Original Research Article

Technology in Cancer Research & Treatment
Volume 23: 1-10
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DOI: 10.1177/15330338241234798
journals.sagepub.com/home/tct

Elevated INHBA Promotes Tumor Progression of Cervical Cancer

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Abstract

Objectives: This study aimed to explore the role of inhibin subunit beta A (INHBA) in the progression of cervical cancer (CCa) and investigate its potential as a therapeutic target. Specifically, the objectives were to assess the expression levels of INHBA in CCa, examine its correlation with patient survival, and elucidate its impact on CCa cell proliferation, cell cycle regulation, migration, invasion, and in vivo tumor growth and metastasis. **Methods:** To achieve the objectives, we conducted a comprehensive set of experimental methods. INHBA expression in CCa was analyzed, and its association with patient survival was assessed using clinical data. In vitro experiments involved the investigation of INHBA's effects on CCa cell proliferation, cell cycle dynamics, migration, and invasion through the epithelial–mesenchymal transition (EMT) process. Additionally, in vivo experiments were performed to evaluate the influence of INHBA on CCa growth and lung metastasis. **Results:** The results of this study revealed upregulated expression of INHBA in CCa, with a significant association between high INHBA expression and poor patient survival. Functionally, INHBA was found to promote the proliferation of CCa cells, regulate the cell cycle, and enhance migration and invasion through the EMT process in vitro. Moreover, in vivo experiments demonstrated that INHBA facilitated the growth and lung metastasis of CCa. **Conclusion:** In conclusion, our findings suggest that INHBA plays a crucial role in the progression of cervical cancer. The upregulation of INHBA is associated with poor patient survival, and its involvement in promoting key aspects of cancer progression makes it a potential therapeutic target for CCa treatment. These results provide valuable insights into the molecular mechanisms underlying CCa and offer a foundation for further exploration of targeted therapeutic interventions.

Keywords

inhibin subunit beta A, cervical cancer, tumor proliferation, tumor metastasis

Abbreviations

CCa, cervical cancer; EMT, epithelial–mesenchymal transition

Introduction

Cervical cancer (CCa) is a significant public health concern worldwide, with approximately 570 000 new cases and 311 000 deaths reported annually.[1] The highest incidence rates are observed in developing countries, where access to screening and treatment is often limited.[1] However, incidence rates have been decreasing in many countries due to the implementation of effective screening programs and the availability of human papillomavirus (HPV) vaccines. Current research efforts focus on improving the accuracy and accessibility of screening methods, developing new treatments for advanced CCa, and increasing HPV vaccination rates. Additionally, studies are underway to identify biomarkers that could improve early detection and prognosis of CCa.[2] Despite these efforts, there is still a need for increased awareness and resources to address the burden of CCa globally.

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The inhibin subunit beta A (INHBA) gene, also known as inhibin beta A, has been the subject of extensive research in the field of cancer. This gene is involved in the regulation of cell growth and differentiation, and its expression is dysregulated in various types of cancer, including ovarian, prostate, breast, and colorectal cancer.[3-5] Studies have shown that INHBA gene expression is associated with tumor progression and poor prognosis in these cancers.[4,6] Furthermore, genetic variations in the INHBA gene have been found to increase the risk of developing certain types of cancer.[7] The potential of INHBA as a therapeutic target for cancer treatment has also been explored, with some promising results in preclinical studies.[8] However, there are gaps in the current knowledge regarding the precise role of INHBA in cancer development and progression, as well as its potential as a target for cancer therapy. Therefore, this study aims to investigate the role of INHBA in affecting tumor progression of CCa, filling the gap in current knowledge and providing a clearer context for the research.

Materials and Methods

CCa Cells and Cell Culture

HeLa and MS751 human CCa cell lines were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% FBS using standard procedures. The cells were maintained at 37°C with 5% CO2.

Cell Transfection

The lentivirus vector pHBLV-CMV-INHBA-3flag-EF1-ZsGreen-T2A-puro and an empty vector were obtained from Hanbio Biotechnology Co., Ltd (Shanghai, China) to induce overexpression of INHBA. Lipofectamine® 3000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection following the manufacturer's instructions. Cells were harvested for further experiments 48 h after transfection.

Reverse Transcription-Quantitative PCR (RT-PCR) Assay

In this study, the RT-PCR assay was used to measure gene expression levels. To extract total RNA, RNAiso Plus (Takara Bio, Inc.) was used. The extracted RNA was then reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Takara Bio, Inc.). The resulting cDNA was used for RT-qPCR, which was performed using SYBR Premix Ex Taq (Takara Bio, Inc.) on a LongGene® Real-Time PCR System Q2000B (LongGene) following the manufacturer's instructions. To calculate the relative expression levels of the target genes, the $2^{-\Delta\Delta Cq}$ method was used with GAPDH as the reference gene. The primer sequences used for RT-qPCR are provided in Supplemental Table 1.

Western Blot Assay

Protein extraction was performed using RIPA lysis buffer (Beyotime Biotechnology), and protein concentrations were determined using a BCA Protein Kit Assay Kit (Thermo Fisher Scientific, Inc.). The extracted proteins (15 μg/lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% nonfat milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies (Supplemental Table 2). The membranes were then incubated with goat anti-rabbit IgG H&L (horseradish peroxidase [HRP] conjugated) or goat anti-mouse IgG H&L (HRP conjugated) (both from Abcam) at a 1:10 000 dilution for 1 h at room temperature. To normalize for loading, β-Actin was used as a loading control. Finally, protein bands were detected using the Pierce ECL System (Thermo Fisher Scientific, Inc.) and analyzed with ImageJ software (National Institutes of Health).

Colony Formation Assay

CCa cells were detached, suspended, and counted for the colony formation assay. Equivalent numbers of cells were seeded into 6-well plates and cultured for 2-3 weeks at 37°C in 5% CO2, with medium changes every 3 days. The formed colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet (Solarbio) for 2 h at room temperature. Colony numbers were calculated visually.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in 96-well plates and incubated for 0, 24, 48, and 72 h. Afterward, 10 μL of CCK-8 solution was added to each well and incubated with the cells for an additional 2 h at 37°C in a humidified atmosphere with 5% CO2. The optical density at 450 nm was measured using a microplate reader (Molecular Devices, LLC).

Wound Healing Assay

After treating cells in all groups for 48 h, $3*10^4$ cells were added to each well. The following day, low-concentration serum-containing medium was used, and a scratch tester was used to push the scratch upward from the central part at the lower end of the 6-well plate. Cells were rinsed twice with serum-free medium, and low-concentration serum-containing medium was added for photography at 0 h. The cells were then incubated in a 5% CO2 incubator at 37°C and photographed at 24 and 48 h. The wound healing rate was calculated for each group.

Transwell Migration and Invasion Assays

The Transwell migration and invasion assays were performed as previously described.[9] The migrated or invaded cells were photographed under a light microscope, and the cell numbers were counted in five random fields per chamber.

Cell Cycle

The Cell Cycle Analysis Kit (Shanghai Yeasen Biotechnology Co., Ltd) were used to measure cell cycle by flow cytometry, following the manufacturer's instructions.

Animals and Experimental Protocol

Twenty-four female BALB/c nude mice (aged 4-5 weeks) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd and housed in a standard environment with free access to food and water. The Ethics Committee of Department of Laboratory Animal Science, Fudan University approved all animal experiments. This animal experiment followed the blind method. The mice were randomly divided into two groups (a random sequence is generated by a computer): Vector and oe-INHBA. A fresh cell suspension was prepared at a concentration of 1×10^7 cells/ml, and six mice from each group were injected subcutaneously with 0.1 ml of cell suspension, while the other six mice were injected with 0.1 ml of cell suspension through the tail vein. Tumor growth was monitored weekly, and tumor size was measured using Vernier calipers. After 5 weeks, mice were euthanized with carbon dioxide (30% vol/min), and tumors (<600 mm^3) were removed, weighed, and photographed. Tumor tissues and lungs were fixed in formaldehyde and embedded in paraffin for hematoxylin and eosin (H&E) staining. The reporting of this study conforms to ARRIVE 2.0 guidelines.[10]

H&E Staining

Paraffin-embedded sections (5-μm) from xenograft samples were pretreated with 0.01% aqueous poly-L-lysin solution to promote adherence to glass slides. The slides were then stained with hematoxylin and eosin for 40 s and examined under a light microscope at ×200 magnification.

Statistical Analysis

Data were analyzed using R version 4.3.0 and GraphPad Prism 5 (GraphPad Software; Dotmatics). Normally distributed data are presented as mean ± standard deviation. Student's t-test was used for the comparison of two groups, while one-way ANOVA was used for the comparison of multiple groups, followed by Tukey's post hoc test. A *P*-value less than 0.05 was considered statistically significant.

Results

INHBA Expression is Upregulated in CCa and Patients with High INHBA Expression Have Poor Survival

First, we explored the expression of INHBA in various cancer cohorts of TCGA, and found that its expression was higher in the tumor group than in the normal group in most cancers (Figure 1A). Next, we analyzed the CCa expression microarray data sets from the GEO database, including GSE9750 and GSE63514, and found that INHBA was significantly highly expressed in CCa tissues compared with normal cervical tissues (Figure 1B and C). The survival analysis curves in Figure 1D and E demonstrate the comparison of overall survival and disease-free survival between patients with high (n = 145) and low (n = 146) INHBA gene expression. In Figure 1D, the high INHBA expression group showed significantly poorer overall survival compared to the low expression group, with a log-rank p-value of 0.00035 and a hazard ratio of 2.5. Similarly, in Figure 1E, the high INHBA expression group exhibited significantly worse disease-free survival compared to the low expression group, with a log-rank p-value of 0.00099 and a hazard ratio of 2.7. These findings suggest a potential correlation between elevated INHBA expression and adverse clinical outcomes in CCa patients, indicating the prognostic significance of INHBA in this context.

Overexpression of INHBA Promotes Proliferation of CCa Cells and Cell Cycle G1/S Transition

We further detected the protein expression of INHBA in five CCa cell lines, and selected HeLa and MS751 cells to continue the follow-up study (Figure 2A). To investigate the role of INHBA in CCa, we established stable cell lines overexpressing INHBA and validated them using RT-PCR and western blot assays (Figure 2B and C). The results of CCK-8 and colony formation assays demonstrated a significant increase in the proliferation capacity of HeLa and MS751 cells following overexpression of INHBA (Figure 2D-G). Flow cytometry analysis was performed to investigate the cell cycle distribution of HeLa and MS751 cells overexpressing INHBA. The results showed a significant increase in the proportion of S phase cells in the overexpression group compared to the control group (Figure 2H and I). Based on the above results, we revealed that INHBA could promote the proliferation of CCa cells and regulate their cell cycle *in vitro* through certain signaling pathways.

INHBA Promotes the Migration and Invasion of CCa Epithelial–Mesenchymal Transition (EMT) In Vitro

To investigate the effect of INHBA expression on the migration and invasion abilities of CCa cells, we further conducted cell migration and invasion assays. Both the wound healing assay and transwell assay results demonstrated a significant increase in the migration and invasion abilities of HeLa and MS751 cells following overexpression of INHBA (Figure 3A-F). Furthermore, western blot analysis of EMT markers, including snail, slug, and vimentin, revealed a significant upregulation of their expression levels after overexpression of INHBA (Figure 3G and H). These findings suggest that overexpression of INHBA can promote the migration and invasion of CCa cells.

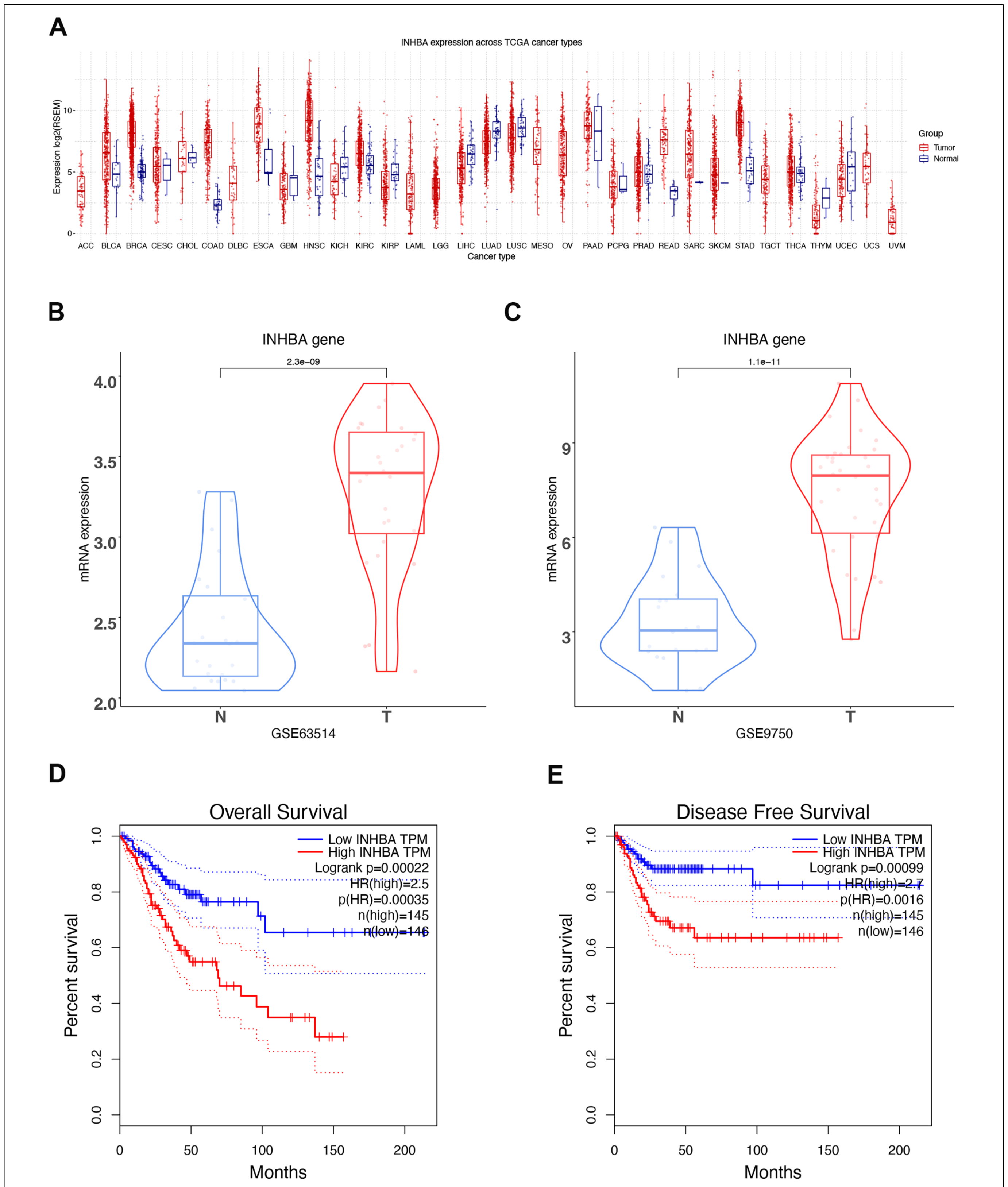

Figure 1. Expression profile of INHBA in CCa and other malignancies, along with survival analysis. (A) Transcriptomic expression landscape of INHBA across diverse human cancer types. (B, C) Analysis of INHBA mRNA expression levels in CCa based on the GSE63514 and GSE9750 datasets. (D, E) Conducting survival analysis associated with INHBA expression utilizing the TCGA-CESC dataset.

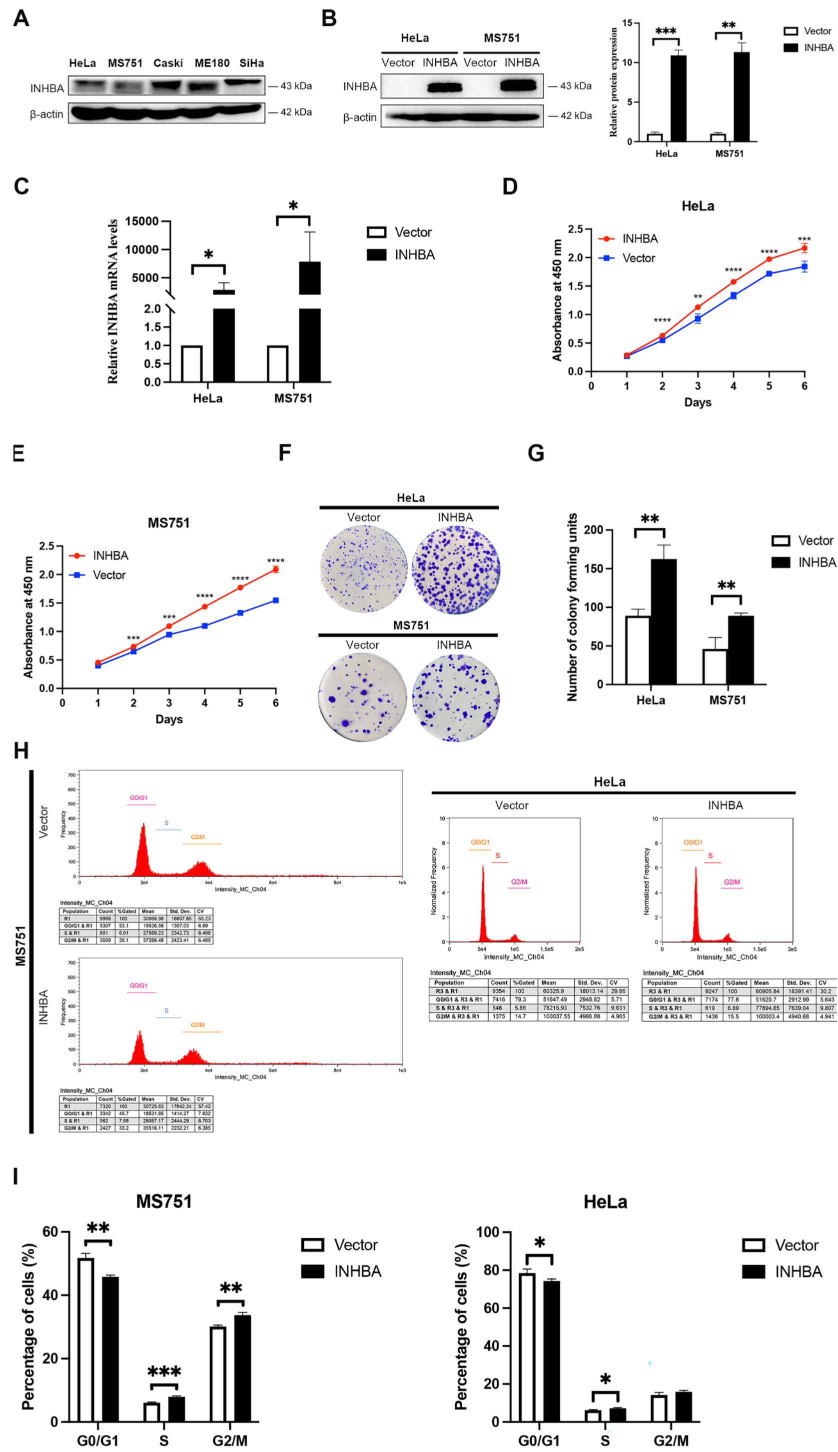

Figure 2. Elevated INHBA expression enhances proliferation of CCa cells and facilitates G1/S cell cycle transition. (A) Protein expression profile of INHBA across five types of CCa. (B, C) Establishment of HeLa and MS751 cell lines overexpressing INHBA, followed by validation through western blotting and qPCR analysis. (D, E) Assessment of the impact of INHBA overexpression on the proliferative capacity of HeLa and MS751 cells through CCK-8 assays. (F, G) Evaluation of the proliferative effects of INHBA overexpression on HeLa and MS751 cells through colony formation assays. (H, I) Investigation of the influence of INHBA overexpression on the cell cycle of HeLa and MS751 cells using flow cytometry analysis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

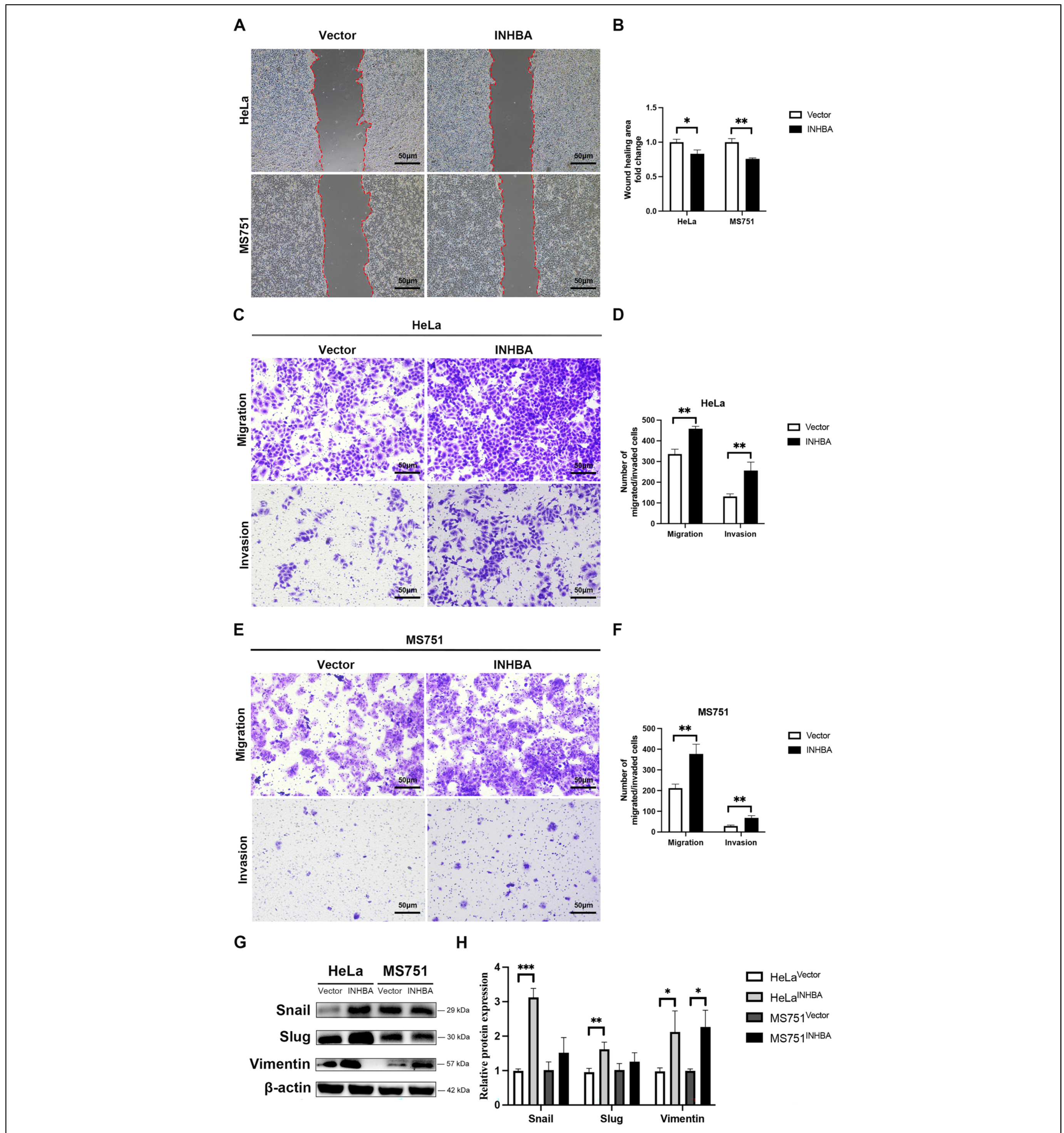

Figure 3. INHBA enhances the migratory and invasive capacities of CCa cells through the induction of EMT. (A, B) Assessment of the impact of INHBA overexpression on the migration abilities of HeLa and MS751 cells through wound healing assays (×100). (C-F) Transwell migration and invasion assays unveil the impact of INHBA overexpression on the malignant phenotype of HeLa and MS751 cells (×100). (G, H) Western blot analysis reveals elevated protein expression levels of mesenchymal phenotypic markers, namely Snail, Slug, and Vimentin, following INHBA overexpression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

INHBA Promotes the Growth and Lung Metastasis of CCa in Vivo Experiments

Based on the *in vitro* results described above, we concluded that INHBA promotes the proliferation, migration, and invasion of CCa cells *in vitro*. To further validate its role *in vivo*, we established subcutaneous tumor and lung metastasis nude mouse models. The animal experiment results showed that overexpression of INHBA significantly increased the size of subcutaneous tumors in mice and had a higher incidence of lung metastasis (Figure 4). However, there was no obvious evidence of metastasis in the liver, heart, or kidneys of the tumor-bearing nude mice.

Discussion

This study aimed to explore the expression, function, and molecular mechanism of INHBA in CCa, as well as its impact on patient prognosis. We found that INHBA was highly expressed in CCa and negatively correlated with survival rate, suggesting its clinical significance. We also found that INHBA could promote the proliferation, migration, invasion, and EMT of CCa cells, as well as the tumor growth and lung metastasis in vivo, revealing its important role in the occurrence and development of CCa. This study provides new clues and evidence for a deeper understanding of the molecular mechanisms of CCa, as well as the development of new therapeutic strategies and prognostic evaluation methods.

First, we discovered that INHBA expression was elevated in CCa, and that worse survival outcomes were associated with high levels of INHBA. Previous literature has shown that INHBA is also upregulated in other types of cancer.[6] In breast cancer, INHBA expression was significantly higher in tumor tissues compared to adjacent normal tissues and was associated with poor patient survival.[6] Liu et al reported that INHBA is linked to the aggressiveness of basal HER2 + tumors, leading to a poor response to HER2-targeted therapy and an invasive phenotype.[5] Li et al found that INHBA expression was specifically localized in the epithelium of ovarian cancer, and higher expression levels were associated with an increased risk of mortality in patients with advanced and higher-grade serous ovarian cancer.[11] Lyu et al demonstrated that INHBA gene expression levels were significantly elevated in cancer tissue of patients with esophageal squamous cell carcinoma compared to normal mucosa. High INHBA expression was significantly associated with lymph node metastasis and poor prognosis.[12] Zeng et al reported that elevated expression of INHBA in CCa patients was correlated with pathological characteristics, anti-tumor immune response, and reduced survival time, resulting in a poor prognosis.[13] Therefore, INHBA is considered as an oncogene, and its high expression is closely associated with poor patient survival.

Next, we found that overexpression of INHBA promoted the proliferation, invasion, and metastasis of CCa cells, as well as regulated the cell cycle. These findings were validated through both *in vitro* and *in vivo* experiments, confirming the oncogenic role of INHBA. The oncogenic role of INHBA in various types of cancer has been previously reported in the literature. An *in vivo* study showed that knockdown of INHBA in cancer cells impaired cancer xenograft growth by reducing the activation of ovarian cancer stromal fibroblasts.[11] The study also revealed that the Smad2 signaling pathway was involved in INHBA-induced activation of stromal fibroblasts, and inhibiting this pathway effectively reversed the activation of stromal fibroblasts.[11] Another study showed that the upregulation of VCAN induced by INHBA promotes the proliferation, migration, and invasion of colon cancer cells.[14] *In vitro* studies demonstrated that INHBA played a crucial role in promoting breast cancer cell proliferation and invasion by inducing EMT and activating TGF-β-regulated genes to accelerate cell motility.[6] Our study findings are consistent with previous literature, suggesting that INHBA affects the biological characteristics of cancer cells through certain potential molecular pathways, resulting in a more aggressive phenotype. Moreover, we developed a xenograft mouse model to investigate the impact of INHBA overexpression on CCa cells. Our observations revealed that heightened INHBA expression significantly enhances the pulmonary metastasis of CCa cells in nude mice. These *in vivo* findings underscore the pivotal role of INHBA in promoting CCa metastasis, shedding light on its significance in the developmental processes of CCa. This suggests that INHBA holds potential as a therapeutic target for impeding intra-body metastasis in CCa.

EMT is a critical process in the development of cancer. Previous literature has demonstrated that INHBA can modulate the malignant phenotype of cancer through the regulation of EMT.[6,15-17] In ovarian cancer, upregulation of INHBA has been shown to suppress the epithelial phenotype of ovarian cancer cells and promote invasive behavior in ovarian cancer cells.[15] In a study conducted by Xiao et al, it was observed that metformin exerts a down-regulatory effect on the expression of INHBA.[8] This modulation subsequently leads to the attenuation of the TGF-β/PI3 K/Akt signaling transduction pathway. As a consequence of this regulatory cascade, the proliferation of colorectal cancer cells is effectively inhibited. An investigation conducted by Howley et al revealed that the translational upregulation of INHBA results in heightened cellular migration and invasion capabilities within cells that have undergone an EMT.[16] Furthermore, this regulatory mechanism was found to exert a promoting influence on cancer progression in an *in vivo* context. In the investigation conducted by Yu et al, it was discerned that INHBA instigates the process of EMT and expedites the migratory capacity of breast cancer cells.[6] This effect is achieved through the activation of genes under the regulation of TGF-β. In the study conducted by Tao et al, evidence emerged suggesting that INHBA potentially functions as an oncogene by facilitating the activation of the Wnt/β-catenin signaling pathway in breast cancer.[17] Our study findings align with prior research outcomes, corroborating the observation that overexpression of INHBA significantly increased the protein levels of various EMT markers in CCa cells. These results suggest that EMT is a critical step in which INHBA affects CCa cells.

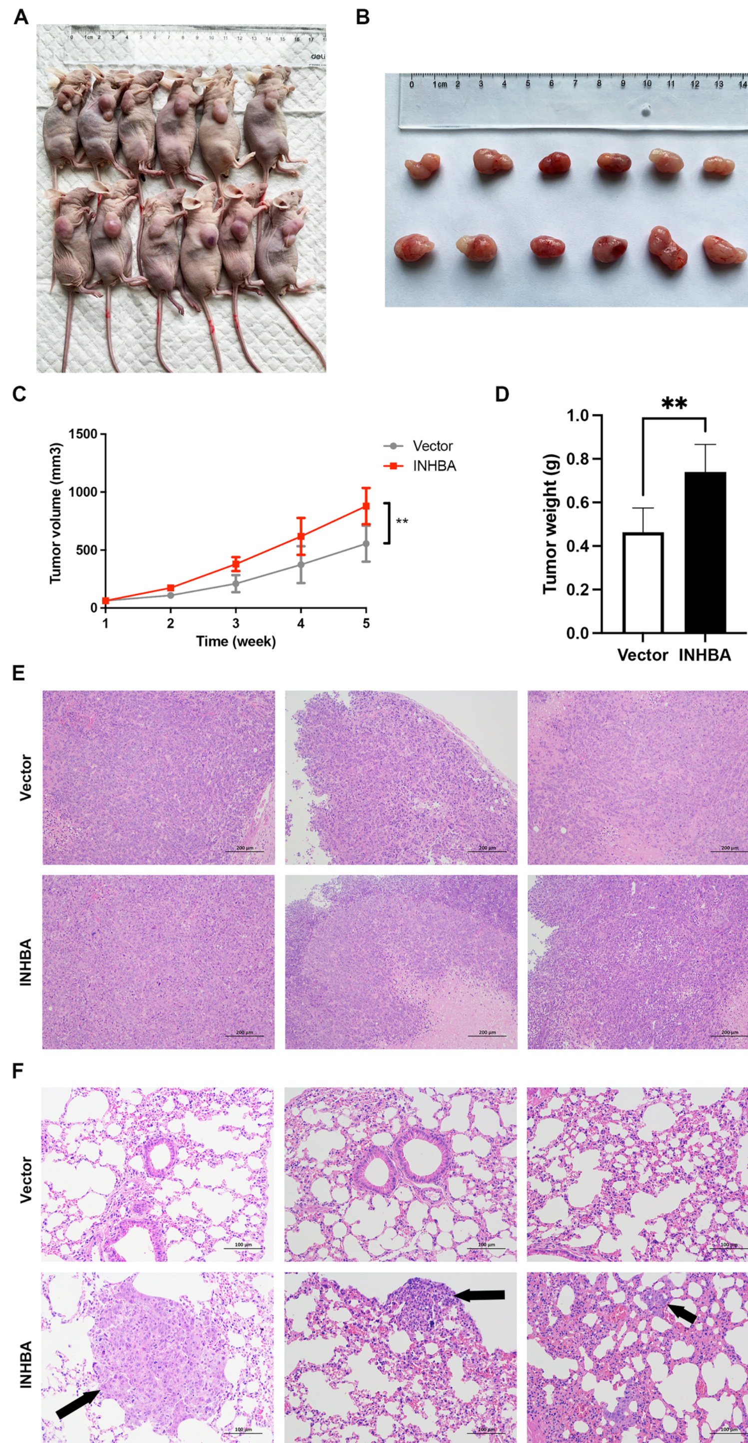

Figure 4. In vivo experiments demonstrate that INHBA facilitates the growth and lung metastasis of CCa. (A, B) Macroscopic specimen images of tumor-bearing mice and subcutaneous tumors. (C) Temporal evolution of subcutaneous tumor volume in the animal model over time. (D) Subcutaneous tumor mass at terminal observation point in the animal model. (E) Representative hematoxylin and eosin (H&E) stained sections of subcutaneous tumors in the animal model (×40). (F) Representative H&E-stained sections of lung tissues in the animal model (×200). Solid black arrows indicate sites of tumor metastasis. $^{**}P < 0.01$.

While our study provides valuable insights into the role of INHBA in CCa progression, it is essential to acknowledge certain limitations. Firstly, the focus on a specific set of experimental conditions and cell lines may limit the generalizability of our findings to the broader CCa population. Additionally, the molecular mechanisms underlying INHBA-mediated effects remain incompletely understood, warranting further investigation into the specific signaling pathways involved. Future research directions could involve comprehensive genomic and proteomic analyses to unravel the intricate networks influenced by INHBA. Additionally, exploring the interplay between INHBA and other key molecular players in CCa pathogenesis could unveil novel therapeutic targets. Addressing these limitations and pursuing these avenues will contribute to a more comprehensive understanding of the role of INHBA in CCa and may guide the development of targeted therapeutic strategies.

Conclusion

In conclusion, our research results indicate that INHBA can enhance the malignant phenotype of CCa by promoting the EMT process. Therefore, targeting the INHBA may be a potential approach for the treatment of CCa, in order to achieve the goal of inhibiting CCa proliferation and metastasis.

Author Contributions

The experiments were designed by Z.W. and R.C. and conducted by R.C., J.C., L.Y., and K.S. Data collection and statistical analyses were performed by F.D., Q.J., and W.L. Z.W. and R.C. wrote the manuscript, which was subsequently revised by Y.C. All authors have read and approved the final version of the manuscript.

Data Availability

The corresponding author can provide the data and materials upon reasonable request.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The Ethics Committee of Department of Laboratory Animal Science, Fudan University approved all animal care and experimental procedures in the study (2022JS-017).

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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