

Molecular mechanisms and clinicopathological characteristics of inhibin β A in thyroid cancer metastasis

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Abstract. The present study aimed to investigate the role and mechanism of inhibin β A (INHBA) in thyroid cancer (TC), and to determine its potential impact on the aggressive behavior of TC cells. The present study employed a comprehensive approach, using public databases, such as the Gene Expression Omnibus and The Cancer Genome Atlas, to identify and analyze the expression of INHBA in TC. Cell transfection, reverse transcription-quantitative PCR, western blot analysis, immunohistochemistry and *in vivo* assays were conducted to investigate the functional effects of INHBA on TC. In addition, the present study explored the molecular mechanisms underlying the effects of INHBA, focusing on the potential impact on the RhoA signaling pathway and associated molecular cascades. Bioinformatics analysis revealed a significant association between INHBA expression and TC, and INHBA expression was markedly upregulated in TC tissues compared with in healthy control tissues. The results of functional studies demonstrated that INHBA overexpression increased the migration and invasion of TC cells, and the opposite result was observed following INHBA knockdown. Mechanistic investigations indicated that INHBA modulated the RhoA pathway, leading to alterations in the phosphorylation status of LIM kinase 1 (LIMK) and cofilin, key regulators of cytoskeletal dynamics and cell motility. Following the introduction of transfected TC cells into zebrafish and nude mouse models, the results of the present study demonstrated that INHBA knockdown attenuated the metastatic potential of TC cells. In conclusion, INHBA may serve a pivotal role in promoting the aggressive phenotype of TC cells through modulating the RhoA/LIMK/cofilin signaling axis. These

findings highlight INHBA as a potential biomarker and therapeutic target for the management of aggressive TC.

Introduction

A survey carried out by the Global Cancer Observatory, under the World Health Organization International Agency for Research on Cancer, indicated that thyroid cancer (TC) is the ninth most prevalent cancer worldwide (1,2). In addition, TC is the most common cancer among adolescents and young adults aged 16-33 years in the United States; however, this trend may vary in other regions globally (3,4). The American Cancer Society estimates that there will be ~44,020 new cases of TC in 2024, with ~2,170 deaths attributed to the disease (5). Similarly, in 2022, the National Cancer Center of China reported that the age-standardized incidence rate for TC increased to 17.7%, along with an upward trend in mortality rates (6). Notably, the majority of TC cases respond well to conventional treatments, such as surgery; however, refractory cases, such as poorly differentiated thyroid carcinoma (PTC), anaplastic thyroid carcinoma (ATC) and some cases of advanced differentiated thyroid carcinoma, often exhibit a poor prognosis due to high levels of malignancy and a lack of effective treatment options (7,8). Thus, identifying target genes in TC is crucial for the development of effective treatment options.

In the present study, TC data were obtained from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. Through a series of bioinformatics analyses, inhibin β A (INHBA) was identified as a pivotal gene in TC, leading to further investigation of its role. INHBA, a member of the transforming growth factor- β superfamily, has been implicated in several vital biological processes, including glucose metabolism (9), cell differentiation (10) and immune response (11). Furthermore, INHBA upregulation is associated with aggressive phenotypes and poor outcomes in various types of cancer, including gastric, colon and esophageal cancer (12-15). Although one study proposed INHBA as a potential biomarker for advanced PTC (16), its specific functions and mechanisms in TC remain to be elucidated.

The present study, aimed to investigate the role of INHBA in TC. Specifically, the study aimed to understand how regulating INHBA expression in TC cells, such as CAL-62 and KTC-1, affects the cytoskeleton and phenotypic changes in

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these cells. The objective was to explore the potential impact of INHBA on the morphological characteristics and behavior of TC cells. The role of the cytoskeleton in cancer, particularly in association with cell metastasis, is both complex and critical (17). Comprising actin filaments, microtubules and intermediate filaments, the cytoskeleton is a dynamic framework that maintains cell shape, structure and motility. Notably, cytoskeletal reorganization is closely associated with the invasiveness and metastatic capacity of cancer cells (18,19). RhoA, a key member of the Rho GTPase family, affects this reorganization, including the configuration of actin filaments and cell migration (20–22). The present study aimed to explore the mechanisms by which INHBA may influence the metastasis of TC cells. Based on the role of cytoskeletal regulatory proteins, such as RhoA, in cell migration and invasion, it was hypothesized that INHBA could affect the metastatic potential of CAL-62 and KTC-1 TC cells through the regulation of the RhoA signaling pathway.

Materials and methods

Public database analysis. Gene expression data for TC were sourced from the GEO online repository (<https://www.ncbi.nlm.nih.gov/geo/>), utilizing the GEO2R online tool (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>) to identify differentially expressed genes (DEGs). The following GEO datasets were used in the present study: GSE33630 (23), GSE605422 (24) and GSE29265, which compared PTC with normal thyroid tissues, and DEGs were identified with a significance cut-off level of FDR <0.05, adjusted P<0.01 and log₂ fold change >0. For the comparison between ATC and PTC, the datasets GSE33630 and GSE27155 (25) were used, and DEGs were also identified with a significance cut-off level of FDR <0.05, adjusted P<0.01 and log₂ fold change >0. In most current studies (26,27), log₂ fold change thresholds >1 or even 2 are commonly used, focusing on genes that exhibit large expression changes. However, the present research design considers the comparison of multiple datasets, with the goal of identifying genes that are differentially expressed across these different datasets. Even if some genes show only small changes in expression in certain groups, they may exhibit consistent trends or larger changes in other groups. Therefore, by setting the threshold at log₂ fold change >0, these potentially key genes can be captured, providing a broader pool of candidate genes for subsequent cross-group analyses. Although the log₂ fold change threshold is relaxed, a strict adjusted P-value standard (P<0.01) was employed. This stringent statistical filtering ensures that all selected genes were highly significant, reducing the likelihood of false positives. Even genes with small expression changes, after multiple testing corrections, still exhibited differential expression across multiple groups, indicating that they may have important biological roles. For the common DEGs obtained through the intersection in the Venn diagram, the STRING database (<https://cn.string-db.org/>) was used to construct a protein-protein interaction network. Expression datasets for TC, along with genes co-expressed with INHBA, were retrieved from cBioPortal (<https://cbioportal.org>). The mRNA expression levels of INHBA in TC tissues were analyzed. The top 25% of patients, based on mRNA expression rank in the TC dataset, were classified as the high INHBA

expression group, and the remaining 75% of patients were classified as the low INHBA expression group. In addition, the Linked Omics database (<https://www.linkedomics.org/login.php>) was employed for further analysis. Genes co-expressed with the INHBA gene in TC were examined, and those with a Spearman's correlation value of >0.5 and a q-value <0.05 were selected for further functional Gene Ontology (GO) enrichment analysis (<https://david.ncicrf.gov/>). For gene annotation, the 'org.Hs.eg.db' R package (<https://bioconductor.org/packages/release/data/annotation/html/org.Hs.eg.db.html>) was utilized, and the 'clusterProfiler' R package (<https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) facilitated the enrichment analysis. The analysis was performed using R software version 4.2.0 (<https://cran.r-project.org/bin/windows/base/old/4.2.0/>). The GO analysis identified the top eight pathways across biological processes, cellular components and molecular functions. P<0.05 was considered to indicate a statistically significant difference.

Human samples. The patients were specifically recruited for the present study between August and November 2022. The age range of the participants was 27 to 67 years, with a mean age of 44 years, and the male-to-female ratio was 1:3. A total of 20 pairs of normal thyroid tissue and tumor tissue samples were collected from the Department of Thyroid Surgery, Shandong ENT Hospital (Jinan, China) and the study was approved by the Ethics Committee of Shandong ENT Hospital (approval no. 20220713). For reverse transcription-quantitative (RT-q)PCR, fresh tissues were directly collected and RNA was extracted. For immunohistochemistry (IHC), the tissues were stored in 4% paraformaldehyde for subsequent processing. All samples were histologically confirmed. The study objectives and procedures were explained to all participants, and written informed consent was obtained from all participants.

TC cell culture. In total, four TC cell lines; namely, BCPAP, CAL-62, KTC-1 and 8305C, were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and were all certified as mycoplasma-free. BCPAP and KTC-1 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), CAL-62 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), and 8305C cells were cultured in MEM (Gibco; Thermo Fisher Scientific, Inc.), all supplemented with 10% fetal bovine serum (FBS) (cat. no. 04-001-1A; Biological Industries), streptomycin (100 mg/ml) and penicillin (100 U/ml). The cells were passaged every 2–3 days and maintained in a humidified incubator at 37°C with 5% CO₂. The BCPAP cell line was authenticated by short tandem repeat profiling and tested for mycoplasma contamination.

Cell transfection. In the present study, short hairpin (sh) RNA was transfected into CAL-62 cells to downregulate INHBA expression, and plasmids were transfected into KTC-1 cells to upregulate INHBA expression. INHBA shRNA (shINHBA) and a scrambled non-targeting negative control (NC) shRNA (shNC) were cloned into a lentiviral vector, LV3-(H1/GFP&Puro). These lentiviral expression vectors were synthesized and purchased from Shanghai GenePharma Co., Ltd. The shRNA sequences were as follows:

shINHBA, sense 5'-CCCTTTGCCAACCTCAAAT-3', antisense 5'-ATTTGAGGTTGGCAAAGGG-3'; and shNC, sense 5'-GTTCTCCGAACGTGTACAGT-3', antisense 5'-ACGTGACACGTTCCGGAGAAC-3'. A pEX-2 plasmid containing human INHBA and an empty pEX-2 vector (NC) were obtained from Shanghai GenePharma Co., Ltd., and were transfected into cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. When the density of KTC-1 cells reached 70-80%, cell transfection was performed in serum-free medium with a plasmid concentration of 1 µg/ml. After incubating the cells at 37°C for 6 h, the medium was replaced with complete medium containing 10% FBS. The cells were cultured for a further 48 h before subsequent experiments were conducted.

shINHBA and shNC were transduced into CAL-62 cells using polybrene at a final concentration of 5 µg/ml (Shanghai GenePharma Co., Ltd.) with a multiplicity of infection (MOI) of 6 for lentiviral vectors. The duration of lentiviral transduction into CAL-62 cells was 24 h at 37°C. Following transduction, the cells were allowed to recover for 48 h before proceeding with subsequent experiments. Stable cell lines were established using puromycin selection at a concentration of 2 µg/ml, with maintenance at 1 µg/ml.

A pEX-2 plasmid containing human RhoA (RhoA-OE) and an empty pEX-2 vector (NC) were also obtained from Shanghai GenePharma Co., Ltd. Initially, the efficacy of the RhoA-OE plasmid was validated in untreated CAL-62 cells. Subsequently, the RhoA-OE plasmid was transfected into CAL-62 cells with INHBA knockdown using Lipofectamine 2000, according to the manufacturer's protocol. When the CAL-62 cells reached 70-80% confluence, transfection was initiated in serum-free medium with a vector concentration of 1 µg/ml. After incubating at 37°C for 6 h, the medium was replaced with complete medium containing 10% FBS. The cells were then cultured for an additional 48 h before proceeding with subsequent experiments. Transfection efficiency was determined using western blotting and RT-qPCR.

RT-qPCR. Total RNA was extracted from cells or tissues using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) according to the conditions: 25°C for 15 min, 42°C for 60 min and 70°C for 10 min. The resulting cDNA was analyzed using a TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (cat. no. RR820B; Takara Bio, Inc.). The primer sequences for INHBA were as follows: Forward, 5'-CCTCCCAAAGGATGTACCCAA-3' and reverse, 5'-CTCTATCTCCACATACCCGTTCT-3' (Sangon Biotech Co., Ltd.). The internal standard gene was β-actin, and its primer sequences were: Forward 5'-AGAGCTACGAGCTGCCTGAC-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3'. qPCR was conducted under the following conditions: 95°C for 2 min (pre-denaturation), followed by 40 cycles at 95°C for 15 sec (denaturation), 52°C for 20 sec (annealing) and 68°C for 20 sec (extension). Gene expression levels were quantified using the 2^{-ΔΔCq} method (28).

Cell Counting Kit-8 (CCK-8) assay. CAL-62 and KTC-1 cells were seeded in 96-well plates (2x10³ cells/well) and

were cultured at 37°C for 24 h. Subsequently, the cells were incubated with 10 µl CCK-8 solution (Beyotime Institute of Biotechnology) for 2 h at 37°C with 5% CO₂ at the indicated time points (0, 24, 48 and 72 h). The absorbance of each well was detected at 450 nm using a spectrophotometer.

Flow cytometric apoptosis detection. CAL-62 cells were digested with 0.25% trypsin to create a single-cell suspension and were washed twice with cold PBS. The experiment was performed using BD Pharmingen™ FITC Annexin V Apoptosis Detection kit I (cat. no. 556547; BD Biosciences). The cells (1x10⁶ cells/ml) were then resuspended in 1X Binding Buffer and 100 µl of this suspension was transferred to a tube. FITC Annexin and PI were then added, vortexed gently, and incubated for 15 min at room temperature in the dark. After adding 400 µl 1X Binding Buffer, the cells were analyzed by flow cytometry within 1 h to distinguish viable, apoptotic and necrotic cells based on their staining patterns. Flow cytometric analysis was performed using a flow cytometer (Accuri C6; BD Biosciences) and analyzed using FlowJo v10 software (FlowJo, LLC).

Cell invasion and migration. To evaluate cell migration, a wound healing assay was performed. A total of 48 h post-transfection, CAL-62 and KTC-1 cells monolayers were cultured until they reached 80-90% confluence. Subsequently, the monolayers were scratched using a sterile 200-µl pipette tip, and the cells were incubated in serum-free medium. Images were obtained after 0 and 24 h using a light microscope. The extent of wound healing was quantified by measuring the wound area using ImageJ software (version 1.53; National Institutes of Health).

Cell invasion was assessed using a Transwell assay. The assay utilized 24-well Falcon® Cell Culture Inserts (pore size, 8.0 µm; cat. no. 353097; Corning, Inc.) and the upper chamber was coated with Matrigel. The 24-well plate was incubated at 37°C for 60 min to allow the Matrigel to solidify. Subsequently, 5x10⁴ cells/well were seeded into the upper chamber in serum-free medium, while the lower chamber was filled with 800 µl medium containing 20% FBS. After incubating for 48 h at 37°C, the cells were fixed with 4% para-formaldehyde at room temperature for 20 min and stained with 0.1% crystal violet (cat. no. G1063; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at room temperature. Images of the invaded cells were subsequently captured using a light microscope (BX53; Olympus Corporation), and invasion was assessed.

Western blot analysis. CAL-62 and KTC-1 cells were lysed using freshly prepared RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.), which was supplemented with 10 µl PMSF solution (cat. no. ST506-2; Beyotime Institute of Biotechnology), 10 µl protease inhibitor cocktail (cat. no. CW2200S; CWBIO), and 10 µl phosphatase inhibitor cocktail (cat. no. CW2383S; CWBIO) per 1 ml RIPA buffer. Protein concentrations were determined using the BCA method following protein extraction. Equal amounts of protein (30 µg) were loaded per lane and subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 8, 10 or 12% gels. Subsequently, proteins

were separated by SDS-PAGE and were transferred to PVDF membranes, which were blocked with 5% skimmed milk powder at room temperature for 2 h, followed by overnight incubation at 4°C with the following primary antibodies: Anti-phosphorylated (p)-LIM kinase 1 (LIMK1; 1:1,000; cat. no. 3841T; CST Biological Reagents Co., Ltd.), anti-LIMK1 (1:1,000; cat. no. ab108507; Abcam), anti-p-cofilin (1:1,000; cat. no. 3313T; CST Biological Reagents Co., Ltd.), anti-cofilin (1:1,000; cat. no. 5175T; CST Biological Reagents Co., Ltd.), anti-INHBA (1:1,000; cat. no. 10651-1-AP; Proteintech Group, Inc.), anti-RhoA (1:1,000; cat. no. 2117T; CST Biological Reagents Co., Ltd.), anti-RhoB (1:1,000; cat. no. 2098T; CST Biological Reagents Co., Ltd.), anti-RhoC (1:1,000; cat. no. 3430T; CST Biological Reagents Co., Ltd.) and anti-GAPDH (1:1,000; cat. no. TA-08; OriGene Technologies, Inc.). After primary antibody incubation for 24 h, the membranes were incubated with HRP-labeled goat anti-rabbit IgG (1:5,000; cat. no. ZB-2301; OriGene Technologies, Inc.) and goat anti-mouse IgG (1:10,000; cat. no. ZB-2305; OriGene Technologies, Inc.) secondary antibodies using 5% skimmed milk powder as the dilution buffer at room temperature for 1.5 h. Immunoreactive proteins were visualized using an ECL kit (cat. no. SQ202L; Epizyme Biotech). The intensity of protein bands was determined using ImageJ software (version 1.53).

Rho/Rho-kinase (ROCK) inhibitor Y27632 treatment. KTC-1 cells were incubated at 37°C. The Rho/ROCK inhibitor Y27632 (cat. no. HY-10071; MedChemExpress) was added to the culture medium at a final concentration of 10 μ M and the KTC-1 cells were treated for 24 h at 37°C with 5% CO₂. After the treatment, the KTC-1 cells were subjected to wound healing and Transwell assays to evaluate the effects of Y27632. Additionally, the downstream signaling proteins of the RhoA/ROCK pathway were examined, including their phosphorylation status, using western blot analysis to assess the impact of the treatment on the signaling cascade.

Immunofluorescence assay. CAL-62 and KTC-1 cells were plated at 2x10⁴ cells/well on coverslips in a 48-well plate. After culturing for 24 h, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 10 min on ice, and blocked with 1% FBS at room temperature for 30 min. Subsequently, 200 μ l 100 nM rhodamine phalloidin (cat. no. R415; Thermo Fisher Scientific, Inc.) was added to each well, and the cells were incubated in the dark at room temperature for 30 min. Nuclei were stained using 2 μ g/ml DAPI in PBS for 10 min and the cells were examined under a confocal inverted microscope (Leica Microsystems, Inc.).

In vivo experiments. Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the National Research Council (29), and were approved by the Ethics Committee of Shandong Provincial ENT Hospital (approval no. 20220713).

Female BALB/c nude mice (age, 4-6 weeks; weight, 18-20 g) were purchased from Charles River Laboratories, Inc., and were housed in a specific pathogen-free environment with controlled temperature (22-24°C) and humidity

(45-55%). Mice had *ad libitum* access to sterilized rodent chow and autoclaved water throughout the experiment, and they were maintained on a 12-h light/dark cycle. A total of 12 mice were used in the experiment. Subsequently, 4x10⁵ CAL-62 cells were seeded into 6-well plates. Lentivirus-encapsulated firefly luciferase reporter plasmids (plasmid backbone, pLV-CMV-Luciferase-T2A-Hygro vector; Shanghai GenePharma Co., Ltd.) were infected into both NC and INHBA-KD CAL-62 cells at a MOI of 6 in the presence of 5 μ g/ml polybrene. The infection was carried out at 37°C in a humidified incubator with 5% CO₂ for 24 h. After infection, the medium was replaced with fresh complete medium, and the cells were further incubated for an additional 48 h before subsequent experiments. The cells infected with the firefly luciferase reporter plasmids were diluted in PBS to a concentration of 1x10⁷ cells/ml. Mice were anesthetized using isoflurane inhalation (3.0% induction; 1.5% maintenance), and 100 μ l luciferase-expressing cell suspension (1x10⁶ cells) was injected into mice via the tail vein. The overall condition of the mice was monitored daily, including vocalization, respiratory difficulty, weight loss, abnormal behavior and physical appearance. Metastatic tumors were monitored weekly using an *in vivo* imaging system (IVIS) to detect luciferase bioluminescence at pre-determined time points. D-luciferin potassium salt solution (15 mg/ml; cat. no. ST196-2g; Beyotime Institute of Biotechnology) was injected intraperitoneally into the mice at a dose of 150 mg/kg body weight. Within 10-15 min of the substrate injection, the mice were placed in the IVIS Spectrum (PerkinElmer, Inc.), and the imaging parameters (exposure time, filter settings, etc.) were adjusted to ensure clear images covering the entire mouse body surface. Humane endpoints included a loss in bodyweight of >20% and a tumor volume of >1,500 mm³. No animals reached the humane endpoints during the experiment and mice were active during all observations. At the end of the experiments, mice were euthanized via cervical dislocation following anesthesia with isoflurane. Death was confirmed by respiratory and cardiac arrest, and after pupil dilation had been observed for $\geq$ 10 min.

Two pairs of AB strain adult zebrafish were generously provided by the Otolaryngology Research Laboratory, Shandong Provincial ENT Research Institute (Jinan, China), and were maintained at 28.5°C under a 14/10-h light/dark cycle. The embryos were obtained from breeding pairs. CAL-62 TC cells were cultured, harvested and suspended in PBS at 1x10⁷ cells/ml. At 48 h post-fertilization, zebrafish embryos were randomly assigned to the NC group (n=10) and the INHBA-KD group (n=10) and were anesthetized by immersion in water containing 0.01% tricaine. Then they were injected with 200-300 fluorescent-labeled CAL-62 cells into the perivitelline space. Embryos were subsequently maintained at 34°C, and metastatic spread was assessed using fluorescence microscopy. All experiments complied with the AVMA Euthanasia Guidelines 2020 (30), and suffering and distress were minimized. At the end of the experiments, the 5-day post-fertilization (dpf) zebrafish were euthanized by immersion in 2-4°C water for $\geq$ 20 min following the loss of operculum movement. Subsequently, a sodium hypochlorite solution with a final concentration exceeding 1% was used. A stock solution of 6.15% sodium hypochlorite was diluted

at a ratio of 1 part bleach to 5 parts system water, achieving a final concentration of ~1.025%. The zebrafish were immersed in this solution for a minimum of 5 min to ensure death.

IHC. Human thyroid tissues and mouse lung tissues were fixed in 10% neutral buffered formalin at room temperature (22–25°C) for 24 h, then dehydrated and embedded in paraffin, and subsequently cut into 4- μ m sections. The paraffin-embedded sections were deparaffinized using xylene and rehydrated through a descending ethanol series (100, 95, 85 and 75%) for 5 min each. Antigen retrieval was conducted at high heat (>100°C) for 10 min, followed by medium to low heat (50–60°C) for 20 min, and then allowed to cool to room temperature for 1 h, using EDTA buffer (pH 9.0; cat. no. ZLI-9068; OriGene Technologies, Inc.). After dewaxing and antigen retrieval, the sections were washed with PBS three times (5 min each). A quenching step was performed using 3% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase activity. For blocking, human thyroid tissues were incubated with 8% normal goat serum (cat. no. SL038; Beijing Solarbio Science & Technology Co., Ltd.) in PBS at 37°C for 30 min to prevent nonspecific binding, with the excess blocking solution gently shaken off without washing. The sections were then incubated with INHBA primary antibody (1:200; cat. no. 10651-1-AP; Proteintech Group, Inc.) overnight at 4°C. Following primary antibody incubation, the tissues were incubated with a goat anti-mouse IgG H&L (HRP) secondary antibody (1:500; cat. no. ab6789; Abcam) at room temperature for 2 h. DAB staining was performed for 5–10 min at room temperature.

H&E staining of the mouse lung tissues was carried out using 0.5% hematoxylin and 0.5% eosin solutions at room temperature (22–25°C) for 2 min each. Tissues were subsequently dehydrated and sealed. Images were captured by light microscope for both IHC and H&E staining. Integrated optical density was determined from IHC images using ImageJ software (version 1.53).

GTPase activation assay. Detection of activated RhoA, RhoB and RhoC was performed using a consistent protocol, with different antibodies used for the final incubation. Cells were lysed using cold IP lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology) and were clarified by centrifugation at 10,000 $\times$ g for 10 min at 4°C. Equal amounts of total proteins (700 μ g) from cell lysates were incubated with 25 μ l Rhotekin-RBD agarose beads (cat. no. P2061; Beyotime Institute of Biotechnology) on a rotator at 4°C for 1 h. The Rhotekin-RBD agarose beads specifically capture activated RhoA, RhoB and RhoC. The beads were then pelleted by centrifugation at 6,000 $\times$ g for 30 sec at 4°C and washed three times with 1X assay buffer (cat. no. P0013; Beyotime Institute of Biotechnology). After the final wash, 25 μ l 1X SDS-PAGE sample buffer was added and the mixture was heated at 95°C for 5 min. The proteins were then separated by SDS-PAGE, transferred to PVDF membranes, and probed with specific antibodies against RhoA (1:1,000; cat. no. 2117T; CST Biological Reagents Co., Ltd.), RhoB (1:1,000; cat. no. 2098T; CST Biological Reagents Co., Ltd.) and RhoC (1:1,000; cat. no. 3430T; CST Biological Reagents Co., Ltd.). This method specifically recognizes and binds to the active

forms of Rho proteins (RhoA, RhoB, RhoC) but not to their inactive GDP-bound forms (Rho-GDP). Secondary antibodies and chemiluminescence were used for protein detection, following the same protocol described for western blotting. For the detection of total RhoA, RhoB and RhoC, the lysates that remained after removing the 700 μ g portion were used, according to the same protocol described for western blotting. The activation levels of RhoA, RhoB and RhoC were normalized to the total levels of RhoA, RhoB and RhoC, respectively.

Statistical analysis. Each experiment was repeated at least three times. All statistical analyses were performed using SPSS software (version 20.0; IBM Corporation) and GraphPad Prism 8 (Dotmatics). Survival analysis was performed using Kaplan-Meier survival curves, with differences between groups assessed using the log-rank test. Hazard ratios and 95% confidence intervals were calculated to evaluate risk. Differences between paired data (e.g., normal and tumor tissues from the same patients) were compared using a paired two-tailed Student's t-test. Differences between two unpaired groups were compared using an unpaired two-tailed Student's t-test, and differences between multiple groups were compared using one-way ANOVA followed by Tukey's post hoc test. χ^2 test or Fisher's exact test was used to compare clinicopathological features between the INHBA high and low expression groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

INHBA is associated with TC. RNA sequencing data were obtained from multiple GEO datasets to identify DEGs between PTC samples and healthy thyroid tissues, and ATC and PTC samples. Using these data, Venn diagrams were constructed, identifying 26 intersecting genes (Fig. 1A–C). The STRING database was used to determine genes that may be associated with TC (Fig. 1D). The results of the present study highlighted that INHBA, a gene implicated in other types of cancer, may have a role in TC. In addition, patient characteristics and clinicopathological data were examined and the results suggested that INHBA expression was significantly associated with clinical stage (Table I). The results of the quantitative analysis revealed significantly higher mRNA expression levels of INHBA in stages T3 and T4 compared with stages T1 and T2. (Fig. 1E), and in patients with N1 stage cancer (Fig. 1F). Collectively, these results demonstrated the potential role of INHBA in TC. Due to the overall low mortality rate and high survival rate of TC, the statistical analysis performed using TCGA data indicated no significant association between INHBA expression and TC survival rates (Fig. 1G). However, the overall trend suggested that lower INHBA expression was associated with better patient survival rates. The present study did not further investigate the association between INHBA expression and TC survival rates.

INHBA is upregulated in TC tissues. To further validate the aforementioned findings, 20 matched pairs of healthy thyroid and cancer tissues were analyzed. INHBA expression was assessed using IHC, and brown coloration was indicative of positive cells (Fig. 2A). The results of the present study

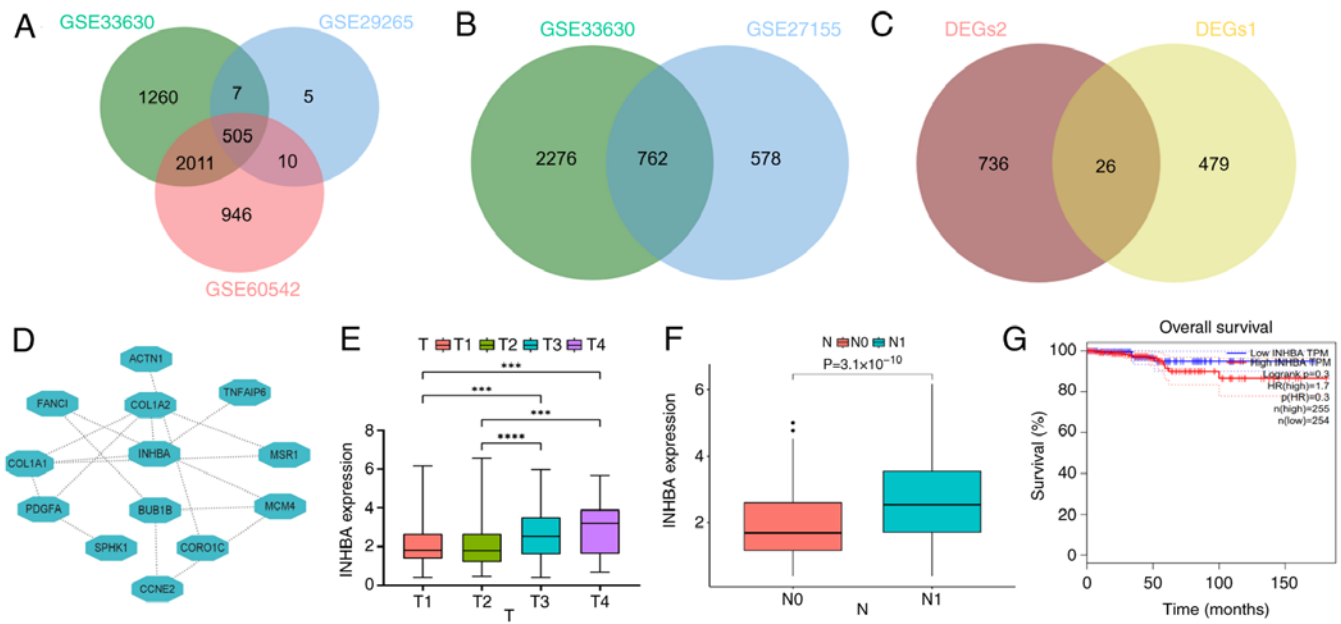


Figure 1. INHBA expression is positively associated with TC tumor staging. (A) A Venn diagram depicting the overlap of DEGs1 (PTC vs. normal) among three GEO datasets (GSE33630, n=105; GSE29265, n=49; GSE60542, n=92). (B) A Venn diagram depicting the overlap of DEGs2 (ATC vs. PTC) between GSE33630 and GSE27155 datasets (GSE33630, n=105; GSE27155, n=99). (C) A Venn diagram depicting the overlap of DEGs1 and DEGs2. (D) Protein-protein interaction network was created using STRING. (E) INHBA expression levels were increased in T3 and T4. ***P<0.001, ****P<0.0001. (F) INHBA expression levels were increased in N1 compared with N0. (G) Relationship between INHBA expression and the prognosis of patients with TC. ATC, anaplastic thyroid carcinoma; DEGs, differentially expressed genes; GEO, Gene Expression Omnibus; INHBA, inhibin β A; PTC, poorly differentiated thyroid carcinoma; TC, thyroid cancer.

indicated that INHBA was predominantly localized in the cytoplasm, with significantly increased expression levels in cancer tissues compared with those in healthy tissues (Fig. 2B). In addition, the results of RT-qPCR analysis demonstrated significantly higher INHBA mRNA expression levels in tumor tissues compared with those in healthy tissues (Fig. 2C).

To further explore the role of INHBA in TC, corresponding expression levels were analyzed in four TC cell lines; namely, BCPAP, CAL-62, KTC-1 and 8305C. INHBA expression was high in CAL-62 cells and low in KTC-1 cells (Fig. 2D and E). Thus, INHBA knockdown was carried out in CAL-62 cells and INHBA overexpression was carried out in KTC-1 cells. INHBA mRNA and protein expression levels were analyzed to confirm transfection efficiency (Fig. 2F-K).

INHBA promotes TC cell migration. Based on clinical observations that distant metastasis significantly impacts the prognosis of patients with TC, the effects of INHBA expression on TC progression were determined in the present study. The results demonstrated that INHBA knockdown or overexpression exerted no marked effect on TC cell proliferation and apoptosis (Fig. 3A-C). The results of the Transwell assay revealed that INHBA knockdown in CAL-62 cells significantly inhibited invasion, whereas INHBA overexpression in KTC-1 cells markedly enhanced invasion (Fig. 3D-G). The results of the migration assay indicated that INHBA knockdown significantly reduced migration in CAL-62 cells, compared with that in the NC group, whereas KTC-1 cell migration was increased following INHBA overexpression, compared with that in the NC group (Fig. 3H-K). Collectively, these results demonstrated that INHBA overexpression exerted no effect on the proliferation of TC cells; however, INHBA overexpression significantly

promoted the migration and invasion of TC cells, providing a theoretical basis for the treatment of TC cell metastasis.

The results of the immunofluorescence staining analysis revealed alterations in the cytoskeleton of cells following INHBA knockdown or overexpression. In the NC group of CAL-62 cells, F-actin, which forms the main cytoskeletal structure, was well-modeled and distributed, and the cell pseudopodia were fully extended; however, in the INHBA knockdown group, the actin filaments were disordered and the cell pseudopodia were reduced (Fig. 3L). Following INHBA overexpression, an increased number of actin filaments accumulated at the leading edge of KTC-1 cells, compared with that in the control group (Fig. 3M). These results indicated that INHBA may impact the arrangement of the cytoskeleton.

INHBA increases activation of the RhoA pathway in TC. In the present study, the functional module of the Linked Omics database was used to determine the molecular role of INHBA in TC, and to analyze the co-expression patterns within the TC cohort. As illustrated in Fig. 4A, genes marked with red dots exhibit those significantly positively correlated with INHBA, whereas those marked with green dots exhibit those significantly negatively correlated with INHBA (false discovery rate <0.01). A heat map was used to display the top 50 genes that were significantly positively associated with INHBA (Fig. 4B). GO analysis categorized the aforementioned proteins into three groups; namely, cellular component, molecular function and biological process. The results of the present study highlighted notable enrichment in the terms 'extracellular matrix organization', 'wound healing' and 'actin cytoskeleton' (Fig. 4C). These results suggested that INHBA may impact TC progression through modulation of cytoskeleton-related

Table I. Association between INHBA mRNA expression and clinical characteristics of patients.

Clinical parameter	Low-INHBA (n=377)	High-INHBA (n=124)	P-value
Age (%)			0.978
<55 years	265 (70.3)	87 (70.2)	
≥55 years	112 (29.7)	37 (29.8)	
Sex (%)			0.135
Female	269 (71.4)	97 (78.2)	
Male	108 (28.6)	27 (21.8)	
Clinical stage (%)			0.009
I	222 (58.9)	61 (49.2)	
II	44 (11.7)	7 (5.6)	
III	75 (19.9)	35 (28.2)	
IV	35 (9.3)	20 (16.1)	
NA	1 (0.2)	1 (0.9)	
Metastasis (%)			0.168
M0	201 (53.3)	78 (62.9)	
M1	7 (1.9)	2 (1.6)	
NA	169 (44.8)	44 (35.5)	
N classification (%)			<0.001
N0	189 (50.2)	37 (29.8)	
N1	149 (39.5)	76 (61.3)	
NA	39 (10.3)	11 (8.9)	
T classification (%)			0.056
T1	114 (30.2)	28 (22.6)	
T2	112 (29.7)	53 (42.7)	
T3	129 (34.2)	40 (32.3)	
T4	20 (5.3)	3 (2.4)	
NA	2 (0.5)	0 (0.0)	

χ^2 test or Fisher's exact test was used to compare clinicopathological features between the INHBA high and low expression groups. INHBA, inhibin β A.

pathways. Notably, cancer cell migration is driven by regulation of the actin cytoskeleton (31). Rho-GTPases are pivotal in regulating cellular morphology, motility and survival, and act as molecular switches through GTP binding and hydrolysis (32). Activated Rho family proteins, such as RhoA, are crucial in reshaping cytoskeletal dynamics, facilitating essential transformations in cell shape and movement that is crucial for tissue development and wound healing (33). Moreover, the results of previous studies highlighted the significant role of Rho-GTPases in cancer cell migration and cell adhesion, highlighting the importance of Rho-GTPases in cytoskeletal regulation (34,35). To further elucidate the role of INHBA in cytoskeletal regulation, western blot analysis was carried out using CAL-62 cells. The results of the present study highlighted that RhoA activation was decreased in the INHBA knockdown group; however, the activation of RhoB and RhoC remained unaltered (Fig. 4D-F). The role of the RhoA/ROCK pathway in various types of cancer is well-documented; for example, in gastric cancer, activation of the RhoA/LIMK/cofilin pathway has been shown to promote tumor metastasis (36); in pancreatic cancer, gastrin can induce focal adhesion formation and

cytoskeletal polarization through the RhoA/ROCK pathway, driving invasive migration (37); and in ovarian cancer, a stiffer matrix activates the RhoA/ROCK pathway, enhancing tumor cell migration and invasion (38). However its involvement in TC remains to be fully elucidated. Thus, downstream targets of the RhoA/ROCK pathway, including LIMK and cofilin, and the corresponding phosphorylation levels, were determined in TC cells. The results revealed that INHBA knockdown led to decreased phosphorylation levels of LIMK and cofilin, while INHBA overexpression resulted in increased phosphorylation levels of these proteins, further highlighting the impact of INHBA on the LIMK/cofilin pathway (Fig. 4G and H). Collectively, these findings further verify the role of INHBA in modulating the cytoskeletal architecture through the RhoA/ROCK signaling axis.

RhoA overexpression and treatment with a RhoA/ROCK axis inhibitor rescue INHBA expression in TC cells. To assess the role of RhoA in INHBA-mediated cell invasion and cytoskeletal protein regulation, the RhoA-OE plasmid was used; its overexpression efficiency in untreated CAL-62

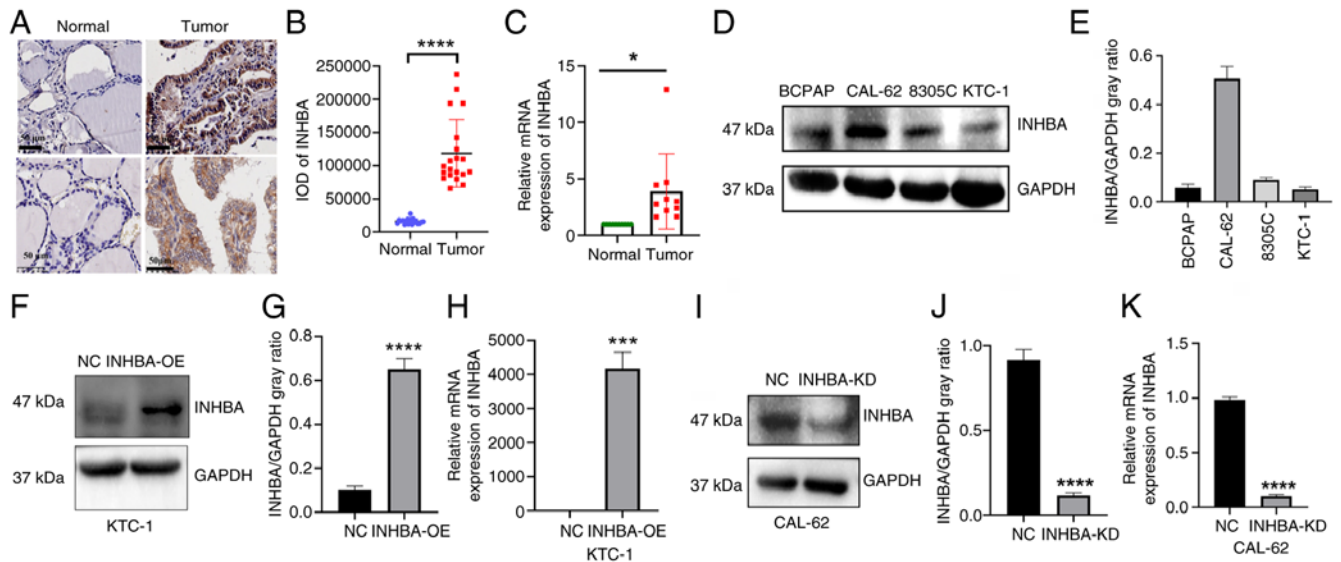


Figure 2. INHBA is highly expressed in TC tissues. (A) Immunohistochemical staining and (B) semi-quantitative analysis of INHBA in TC tissues (n=20). ****P<0.0001. (C) Reverse transcription-quantitative PCR demonstrated the increased expression of INHBA in TC tissues. *P<0.05. (D) INHBA expression was analyzed in TC cell lines using western blotting. (E) Semi-quantitative analysis of INHBA protein levels in TC cell lines from western blotting. (F) Transfection efficiency of INHBA-OE vectors in KTC-1 cells was analyzed using western blotting. (G) Semi-quantitative analysis of INHBA in KTC-1 cells. (H) Transfection efficiency of INHBA-OE vectors was analyzed using reverse transcription-quantitative PCR in KTC-1 cells. (I) Transfection efficiency of INHBA-KD was analyzed in CAL-62 cells using western blotting. (J) Semi-quantitative analysis of INHBA in CAL-62 cells. (K) Transfection efficiency of INHBA-KD was analyzed using reverse transcription-quantitative PCR in CAL-62 cells. ***P<0.001, ****P<0.0001 vs. NC. Data are presented as the mean $\pm$ SD. INHBA, inhibin β A; IOD, integrated optical density; KD, knockdown; NC, negative control; OE, overexpression; TC, thyroid cancer.

cells was validated using western blotting (Fig. 5A and B). Subsequently, RhoA overexpression was induced in CAL-62 cells with INHBA knockdown. The results demonstrated that p-LIMK and p-cofilin levels were rescued following RhoA overexpression (Fig. 5C). In addition, RhoA/ROCK inhibition was carried out using the Y27632 inhibitor at a concentration of 10 μ M for 24 h in KTC-1 cells with INHBA overexpression, and the results revealed that p-LIMK and p-cofilin levels were decreased (Fig. 5C). These results further highlighted the role of RhoA in this pathway. In addition, the present study revealed that migration and invasion was increased in CAL-62 cells with INHBA knockdown following RhoA overexpression (Fig. 5D, F, H and I), whereas migration and invasion was decreased in KTC-1 cells with INHBA overexpression treated with the RhoA/ROCK inhibitor Y27632 (Fig. 5E, G, H and J). Since RhoA is a downstream molecule of INHBA, these results suggested that INHBA may affect the phosphorylation and signaling of RhoA-LIMK and cofilin, ultimately promoting a more aggressive phenotype of TC.

INHBA knockdown significantly suppresses the metastasis of TC cells *in vivo*. To elucidate the biological function of INHBA *in vivo*, zebrafish and nude mouse xenograft models were used in the present study. INHBA knockdown significantly reduced the metastatic ability of TC cell-derived xenografts in 5-dpf zebrafish (Fig. 6A and B). Moreover, INHBA knockdown notably decreased the metastasis of CAL-62 cells injected into mice, resulting in fewer and smaller metastatic nodules, compared with the control group (Fig. 6C-F). H&E staining further confirmed the reduced metastatic ability of CAL-62 cells following INHBA knockdown (Fig. 6G). Collectively, these findings revealed that INHBA is a crucial regulator of distant metastasis in TC.

Discussion

The management of TC has advanced in recent years. Gene sequencing and mechanistic analysis have led to the identification of effective molecules for the diagnosis and treatment of TC. Multiple kinase inhibitors targeting key pathways may prolong the progression-free survival of patients, and these have been approved as a treatment option. For example, sorafenib and lenvatinib target the VEGF receptor and platelet-derived growth factor receptor pathways, and have been shown to be effective in treating radioiodine-refractory differentiated thyroid cancer (39,40). Additionally, dabrafenib and trametinib specifically target the MAPK/ERK pathway, particularly in patients with BRAF V600E mutations, helping to inhibit tumor growth and proliferation (41). Using the GEO database analysis, the results of the present study revealed that INHBA was markedly upregulated in TC. Further analysis of TC clinical data obtained from TCGA database demonstrated that the expression of INHBA was significantly positively associated with the T stage and N stage of patients with TC, suggesting that INHBA may be a key regulator driving TC aggressiveness. IHC and RT-qPCR analyses also revealed that INHBA expression levels were increased in TC tissues.

Shibata *et al* (16) used the nCounter PanCancer Progression panel to examine the expression levels of 740 genes associated with tumor progression in samples obtained from six low-risk and six high-risk patients with PTC. The results of this previous study indicated a notable increase in INHBA expression in patients with high-risk PTC, suggesting that INHBA exhibits potential as a biomarker for advanced PTC. Notably, these results are comparable with those of the present study, which demonstrated that INHBA was associated with the N stage of patients with TC. This association suggests that INHBA might

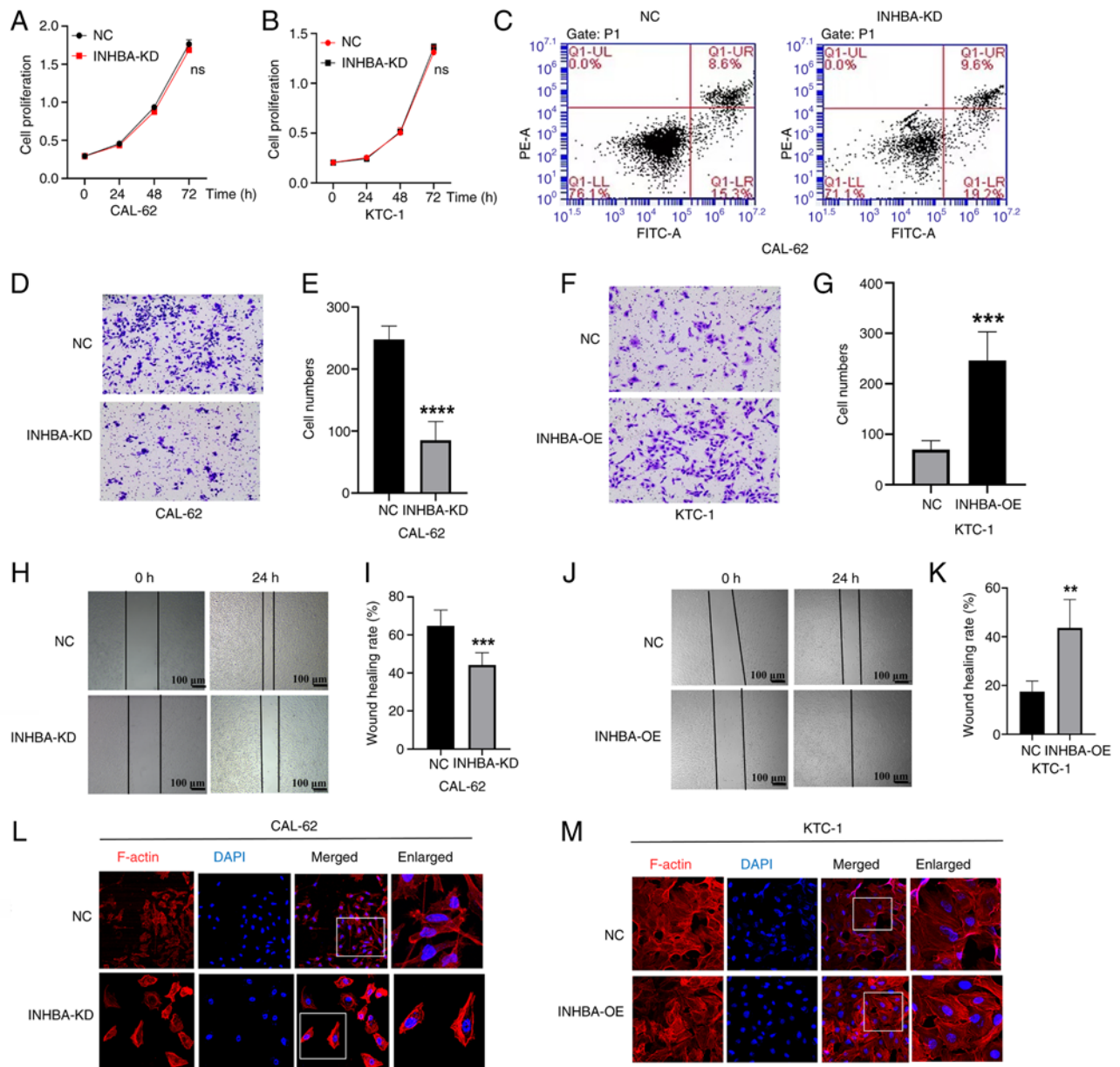


Figure 3. INHBA expression promotes the migration and invasion of TC cells. In (A) CAL-62 cells and (B) KTC-1 cells, the results of the Cell Counting Kit-8 assay demonstrated that INHBA expression had no effect on the proliferation of TC cells. (C) Effect of INHBA expression on apoptosis of CAL-62 TC cells was detected using the Annexin V assay. (D) INHBA-KD inhibited CAL-62 cell invasion; (E) corresponding statistical analysis. (F) INHBA-OE promoted KTC cell invasion; (G) corresponding statistical analysis. Magnification, x200). (H) INHBA-KD inhibited the migration of CAL-62 cells; (I) corresponding statistical analysis. (J) INHBA-OE promoted the migration of TC cells; (K) corresponding statistical analysis. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. NC. (L) Cytoskeleton of CAL-62 cells was visualized using confocal microscopy. (M) Cytoskeleton of KTC-1 cells was visualized using confocal microscopy. Magnification, x400. Data are presented as the mean $\pm$ SD. INHBA, inhibin βA ; KD, knockdown; NC, negative control; OE, overexpression; TC, thyroid cancer.

serve a crucial role in the metastatic process of TC. Previous studies have shown that INHBA expression is dysregulated in some types of cancer. For example, INHBA has been reported to be upregulated in colorectal cancer, and may promote tumor growth and metastasis through upregulating VCAN (42). A study on pancreatic ductal adenocarcinoma highlighted that INHBA promotes pancreatic cancer progression by enhancing tumor cell proliferation through a SMAD3-dependent signaling pathway (43). Additionally, a study on upper tract urothelial carcinoma emphasized that INHBA, via promoter hypomethylation, can significantly promote tumor progression by enhancing cell proliferation and migration (44). However, the specific roles and mechanisms of INHBA in TC remain to

be fully elucidated. In the present study, CAL-62 and KTC-1 cell lines, which respectively exhibit high and low INHBA expression levels, were used to investigate the biological functions of INHBA. Notably, INHBA knockdown reduced the migration of CAL-62 cells, whereas the opposite effect was observed in KTC-1 cells following INHBA overexpression. Moreover, *in vivo* studies confirmed that the downregulation of INHBA expression in TC cell lines markedly reduced tumor metastasis in xenograft models, including zebrafish and nude mice. These findings are consistent with those of previous reports on the pro-tumorigenic role of INHBA in other types of cancer (43-46). These findings indicated a broad relevance of INHBA in tumor progression and metastasis, and underscore

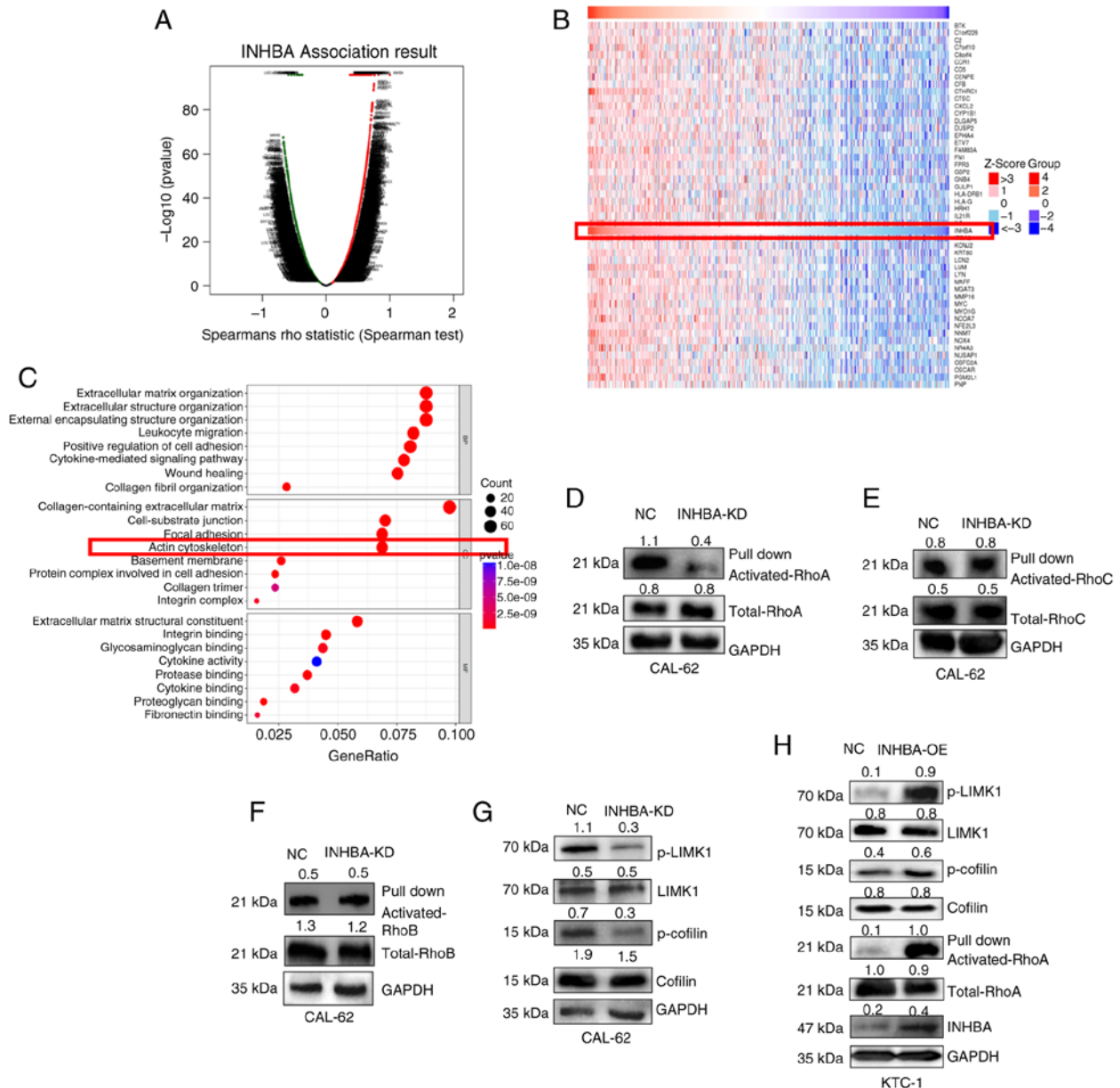


Figure 4. INHBA-KD affects cytoskeleton-related RhoA pathway proteins in TC cells. (A) Volcano plot of genes highly correlated with INHBA. (B) A heatmap demonstrated the top 50 genes positively correlated with INHBA in TC. (C) Gene Ontology analysis revealed that INHBA expression exerted a significant regulatory effect on 'actin cytoskeleton'. (D) RhoA, (E) RhoC and (F) RhoB expression and activation in CAL-62 TC cells with INHBA-KD. (G) Effects of INHBA-KD on the phosphorylation of proteins downstream of the RhoA/ROCK axis in CAL-62 cell lines. (H) Effects of INHBA-OE on phosphorylation of RhoA/ROCK pathway proteins in KTC-1 cells. INHBA, inhibin β A; KD, knockdown; LIMK1, LIM kinase 1; NC, negative control; OE, overexpression; p-, phosphorylated; TC, thyroid cancer.

the critical need for further investigation into the molecular mechanisms of the involvement INHBA of in TC biology and its potential as a therapeutic target in cancer treatment.

Since INHBA has been shown to promote cell migration *in vitro* and *in vivo*, further investigations into the specific mechanisms underlying the effects of INHBA on TC cell migration are required. In the present study, proteins associated with actin cytoskeleton regulation were determined using TCGA database and GO analysis, and the results of the present study revealed that INHBA knockdown or overexpression altered the morphology of TC cells.

Key processes in tumor metastasis, such as migration and invasion, require cytoskeletal rearrangement (47). Invasive cancer cells migrate along the basal membrane through a series

of forward protrusions and tail retractions, ultimately invading nearby tissues or distant organs. This process relies on the rearrangement of the actin cytoskeleton at the leading edge of the cell. The coordinated action of various actin regulators is crucial for the reorganization of the actin cytoskeleton (48,49). The cytoskeleton is composed of actin, microtubules and intermediate filaments. In cancer cells, mutations and the abnormal expression of cytoskeletal proteins reduce the sensitivity of cells to chemotherapy, and affect cell proliferation and migration. These aberrations disrupt regular cellular functions, thus facilitating the aggressive behavior typical of cancer cells (17,50,51). Activation of specific receptor proteins on the plasma membrane initiates cytoskeletal reorganization. These signals are mediated by the Rho GTPase family and its downstream effector,

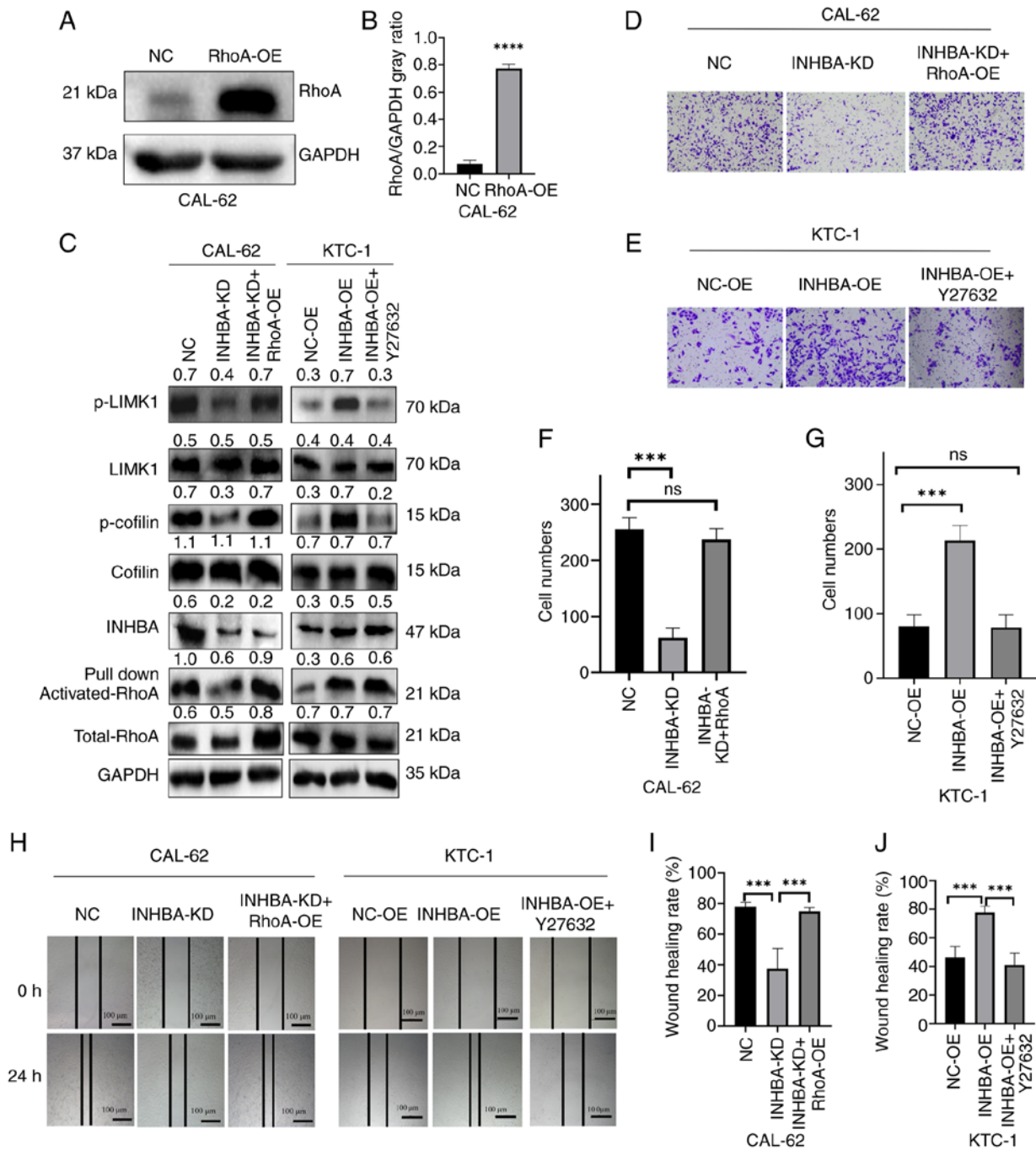


Figure 5. INHBA-mediated TC cell migration and invasion were regulated by the RhoA/ROCK/LIMK/cofilin pathway. (A) Transfection efficiency of RhoA-OE in CAL-62 cells; (B) corresponding semi-quantitative analysis. **** $P < 0.0001$ vs. NC. (C) RhoA OE or use of the RhoA downstream inhibitor Y27632 altered proteins downstream of the RhoA/ROCK axis in TC cell lines. (D) Rescue experiments were carried out in CAL-62 cells and analyzed using Transwell assays. (E) Statistical analysis of CAL-62 cell invasion from the rescue experiments. (F) Rescue experiments were carried out in KTC-1 cells and analyzed using Transwell assays. (G) Statistical analysis of KTC-1 cell invasion from the rescue experiments. Magnification, $\times 200$. (H) Rescue experiments were carried out in CAL-62 and KTC-1 cells and analyzed using wound healing assays. (I) Statistical analysis of CAL-62 cell migration from the rescue experiments. (J) Statistical analysis of KTC-1 cell migration from the rescue experiments. *** $P < 0.001$. Data are presented as the mean $\pm$ SD. INHBA, inhibin βA ; KD, knockdown; LIMK1, LIM kinase 1; NC, negative control; NS, not significant; OE, overexpression; p-, phosphorylated; ROCK, Rho-kinase; TC, thyroid cancer.

ROCK, constituting the Rho/ROCK signaling pathway (52,53). Notably, as a classical signaling pathway implicated in cytoskeletal reorganization, the RhoA/ROCK pathway impacts the metastasis of several types of cancer through regulation of the cytoskeleton (54,55). RhoA, the most extensively studied member of the Rho family (56), activates ROCK, leading to the phosphorylation of LIMK1 and LIMK2, at Thr508 and Thr505,

respectively. LIMKs are also associated with the progression and metastasis of various cancer types, such as glioma and gastric cancer (57,58). Cofilin, a direct downstream target of LIMK, is phosphorylated in its actin-binding domain, inhibiting its interaction with actin filaments and therefore regulating cytoskeletal dynamics. Previous studies have demonstrated that LIMK promotes cancer cell migration through regulating the

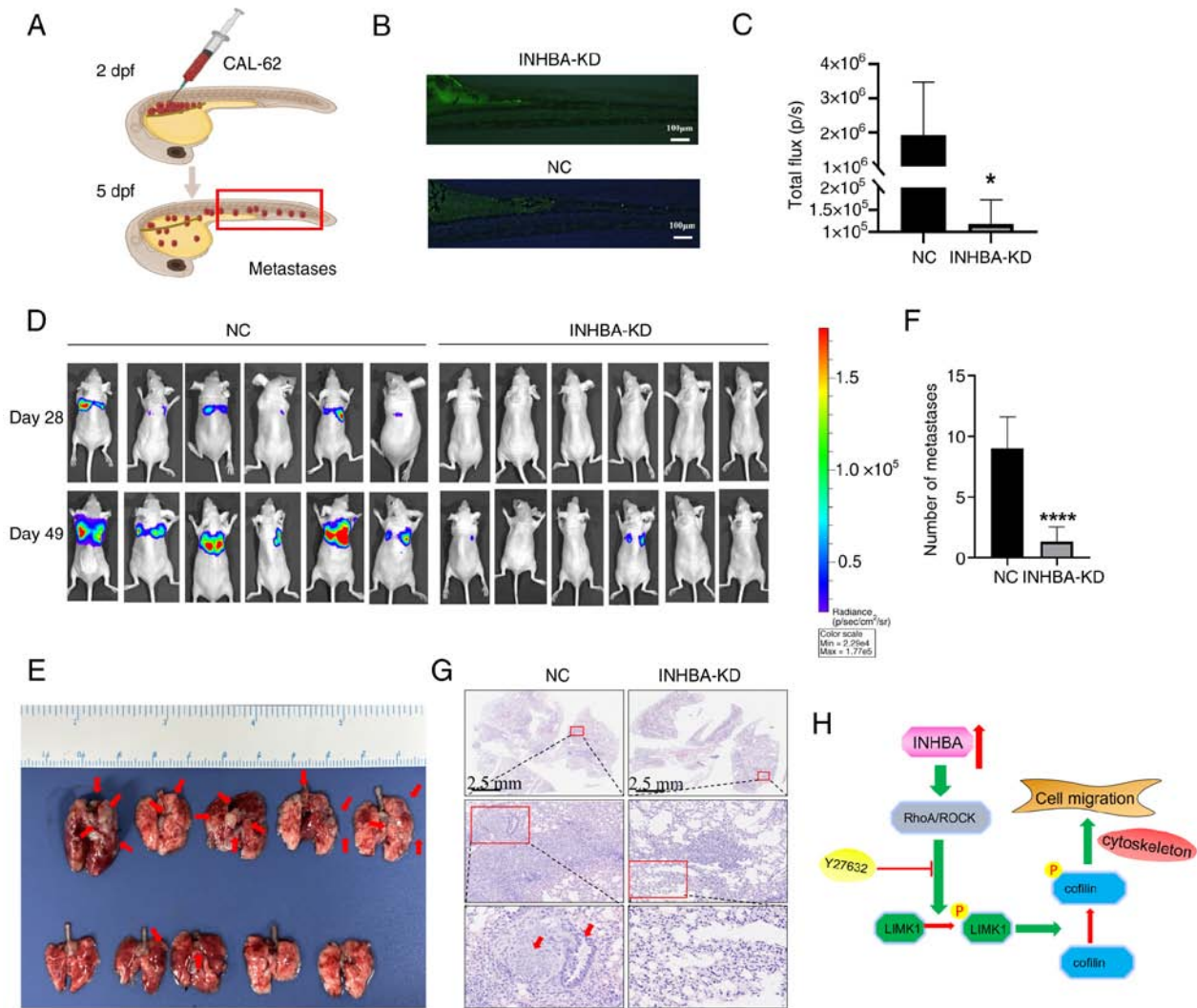


Figure 6. INHBA knockdown decreases the metastasis and growth of TC *in vivo*. (A) Schematic of the experimental procedure. CAL-62 cells (red) were implanted into the perivitelline space of each zebrafish. (B) Cell metastasis was investigated using a fluorescence microscope. (C) Quantitative analysis of the metastasis of CAL-62 cells in mice following INHBA knockdown. (D) IVIS of mice following an intravenous injection with CAL-62 cells. (E) Pulmonary metastasis in the hematogenous metastasis model using CAL-62 cells. Nodules are indicated using a red arrow. (F) Number of metastasis nodules (n=6). (G) Hematoxylin and eosin staining revealed the metastases of TC cells in lung tissues and metastatic carcinoma are indicated using red arrows. (H) Schematic diagram illustrating how INHBA might promote the metastasis of TC through the RhoA/ROCK pathway. * $P < 0.05$, **** $P < 0.0001$ vs. NC. Data are presented as the mean $\pm$ SD. INHBA, inhibin β A; KD, knockdown; LIMK1, LIM kinase 1; NC, negative control; ROCK, Rho-kinase; TC, thyroid cancer.

phosphorylation of cofilin, affecting the stability of actin filaments (59,60). The results of the present study indicated that phosphorylation of LIMK/cofilin and TC cell migration were induced by INHBA-mediated activation of the RhoA/ROCK signaling pathway.

To further substantiate that RhoA/ROCK is the downstream target influenced by altered INHBA expression, rescue experiments were performed in the present study. Y27632 was applied as an inhibitor of the RhoA/ROCK pathway. Notably, Y27632 is capable of impeding cytoskeletal reorganization, cell migration and proliferation, and it is well known that Y27632 can selectively inhibit ROCK, with minimal effects on other kinases (26). In the field of drug discovery, particularly in cancer research, Y27632 has demonstrated potential in multiple areas (61). The results of the rescue study revealed that Y27632 treatment reversed the phosphorylation of LIMK and cofilin induced by INHBA overexpression, leading to a significant reduction in TC cell migration. This highlights the critical role of the RhoA/ROCK pathway

in mediating the effects of INHBA on TC; INHBA-mediated activation of RhoA may be a key driver of cytoskeletal reorganization and cellular motility in TC. Collectively, these results confirmed the involvement of the RhoA/LIMK/cofilin pathway in INHBA-induced TC cell migration. Activation of the RhoA/LIMK/cofilin signaling pathway has been shown to promote tumor progression through the cytoskeleton in various cancer types (62,63); however, to the best of our knowledge, the activation of RhoA induced by INHBA overexpression has not been previously studied. The results of the present study demonstrated that INHBA overexpression may lead to the activation of RhoA, which in turn could impact phosphorylation of the LIMK/cofilin pathway, thereby promoting TC cell migration.

In conclusion, the present study demonstrated that INHBA is associated with TC metastasis by inducing RhoA activation, leading to phosphorylation changes in the LIMK/cofilin pathway, as illustrated in the hypothetical model (Fig. 6H). Clinical and cell line analyses, alongside *in vivo* experiments in xenografted

zebrafish and nude mice, showed that INHBA knockdown could significantly reduce metastasis. These findings suggested that INHBA may enhance the aggressive phenotype of TC cells through the RhoA/LIMK/cofilin signaling axis. However, the primary focus of the present study was on elucidating the role of INHBA in promoting TC metastasis at both cellular and animal levels. While functional validation of Y27632 was conducted at the cellular level, its effects on metastasis in mice were not within the scope of the current research, which represents a limitation of the study. Another limitation of the present study is the lack of information on the histological type and stage of the cancer tissues used for target validation. We did not further classify or stage the tissue samples, partly due to the limited sample size. Future studies will include this information to enhance the comprehensiveness and accuracy of the present findings. Despite this, INHBA shows potential as a biomarker and therapeutic target for aggressive TC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

WZ, ZL and WX designed the present study. WZ and YZ conducted the experiments. Statistical analysis was conducted by WW and WZ wrote the manuscript. WX and ZL revised the manuscript. WX and WZ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with ethical standards and was approved by the Ethics Committee of Shandong Provincial ENT Hospital. The ethics committee separately reviewed and approved the human studies and the animal studies as part of this project. Each study was subject to its respective ethical review standards. Despite the different ethical standards for human and animal research, both were rigorously reviewed and approved under the same project approval number (20220713). This unified approval number reflects the comprehensive review and approval by the ethics committee for the entire project. Written informed consent was obtained from the patients.

Patient consent for publication

Written informed consent was obtained for publication of the patient data and images.

Competing interests

The authors declare that they have no competing interests.

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