coated with confluent monolayers

of cells for wounding. Wounds were made using a pipette tip, and photographs taken immediately (time zero) and 24 or 48 hours after wounding. The distance migrated by the cell monolayer was measured. Experiments were repeated a minimum of 3 times.

The invasion assay was carried out using a Boyden chamber (Corning, Tewksbury, MA, USA) assay, which measures the ability of cells to invade a Matrigel matrix (BD, Franklin lake, NJ, USA) overlying a membrane containing 8- $\mu$ m pores. The above treated cells with INHBB were added to the bottom chamber. After 48 hours cultivation, the chambers were fixed with methanol and acetone, stained with hematoxylin. The number of cells was enumerated at 100 $\times$  magnification for each filter and decolorized to detect OD value for three times, the data are presented as the mean  $\pm$  SD.

## 2.8 | Western blot analysis

The experimental cells of the same growth time and good condition were collected. After washing with ice-cold PBS, cells were added to the RIPA lysis buffer for 20 minutes. After centrifuged (13 188 g) with 4°C, protein was determined using a BCA protein assay kit. Western blotting was carried out according to standard protocol. The positive stripe was analyzed by Gel Pro 4.0 optical density analysis software (Media Cybernetics, Rockville, MD, USA), and the accumulated optical density reference value of integrated optical density was measured. The following antibodies were used in this study: anti-INHBB (ab69286; Abcam, Cambridge, UK), anti-E-cadherin (YT1454; ImmunoWay, Plano, TX, USA), anti-vimentin (YT4880; ImmunoWay), anti-VEGF-A (YT5108; ImmunoWay), anti-MMP-9 (YT1892; ImmunoWay), anti-Smad2/3 (YT4332; ImmunoWay), anti-Smad4 (YT4337; ImmunoWay), anti-p-Smad2/3(T8) (YPO362; ImmunoWay), anti-TGF- $\beta$ 1 (YT4632; ImmunoWay), anti- $\beta$ -actin (20270; ProMab, Richmond, CA, USA), anti-p53 (10442-1-AP; Proteintech, Rosemont, IL, USA).

## 2.9 | Statistical analysis

Data were analyzed with SPSS 16.0 (SPSS, Chicago, IL, USA). Student's *t* test was applied for comparison of the differences between two groups, one-way ANOVA was used to evaluate multiple groups, the clinicopathological parameters were analyzed by the  $\chi^2$  test or Fisher's exact test, event-free survival and overall survival were plotted by Kaplan-Meier survival curves, and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).  $P \leq 0.05$  indicated statistical significance.

## 3 | RESULTS

### 3.1 | Inhibin B downregulated in nasopharyngeal carcinoma tissues and associates with clinical prognosis

We previously detected the INHBB expression levels in a TMA using IHC staining (Figures 1A,B, S1). The results showed that INHBB mRNA in nasopharyngeal epithelium tissue with a positive signal was

mainly located in the nucleus. The INHBB expression was positive in epithelium with chronic inflammation and strongly positive in dysplastic nasopharyngeal epithelium, whereas major NPC tissues presented a negative signal. Inhibin B expression was downregulated in NPC tissue (181 positive cases of 356 cases, 50.8%) compared with dysplastic nasopharyngeal epithelium (39 of 53, 73.6%), with statistical significance ( $P < 0.05$ ), and lower expression than that in the adjacent epithelium of NPC (47 of 63, 74.6%).

We analyzed INHBB expression and clinicopathological parameters (Figure 1C,D, Table 1). In clinical stage III and IV, WHO II, and T3 and T4 patients, INHBB expression was lower than in clinical stage I and II, WHO I, and T1 and T2 patients ( $P = 0.000, 0.003$ , and  $0.043$ , respectively). Furthermore, INHBB expression in patients with NPC metastases was significantly decreased compared to those without metastases ( $P = 0.009$ ). In NPC patients, there was an obviously lower overall survival rate in the INHBB-negative group (59.3%) than in the INHBB-positive group (83.3%) ( $P = 0.038$ ).

### 3.2 | Anoikis-resistant cell establishment

The NPC cell lines were cultured in suspension for 24, 48, and 72 hours with 2% FBS medium using a coated poly-HEMA plate. Then the MTT test was undertaken separately. The percentages of HNE1 and 5-8F cells were found to decrease rapidly over time. However, the percentages of cells did not shift after 48 hours (Figure 2A,B). Therefore, these cells of suspension cultured for 48 hours were identified as anoikis-resistant cells (HNE1<sup>SUS</sup> and 5-8F<sup>SUS</sup>). The anoikis-resistant cells were reattached cultures, so re-adhesion of anoikis-resistant cells (HNE1<sup>re-ad</sup> and 5-8F<sup>re-ad</sup>) was obtained. Since then, we have achieved three different cell conditions: adhesion cells, anoikis-resistant cells, and re-adhesion anoikis-resistant cells.

**TABLE 1** Correlations between the expression of inhibin B (INHBB) and the clinicopathological parameters of nasopharyngeal carcinoma tissues

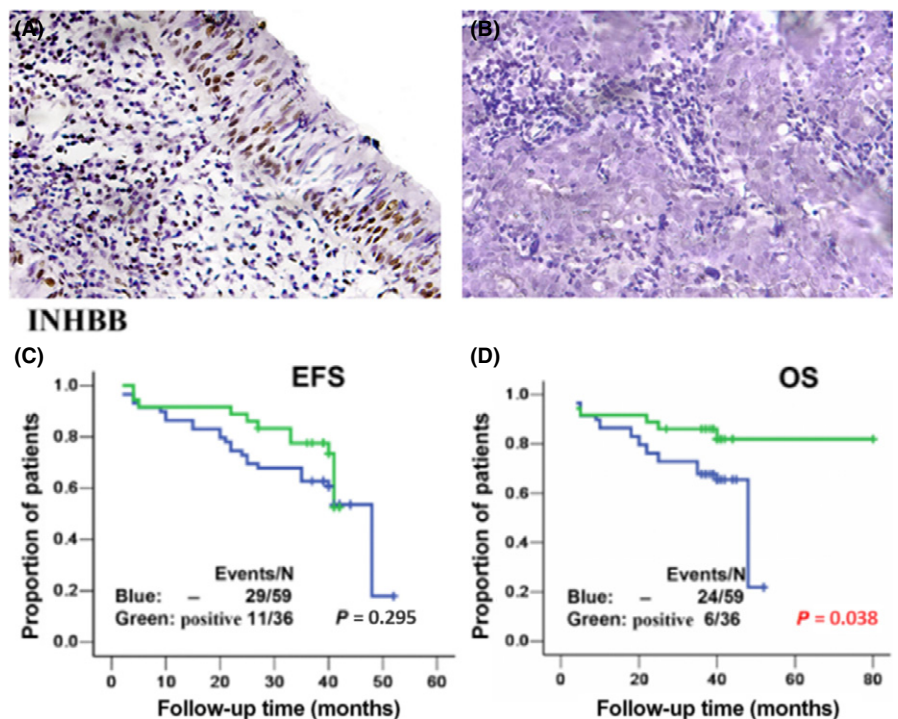
Characteristics	INHBB, +/-	INHBB positive rate, %	P value
Gender			
Male	122/124	49.6	0.483
Female	37/45	45.1	
Metastasis	94/117	44.5	0.009
No metastasis	79/55	59.0	
Stages I + II	63/31	67.0	0.000
Stages III + IV	113/140	44.7	
WHO I-a	13/2	86.7	a-b: 0.003
WHO II-b	152/169	47.4	
WHO III-c	16/4	80.0	b-c: 0.005
T1 + T2	104/92	53.1	0.043
T3 + T4	55/77	41.7	

Fisher's  $\chi^2$  method:  $P < 0.05$  statistically significant.

### 3.3 | Anoikis-resistant cells change EMT markers and the cell cycle

Under the microscope, the suspension cells were clustered, and the boundary was not clear. The re-adhesion anoikis-resistant cells were evacuated and dwindled (Figure 2C). Due to cell deformation, we questioned whether HNE1<sup>SUS</sup> and 5-8F<sup>SUS</sup> possessed EMT characteristics. We found that the epithelial cell markers of E-cadherin disappeared, and EMT marker vimentin increased; however, the HNE1<sup>re-ad</sup> and 5-8F<sup>re-ad</sup> cells had no change (Figure 2D). These results indicated that we had successfully induced anoikis-resistant NPC cell lines.

**FIGURE 1** Inhibin B (INHBB) is downregulated in nasopharyngeal carcinoma (NPC) tissues and is associated with clinical prognosis. A, INHBB protein was localized in the nucleus of columnar epithelial cells (brown denotes a positive signal). B, INHBB staining was negative in the differentiated non-keratinizing NPC. C, D, Kaplan-Meier survival curves in NPC patients with varying INHBB expression. The difference in INHBB expression levels was not statistically significant between the event-free survival (EFS) of the two groups, but was statistically significant between the overall survival (OS) of the two groups. Blue, negative expression; green, positive expression. EFS,  $P = 0.295$ ; OS,  $P = 0.038$ , log-rank test



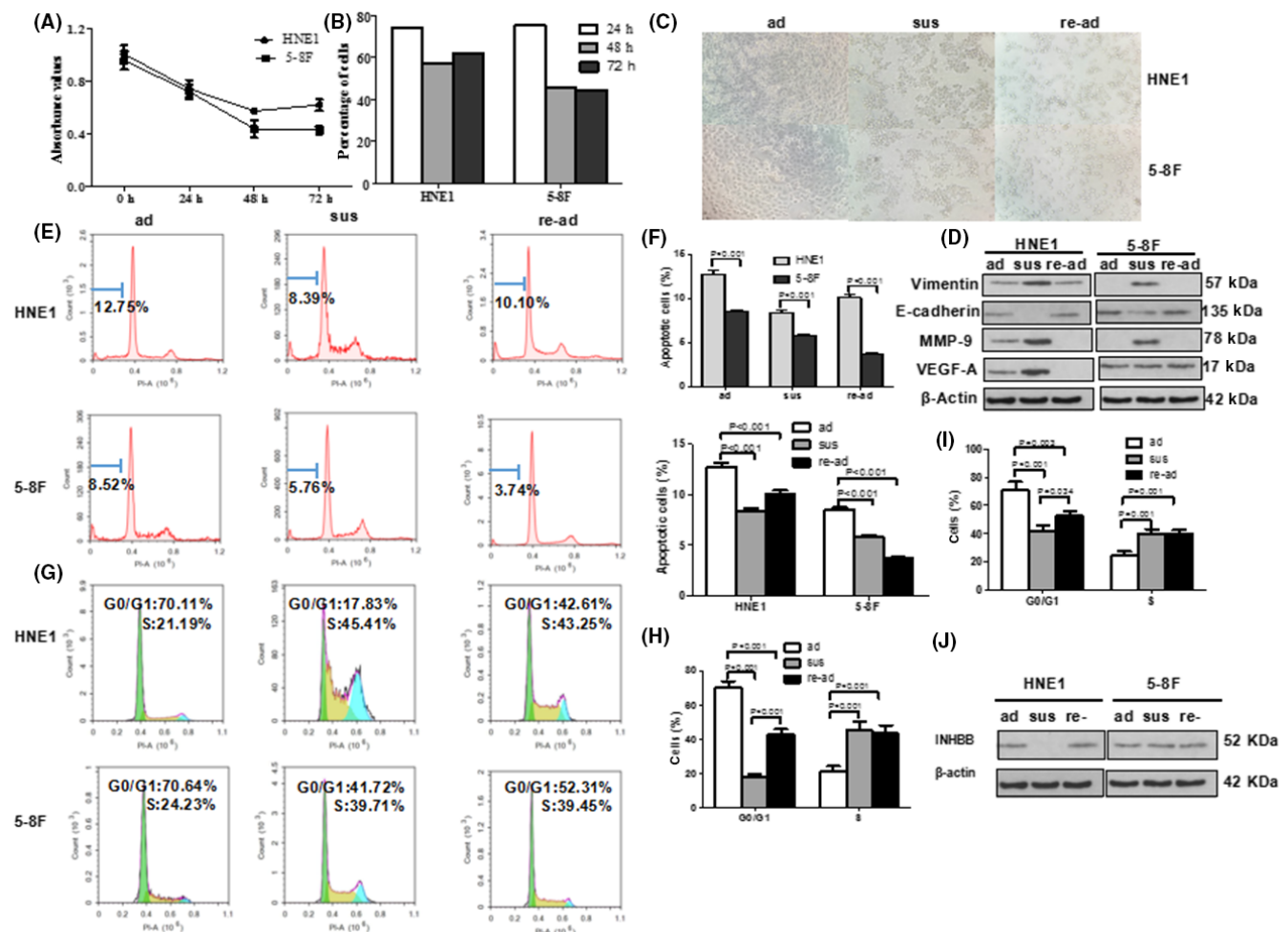
Anoikis-resistant cells enhanced resistance to apoptosis and invasion. Apoptosis detection was carried out in HNE1 and 5-8F cells by flow cytometry. The results showed that HNE1<sup>SUS</sup> and 5-8F<sup>SUS</sup> cell apoptosis ratios decreased (8.39% vs 12.75%, 5.76% vs 8.52%, respectively), and 5-8F<sup>SUS</sup> had stronger anti-apoptotic ability than HNE1<sup>SUS</sup> (Figure 2E,F). Expression of MMP-9 and VEGF in HNE1<sup>SUS</sup> and 5-8F<sup>SUS</sup> cells was upregulated (Figure 2D), which increased the cell invasion capacity. HNE1<sup>re-ad</sup> and 5-8F<sup>re-ad</sup> apoptosis decreased, and the anti-apoptotic ability increased (Figure 2E,F). However, the expression of MMP-9 and VEGF did not increase (Figure 2D), and the cell invasion force did not strengthen.

Cell proliferation is mainly mediated by modification of the cell cycle, so we detected whether anoikis-resistant NPC cells changed the cell cycle. Compared to untreated parental cells, the G<sub>0</sub>/G<sub>1</sub> phase cells of HNE1<sup>SUS</sup> and HNE1<sup>re-ad</sup>, and 5-8F<sup>SUS</sup> and 5-8F<sup>re-ad</sup> cells decreased

(17.83% and 42.61% vs 70.11%; 41.72% and 52.31% vs 70.64%, respectively), while the S phase increased (45.41% and 43.25% vs 21.19%; 39.71% and 39.45% vs 24.23%, respectively) (Figure 2G-I). These results verified that the DNA synthesis of these cells increased, which indicated that anoikis-resistant cells enhanced anti-apoptosis ability, induced DNA synthesis, and promoted proliferation and growth.

### 3.4 | Inhibin B induces apoptosis of anoikis-resistant cells

Results of our preliminary experiments showed that INHBB mRNA presented a negative signal in major NPC tissues, and INHBB expression was lower in NPC tissues. Studies have shown that INHBB has an anticancer effect. This study attempted to reveal whether INHBB can inhibit anoikis-resistant NPC cell growth. The



**FIGURE 2** Establishment of anoikis-resistant nasopharyngeal carcinoma (NPC) cells. A, B, Suspension culture of NPC HNE1 and 5-8F cells over 72 hours of MTT test (A) and showing the number of cells (B). C, HNE1 and 5-8F cell morphology after 48 hours in 3 different culture conditions: adhesion (ad), suspension (sus), and re-adhesion after suspension for 48 hours (re-ad). D, Cell apoptosis flow cytometry analysis. X-axes, propidium iodide (PI)-A staining; Y-axes, cell counts. MMP-9, matrix metalloproteinase-9; VEGF-A, vascular endothelial growth factor-A. E, Apoptotic cell comparison of HNE1 and 5-8F cells, and that of 3 different culture condition cells. F, Epithelial-mesenchymal transition marker changes and migration of 3 different culture condition cells. G, Cell cycle flow cytometry analysis. X-axes, PI-A staining; Y-axes, cell counts. H, G<sub>0</sub>/G<sub>1</sub> and S cell comparison of HNE1 cells in three different culture conditions. HNE1<sup>SUS</sup> and HNE1<sup>re-ad</sup> G<sub>0</sub>/G<sub>1</sub> cells were reduced, and S cells were increased. I, G<sub>0</sub>/G<sub>1</sub> and S cells comparison of 5-8F cells in three different culture conditions. 5-8F<sup>SUS</sup> and 5-8F<sup>re-ad</sup> G<sub>0</sub>/G<sub>1</sub> cells were reduced, and S cells were increased. J, Inhibin B (INHBB) expression for HNE1 and 3 different cultured conditions of 5-8F cells

results of untreated NPC cells showed that the INHBB expression of 5-8F cells was not different from that of HNE1 (Figure 2J). The HNE1 cell line was used for INHBB transfection tests, and the 5-8F cell line was used for shRNA-INHBB transfection tests.

Inhibin B expression was absent in HNE1<sup>SUS</sup> and unchanged in 5-8F<sup>SUS</sup> cells (Figure 2J). We considered that the function change might be related to INHBB. Compared with untreated cells, HNE1<sup>ad</sup> and HNE1<sup>SUS</sup> cells overexpressing INHBB had increased apoptosis sensitivity (33.28% vs 11.69% and 27.60% vs 8.14%). Although HNE1<sup>re-ad</sup> and HNE1<sup>SUS</sup> cells treated with INHBB still had stronger anoikis resistance than HNE1<sup>ad</sup> (13.02% and 27.60% vs 33.28%), the resistance intensity was significantly suppressed (Figure 3A-C).

Compared with untreated cells, 5-8F<sup>ad</sup>, 5-8F<sup>SUS</sup>, and 5-8F<sup>re-ad</sup> cells knocking down INHBB had higher levels of apoptotic resistance (3.96% vs 7.35%, 2.55% vs 4.64%, and 2.67% vs 3.70%). 5-8F<sup>re-ad</sup> and 5-8F<sup>SUS</sup> cells with shRNA-INHBB had still stronger resistance than 5-8F<sup>ad</sup> (2.67% and 2.55% vs 3.96%), and the resistance intensity was significantly strengthened (Figure 3D-F).

The above indicated that INHBB could induce apoptosis of cells and enhance anoikis sensitivity.

### 3.5 | Inhibin B inhibits DNA synthesis in anoikis-resistant NPC cells

Inhibin B could induce apoptosis of anoikis-resistant cells, which focused our attention on the cell cycle. Compared with untreated cells, the G<sub>0</sub>/G<sub>1</sub> phase of HNE1<sup>ad</sup>, HNE1<sup>SUS</sup>, and HNE1<sup>re-ad</sup> cells overexpressing INHBB were unchanged, whereas the S phase was reduced (16.5% vs 21.74%, 24.04% vs 38.09%, and 23.6% vs 41.52%, respectively) (Figure 4A-C). Inhibin B weakened the S phase cell increase in anoikis-resistant NPC cells. The G<sub>0</sub>/G<sub>1</sub> phase of NPC cells with INHBB knockdown decreased, while the S phase of 5-8F<sup>SUS</sup> cells increased (65.14% vs 48.10%, *P* = 0.009) (Figure 4D,E). The 5-8F<sup>SUS</sup> and 5-8F<sup>re-ad</sup> cells with shRNA-INHBB still promoted DNA synthesis compared to 5-8F<sup>ad</sup> cells (65.14% and 45.72% vs 24.81%), especially in 5-8F<sup>SUS</sup> cells (Figure 4F).

### 3.6 | Inhibin B attenuates EMT changes of anoikis-resistant cells

We continued to observe whether INHBB affected the EMT markers. The study found that the expression of E-cadherin was upregulated in the INHBB-overexpressing NPC cells, although the expression of E-cadherin in the INHBB over-expression HNE1<sup>SUS</sup> cell was lower than that of HNE1<sup>ad</sup> cell, which indicated that INHBB significantly promoted the E-cadherin expression of HNE1<sup>SUS</sup> cell. The opposite happened in vimentin, which revealed that INHBB reduced the ability of HNE1<sup>SUS</sup> cells to undergo EMT changes. In the wound-healing assay, our results showed that anoikis-resistant NPC cells healed wounds at a much faster rate than adhesion cells. Inhibin B could also inhibit migration of NPC cells (Figure S2A,C). An invasion assay was carried out using a Boyden chamber. Apart from being migratory in nature, anoikis-resistant NPC cells were also highly invasive compared to

adherent cells. Inhibin B could reduce the invasiveness of NPC cells (Figure S2B,D). Furthermore, the expression of MMP-9 and VEGF-A in HNE1<sup>SUS</sup> cells was greater than that of HNE1<sup>ad</sup> cells; these expressions were downregulated in overexpressing INHBB cells, which indicated that INHBB significantly abated the powerful invasion ability of HNE1<sup>SUS</sup> cell and vice versa in anoikis-resistant NPC cells with INHBB knockdown (Figure 5A).

### 3.7 | Inhibin B affects the TGF- $\beta$ /Smads signaling pathway in NPC

Transforming growth factor- $\beta$  was reported to lead to high microRNA-181a expression in 3-D culture, induce EMT, and promote invasion and metastasis of breast cancer cells.<sup>16</sup> However, the role of INHBB in the interference with anoikis-resistant cells was unclear. We suspected that the effect of INHBB might be achieved by TGF- $\beta$ . The expression of TGF- $\beta$ 1 was inhibited by INHBB overexpression anoikis-resistant NPC cells, TGF- $\beta$ 1 expression in INHBB knockdown cells was increased (Figure 5B), and the expression of downstream Smads proteins also changed. Diminished INHBB could upregulate Smad2/3, p-Smad2/3, and Smad4 to activate the TGF- $\beta$ /Smads pathway and prompt all of the above changes. The TGF- $\beta$ /Smads pathway also played a role in the re-adhesion of anoikis-resistant cells, although the function weakened, which was consistent with the previous inconspicuous changes of re-adhesion anoikis-resistant cells. Diminished INHBB could increase the TGF- $\beta$  expression of adhesion cells, but the downstream Smad4 had no change, which indicated that it was not affected by the TGF- $\beta$ /Smads pathway (Figure 5B).

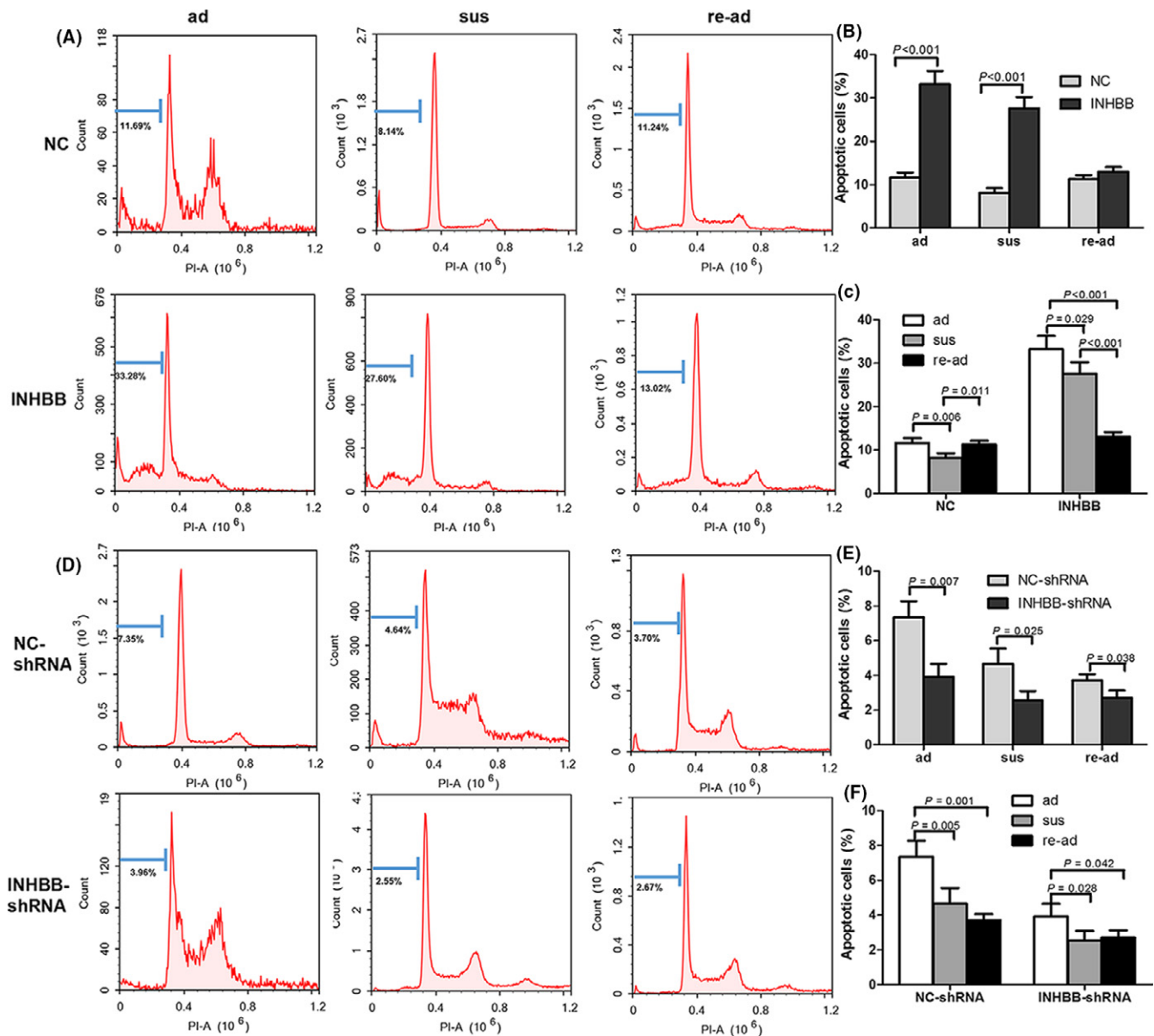
### 3.8 | Inhibin B influences the expression of p53 in anoikis-resistant NPC cells

It has been recognized that the mutated p53, a tumor promoter, leads to tumor formation or cell transformation, and p53 is closely related to TGF- $\beta$  signal transduction. We detected p53 expression in NPC cells and the results showed p53 expression was upregulated in anoikis-resistant NPC cells; the expression of p53 was decreased in anoikis-resistant NPC cells with INHBB overexpression, and knocking down INHBB could increase p53 expression (Figure S3). Inhibin B could affect the TGF- $\beta$  pathway, and regulating the expression of p53, which could provide new ideas for the treatment of NPC.

Therefore, loss of INHBB activated the TGF- $\beta$ /Smads pathway in the anoikis-resistant NPC cells, which could accelerate anoikis resistance and the synthesis of cellular DNA, promote cell proliferation and tumor growth, increase the expression of MMP-9 and VEGF, enhance tumor invasion metastasis (Figure 6), and further increase p53 expression. Inhibin B could serve as a potential biomarker for NPC metastasis and prognosis, as well as a therapeutic application.

## 4 | DISCUSSION

As one of the TGF- $\beta$  super-family members, INHBB is commonly described as inhibitor of gonadal follicle-stimulating hormone

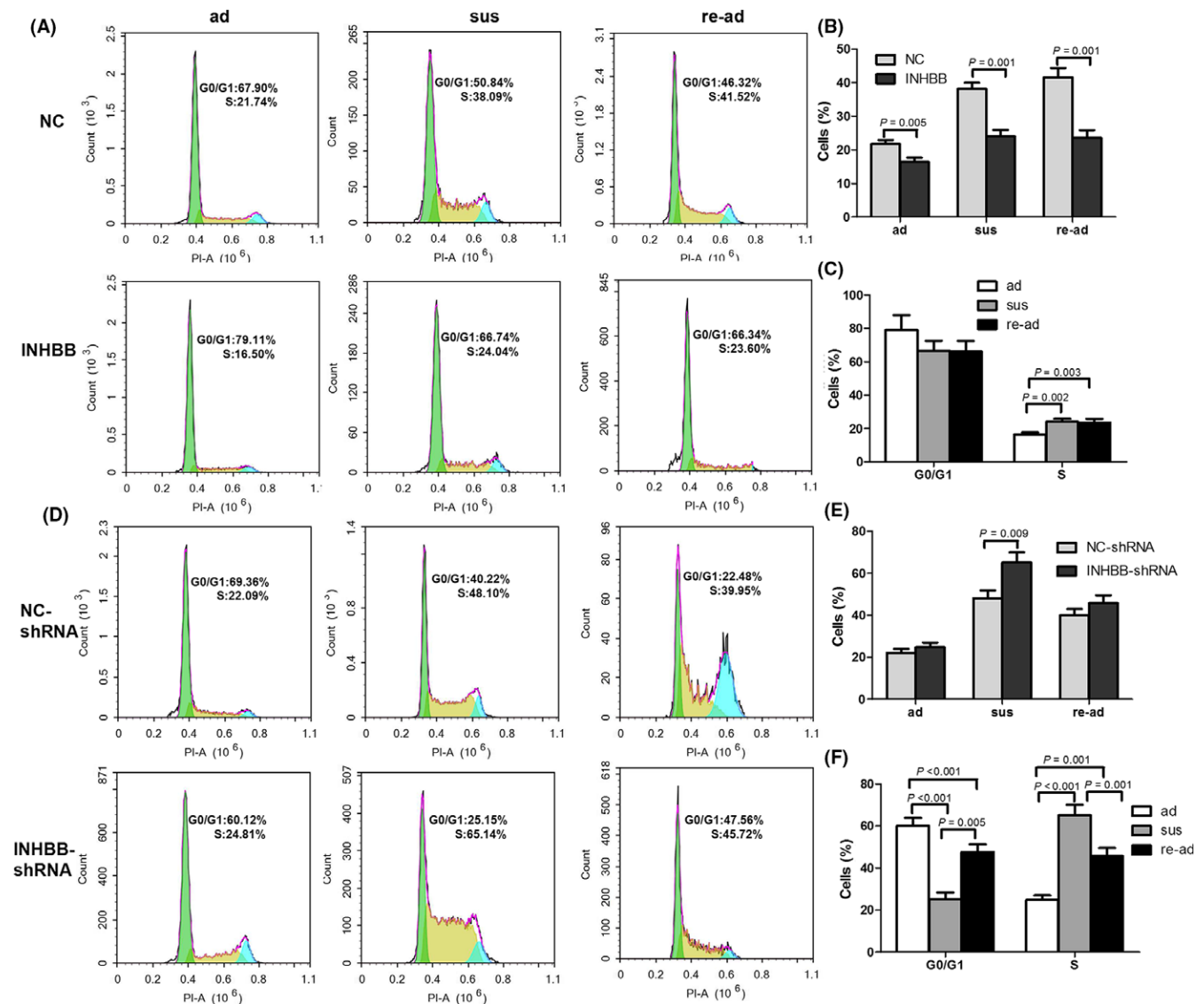


**FIGURE 3** Inhibin B (INHBB) induces apoptosis in anoikis-resistant nasopharyngeal carcinoma cells. A, Overexpression of INHBB cells for apoptosis flow cytometry analysis. X-axes, propidium iodide (PI)-A staining; Y-axes, cell counts. B, Overexpression of INHBB promoted apoptosis. Number of apoptotic cells from HNE1 adhesion culture (HNE1<sup>ad</sup>) and suspension culture (HNE1<sup>sus</sup>) treated with INHBB increased. C, INHBB induced apoptosis of anoikis-resistant cells. Cells from HNE1 re-adhesion culture (HNE1<sup>re-ad</sup>) and HNE1<sup>sus</sup> treated with INHBB had stronger resistance than HNE1<sup>ad</sup>. D, INHBB knockdown cells for apoptosis flow cytometry analysis. X-axes, PI-A staining; Y-axes, cell counts. E, INHBB knockdown cells lowering apoptosis. Apoptotic cells of 5-8F<sup>ad</sup> and 5-8F<sup>sus</sup> with shRNA-INHBB were decreased. F, Knockdown of INHBB inhibited apoptosis of anoikis-resistant cells. 5-8F<sup>re-ad</sup> and 5-8F<sup>sus</sup> cells with shRNA-INHBB showed stronger resistance than 5-8F<sup>ad</sup> cells

secretion, and INHBB has been evaluated for pregnancy-related conditions<sup>17,18</sup> and reproductive system tumors.<sup>19–21</sup> Risbridger et al<sup>22</sup> reported that INHBB was elevated in testicular Sertoli cell tumor, adrenocortical carcinoma, and placental tumors, and lower in renal cell, hepatocellular, pancreatic, and prostate carcinomas. Evidently, it is necessary to make clear inhibin function in oncology field for the contradiction. However, to our knowledge, there has been no report on the relationship between INHBB and NPC. Our pilot studies have shown that INHBB expression was downregulated in NPC tissue by

IHC staining in TMA (Figures 1, S1), and INHBB was negatively associated with lymph node metastasis, stage of disease, and clinical progression (Table 1). We confirmed for the first time the correlation between lower INHBB expression and negative clinical prognosis in NPC.

In this study, in order to determine the reason for INHBB influence on the clinical progression and prognosis of NPC, we simulated the high metastasis characteristics of NPC, induced highly metastatic NPC cells, and studied INHBB molecular mechanisms. Infection of



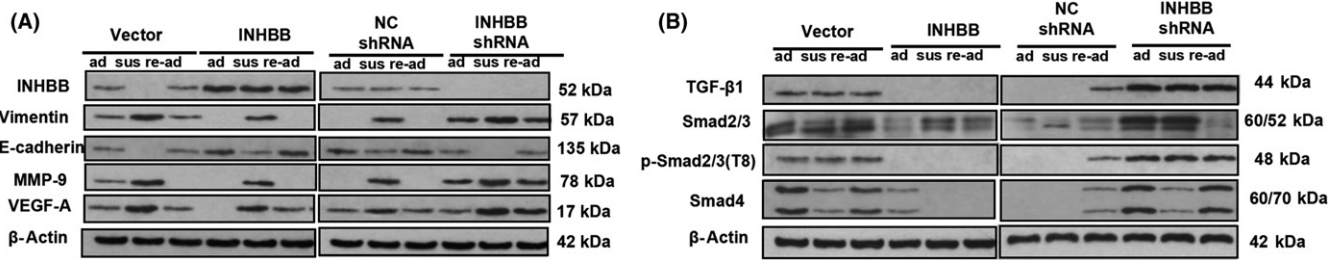
**FIGURE 4** Inhibin B (INHBB) inhibits DNA synthesis in anoikis-resistant nasopharyngeal carcinoma cells. A, Overexpression of INHBB cells for cell cycle flow cytometry analysis. X-axes, propidium iodide (PI)-A staining; Y-axes, cell counts. B, INHBB suppressed S phase cell growth. S phase cells of adhesion culture (HNE1<sup>ad</sup>) and suspension culture (HNE1<sup>sus</sup>) treated with INHBB decreased. C, INHBB weakened the S phase cell increase of anoikis-resistant cells, and there was no change in G<sub>0</sub>/G<sub>1</sub> phase cells. D, INHBB knockdown cells for cell cycle flow cytometry analysis. X-axes, PI-A staining; Y-axes, cell counts. E, 5-8F<sup>sus</sup> with shRNA-INHBB S phase cells increased. F, Anoikis-resistant cells with shRNA-INHBB G<sub>0</sub>/G<sub>1</sub> phase cells decreased, and S phase cells increased

EBV is frequently associated with NPC. Ng et al<sup>6</sup> showed that EBV-related NPC cells possessed the innate ability to survive and proliferate in an ultra-low attachment cell culture plate covalently bounded with hydrogel and induced the anoikis-resistant cells HONE-1-EBV, HK1-LMP1, and C666-1. Two NPC cell lines are associated with EBV infection: HNE1 is a poorly differentiated squamous carcinoma cell line, and 5-8F is a SUNE-1 substrain, which is a poorly differentiated squamous carcinoma cell line with high metastasis and high tumorigenesis capacity. The two cell lines were induced in a suspended growth system, and were proved to be highly transferable and anti-apoptotic. Compared with adhesion growth cells, the apoptosis ratios of suspension growth cells HNE1<sup>sus</sup> and 5-8F<sup>sus</sup> decreased, whereas MMP-9 and VEGF-A expression in those cells

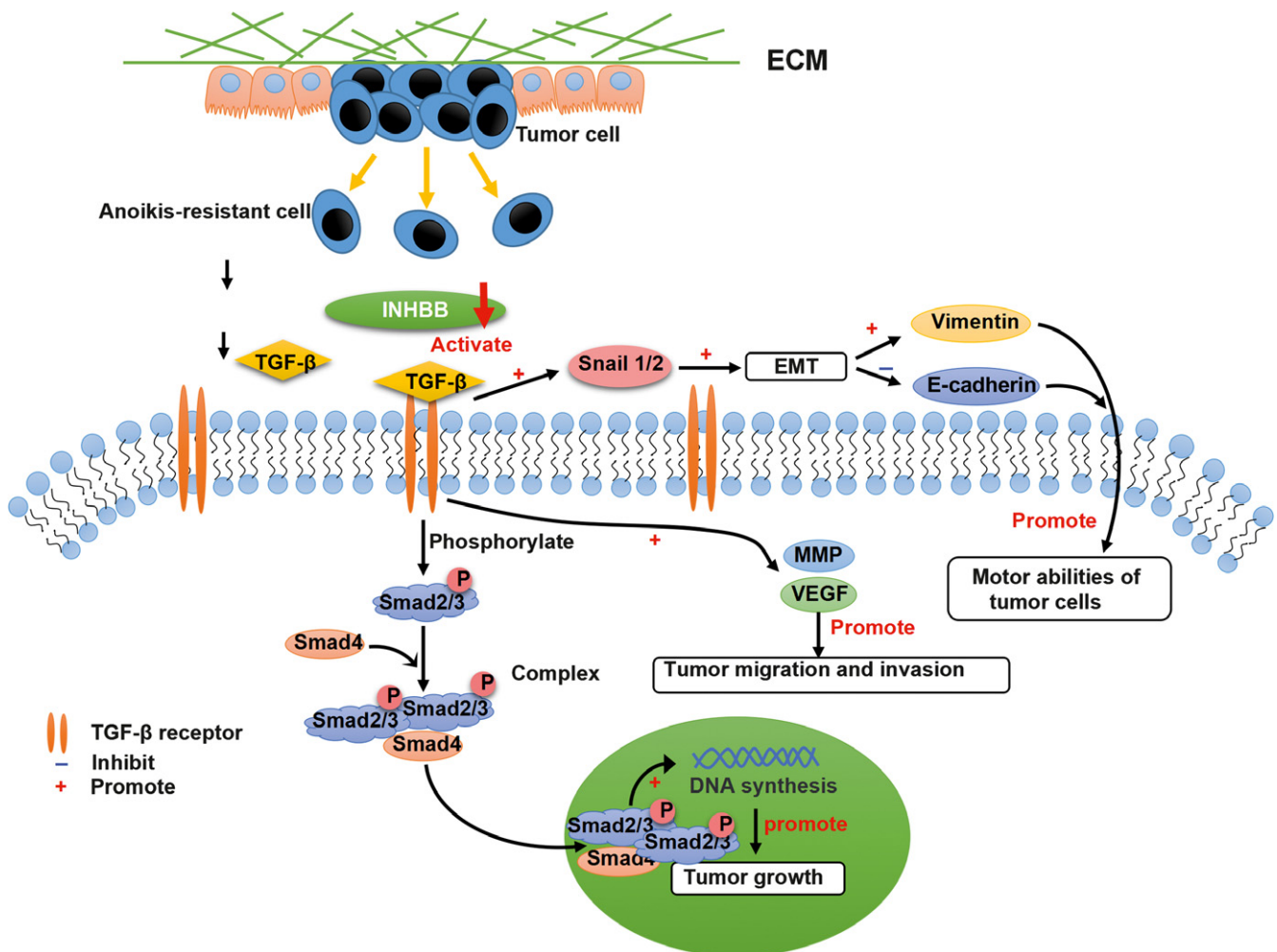
were upregulated (Figure 2). The suspension cells had the properties of anoikis resistance.

Anoikis resistance is essential for tumor metastasis. Tumor cells acquired metastatic characteristics by suppressing the death receptor and mitochondrial pathways.<sup>23</sup> We successfully induced anoikis-resistant characteristics of NPC cells, compared with parental cells, in which the growth pattern changed, epithelial cells marking E-cadherin disappeared, and the mesenchymal cell marker vimentin was upregulated. These suggested that the anoikis-resistant cells led to EMT modification, and these anoikis-resistant NPC cells healed wounds at a much faster rate and were highly invasive compared to adhesion cells (Figure S2). Furthermore, these EMT changes enhanced anti-apoptosis and invasion (Figure 2).





**FIGURE 5** Inhibin B (INHBB) is involved in epithelial-mesenchymal transition (EMT) and metastasis by influencing the transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway. A, Western blot analysis of EMT marker protein (vimentin and E-cadherin) and metastasis marker protein (matrix metalloproteinase-9 [MMP-9] and vascular endothelial growth factor-A [VEGF-A]) expression for HNE1 and 3 different culture conditions of 5-8F cells with INHBB and shRNA-INHBB. B, Western blot analysis of TGF- $\beta$  pathway protein (TGF- $\beta$ 1, Smad2/3, p-Smad2/3(T8), Smad4) expression for HNE1 and 3 different culture conditions of 5-8F cells with INHBB and shRNA-INHBB. ad, adhesion; re-ad, re-adhesion after suspension for 48 hours; sus, suspension



**FIGURE 6** Diminished inhibin B (INHBB) expression activates the transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway to promote anoikis resistance and migration. Schematic of diminished INHBB reprogramming by activation of the TGF- $\beta$  pathway in anoikis-resistant cells, accelerating the anoikis sensibility and DNA synthesis of cells, promoting the proliferation of cells and the growth of the tumor, inducing epithelial-mesenchymal transition (EMT) changes to enhance motor abilities of tumor cells, upregulating matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor-A (VEGF-A), and improving tumor invasion and metastasis

Epithelial-mesenchymal transition involves tumor pathological processes, especially tumor invasion and metastasis. In the process of EMT, E-cadherin was replaced by N-cadherin. This process of

“cadherin switching” greatly changes the cell adhesion connectivity<sup>24</sup> and improves the mobility of tumor cells. A study showed that endothelial cells secreted epidermal growth factor, induced Snail

through PI3k-Akt pathways to form EMT in squamous epithelium cell carcinoma, and obtained stem cell-like features (aldehyde dehydrogenase and CD44).<sup>3</sup> Chunchacha et al<sup>25</sup> obtained tumor cells from Thai patients with primary lung cancer with EMT characteristics, showing that tumor suppressor gene *p53* effector protein ASPP1 promoted apoptosis protein Bim downregulation and achieved anoikis resistance.<sup>26</sup> These results showed that EMT was one crucial step in tumor metastasis.

We found that INHBB could affect anoikis resistance, EMT changes, and metastasis in NPC cells. Through overexpression of INHBB in the anoikis-resistant NPC cells, the anoikis resistance was significantly inhibited, and the number of apoptotic cells increased (Figure 3). After INHBB treatment of anoikis-resistant NPC cells, cells in S phase cycle were suppressed and the enhanced DNA synthesis ability was weakened (Figure 4), which inhibited the proliferation of tumor cells. Inhibin B decreased the invasiveness and migration of anoikis-resistant NPC cells (Figure S2). These results might provide further powerful evidence for NPC treatment; nevertheless, there is little research on the clinical application of INHBB in NPC.

As we know, TGF- $\beta$  super-family members include TGF- $\beta$  itself, activin, inhibin, and bone morphogenetic proteins, with interactions between their receptors.<sup>27</sup> Inhibin subunits exist in female endocrine tumors and play an important role in the malignant cell transformation.<sup>28</sup> Inhibin  $\alpha$ -subunit promoter (*INH $\alpha$* ) gene deletion mice were used to identify for the first time that the INH  $\alpha$ -subunit showed tumor-inhibiting activity.<sup>13,29</sup> However, studies have found that INHB subunits have a two-sided effect on tumors. Overexpression of *INH $\alpha$*  could inhibit the growth of prostate cancer LNCaP cells, while promoting the growth and transfer of PC3 cells.<sup>30</sup> Recently, interest has developed into the clinical application of INHBB as tumor therapy. Studies have found that INHBB genes were downregulated in squamous cell lung cancer patients who smoke and cervical endometrium adenocarcinoma patients,<sup>31,32</sup> and INHBB-negative expression of cervical endometrium adenocarcinoma had higher cervical invasion.<sup>33</sup> Mylonas et al<sup>34</sup> also confirmed that high INHBB expression in endometrial carcinoma was accompanied by a high survival rate. Therefore, INHBB was considered as a tumor suppressor.<sup>13</sup>

However, there are few studies on the function, effect, and mechanism of INHBB in tumor cells, and only some research about activin B. Studies have shown that activin B could increase the adhesion, migration, and invasion of endometrial cancer cells through the activin B-Smad2/3-integrin  $\beta$ 3 pathway.<sup>35</sup> With inhibin and activin mutually inhibited, INHBB could bind to and inactivate the membrane-bound ActRII, preventing activin access to the receptor,<sup>36</sup> which inhibited the TGF- $\beta$ /Smads signaling pathway activation. Another study reported that expression of activin B was upregulated in oral squamous cell carcinoma tissue, modulating EMT genetic modifications, which upregulated the expression of E-cadherin and downregulated Snail. Knockdown of activin B could also promote tumor cell adhesion and suppress invasiveness and migration.<sup>37</sup> So INHBB plays roles in restraining tumor proliferation, invasion, and metastasis by limiting the actions of activin.

As far as we know, the TGF- $\beta$  signaling pathway is critical for EMT. During the EMT process, the polar protein could not be accurately positioned on the membrane, which could remove the blocking of Smads and promote the activation of the TGF- $\beta$  signal, which preserved the stability of the EMT phenotype and the ability to resist anoikis.<sup>38</sup> A low dose of paraquat could induce pulmonary epithelial cell A549 EMT alteration, and the EMT-like reaction and fibrinogenesis of the cells were promoted through the TGF- $\beta$  pathway.<sup>39</sup> Flotillin-1 could induce the expression and secretion of TGF- $\beta$ 1 and promote the activation of the TGF- $\beta$ /Smads signaling pathway, leading to EMT changes in NPC cells.<sup>40</sup> These findings indicated that EMT changes could be induced by activating the TGF- $\beta$  pathway. In this study, after treatment with shRNA-INHBB, diminished INHBB weakened binding to ActRII, which activated the TGF- $\beta$  pathway and Smad2/3 phosphorylation. Epithelial-mesenchymal transition was induced in the anoikis resistance of NPC cells, and then the anoikis resistance of NPC cells obtained stronger anti-apoptosis, invasion, and metastasis ability (Figures 5, 6). As with INHBB, the absence of tumor suppressor DEAR1 led to Smad3 phosphorylation in tumor cells, activating the TGF- $\beta$  signaling pathway and inducing EMT, which restrained anoikis and accelerated tumor invasion and metastasis.<sup>41</sup>

Using high-throughput microarray analysis, we found that the TGF- $\beta$ 1 receptor (also called activin receptor-like kinase 1, ALK1) and TGF- $\beta$ 2 were downregulated in NPC, and could be used in the tumorigenesis of independent factors.<sup>42</sup> We have data suggesting that INHBB expression is positively correlated with that of ALK1 and TGF- $\beta$ 2 (unpublished data, 2009). In endothelial cells, TGF- $\beta$  activated ALK1, which transmits signals through the ALK1/Smad1/5 pathways and, to a lesser degree, the Smad2/3 signaling pathways, stimulated endothelial cell proliferation and migration.<sup>43-45</sup> Thus, we have reason to believe that INHBB can induce the anoikis-resistant cell EMT modification through the TGF- $\beta$ /Smads pathway to affect tumor growth, invasion, and metastasis (Figure 6).

We suggest that INHBB could serve not only as a potential metastatic and prognostic biomarker for NPC, but also as a therapeutic application. Inhibin B is rarely reported as a therapeutic target. Our results revealed *p53* expression was upregulated in anoikis-resistant NPC cells, and *p53* expression was enhanced in the anoikis-resistant NPC cells with INHBB knockdown (Figure S3). Studies have shown that *p53* is the cellular gatekeeper for cell growth and proliferation, and contributes to cell cycle control and cell apoptosis. The mutant *p53* gene might cause the development of malignant tumors. Wild-type *p53* is considered to be a cancer suppressor; *p53* is transfected into tumor cells with an adenovirus as the carrier, which can inhibit tumor growth and cell proliferation.<sup>46,47</sup> Furthermore, rAd-*p53* combined with chemoradiotherapy for the treatment of recurrent NPC patients, which reportedly enhanced survival and provided better efficacy and lower toxicity than rAd-*p53* or chemoradiotherapy alone.<sup>48</sup> However, mutated *p53* has been suggested to switch TGF- $\beta$  to a tumor impact factor. The functional switching of TGF- $\beta$  is partially caused by *p53* mutation or *p53* inactivation during cancer progression.<sup>49</sup> A significant correlation existed

between p53 overexpression and poor prognostic factors, an increased frequency of regional recurrence, and visceral metastasis in breast cancer patients,<sup>50</sup> and patients with triple-negative breast cancer showed p53 protein overexpression, which resulted in lower survival.<sup>51</sup> In our study, the expression of p53 was upregulated in anoikis-resistant NPC cells with highly invasive and metastatic characteristics. Inhibition of INHBB can activate TGF- $\beta$  function through the interaction of TGF- $\beta$  and p53,<sup>52</sup> which could further improve p53 levels in metastatic NPC cells (Figure S3). We speculated that INHBB could achieve a good effect by downregulation of mutant p53 in the treatment of metastatic NPC patients. We will verify the hypothesis in the next study.

In conclusion, diminished INHBB can activate the TGF- $\beta$ /Smads signaling pathway and promote EMT modification, enhance greater invasion and metastasis abilities in anoikis-resistant NPC cells, and further increase p53 expression. Inhibin B could be used as a candidate biomarker for the clinical progression of NPC, especially as a candidate marker for lymph node metastasis of NPC, as well as a therapeutic application.

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## DISCLOSURE

The authors declare that they have no competing interests.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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