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The regulatory effect of CoL10A1 to the intracranial vascular invasion and cell proliferation in breast cancer via EMT pathway

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With advances in breast cancer (BC) treatment technology, although it could prolong the BC patients' survival, brain metastasis (BM) is increasing gradually. Patients with brain metastasis of breast cancer (BMBC) could have the decline of survival rate and quality of life. Investigate the regulatory role of Collagen Type X Alpha 1 Chain (CoL10A1) in BMBC process was the aim of this study. CoL10A1 expression was analyzed from TCGA database and clinical tissues, and then detected the regulation of CoL10A1 on BC cells proliferation, migration, and invasion in BC cell lines and mouse models. Our findings indicated that BMBC tissues have significant levels of CoL10A1 expression. BC cells proliferation, migration and invasion may be inhibited by knocking down Co10A1 in vitro and in vivo. In addition, we found that knocking down CoL10A1 could reduce the penetration of 468 cells into hCMEC/D3 cells. Knocking down CoL10A1 regulated the epithelial-mesenchymal transition (EMT) pathway related proteins expression. CoL10A1 could regulate BC cells proliferation, migration and invasion, affect the penetration into hCMEC/D3 cells in vitro, and inhibit the intracranial vascular invasion in mouse models. These results suggested that CoL10A1 may be a new target for treating human BMBC.

Keywords Breast cancer, CoL10A1, Breast metastasis, Epithelial–mesenchymal transition, Invasion

BC was often referred to as "pink killer", and has the highest incidence rate of malignant tumors in woman in the world, with male BC being comparatively uncommon, which had a great impact on women's physical and mental health[1–4]. Currently, commonly used treatment methods include surgery, radiotherapy and chemotherapy[5], could reduce the mortality of breast cancer, but recurrence and metastasis were still important and difficult points in clinical treatment[6]. In addition, target therapy[7] and immunotherapy[8] are becoming increasingly common. Brain metastasis (BM) of cancer mainly occurs in lung adenocarcinoma, melanoma and breast cancer[9]. Breast cancer could metastasis to the nervous system (CNS)[10,11]. When cancer cells travel form the primary tumors through the bloodstream to the brain and begin to proliferate, BM tumors will develop. Epidemiological survey showed that there are about 15–25% of brain metastasis comes from the breast cancer[12]. In recent years, the incidence of BMBC was still very high, which is the main risk factor threatening the health of patients. So, it is necessary to find early treatment of BMBC to improve prognosis.

The molecular mechanisms involved in BMBC, including the survival, migration, and adhesion of cancer cells, the role of the blood-brain barrier (BBB) in extravasation, and the interactions between cancer cells and resident cells in the brain[13]. Current treatment options for BMBC include surgery, whole-brain radiation therapy, stereotactic radiosurgery, and chemotherapy[14]. However, the prognosis for patients with BMBC remains poor due to the limited permeability of the BBB, chemoresistance, and the heterogeneity between primary breast cancer and BMBC[15]. New therapies include innovative research on novel targeted therapies, immunotherapy, and nanotherapies to improve the prognosis of patients with brain metastases[16]. The complexity of BMBC indicates that developing new effective treatment methods is necessary.

CoL10A is a secreted collagen protein and belongs to the collagen family[17]. Research has found that CoL10A1 has limited expression in normal tissues and increased expression in many tumor tissues[18,19]. The reports showed that CoL10A1 in colon cancer tissue was highly expression[19]. Tissue microarray (TMA) analysis found

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that overexpression CoL10A1 was linked to poor prognosis, and may enhance the cell proliferation, migration, and invasion in colorectal cancer[19]. CoL10A1 promoted the invasion of gastric cancer cell via TGF-β1-SOX9 axis[20]. CoL10A1 promoted choroidal neovascularization (CNV formation), which may be a candidate target for the diagnosis and therapy of other neovascular diseases in age-related macular degeneration (AMD)[21]. So, we speculate that CoL10A1could also promote cells proliferation, migration, and invasion in BMBC.

The role of CoL10A1 in various cancer types and its potential association with BMBC could be understood through several research studies. For example, one study reveals that CoL10A1 was involved in the EMT process in gastric cancer[20], which is a key mechanism that enables cancer cells to metastasize. Since EMT is a common process in various cancers, including BC, it's plausible that CoL10A1 could contribute to the metastatic spread of BMBC. Another study directly indicated that CoL10A1 is associated with cancer progression[22]. Given that CoL10A1 was associated with poor prognosis and may play a role in the process of metastasis. While these studies do not focus on BMBC, they collectively highlight the multifaceted role of CoL10A1 in cancer progression, suggesting that it could be a factor in the spread of BC to various organs, including the brain.

In our study, we discovered that CoL10A1 was highly expressed in BMBC tissues of clinical samples. To demonstrate the potential function of CoL10A1 in BMBC, we knockdown CoL10A1 in BC cell lines. We investigate the regulation of cell proliferation, invasion and metastasis by knocking down CoL10A1. In addition, we found that the EMT signaling pathway is involved in BC regulation, and investigated the correlation between CoL10A1 and EMT pathway. Our studies may provide new therapeutic strategies for BC.

Materials and methods

Bioinformatics analysis

The mRNA expression data of human BC were extracted from TCGA (The Cancer Genome Atlas, http://portal.gdc.cancer.gov) database, which included 291 normal tissues and 1085 BC tissues. The expression of CoL10A1 was analyzed between BC tissues and normal tissues by software R (4.2.1) version, the data processing method is Log2 (value + 1), and the statistical method is Wilcoxon rank sum test.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA, http://www.broad.mit.edu/gsea) was used to analyze CoL10A1 expression associated with the biological processes or pathway. In the process of gene set selection, the MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp) database was used to identify relevant gene sets based on their biological correlation with CoL10A1. Samples were divided into high- and low- expression groups using the median as the cutoff. We chose the median as the dividing criterion because it is a robust measure of central tendency that minimizes the influence of outliers. The Benjamini Hochberg program was used to control the False Discovery rate (FDR) for handing multiple hypothesis testing. FDR < 0.25 indicates that this gene set is significantly enriched.

Clinical sample collection

The BMBC samples were collected from patients who were with brain metastasis from brain cancer requiring surgery, non-tumor samples from traumatic brain injury requiring surgery were collected as control group, the BC samples and paired adjacent samples were removed from patients who were having surgery. All the BMBC ($n = 19$), non-tumor samples ($n = 5$), the BC sample ($n = 4$) and paired adjacent ($n = 4$) were collected at Beijing Tiantan Hospital, and forzen in liquid nitrogen as soon as the tumor was removed. The preliminary processing of RNA extraction was carried out before cryopreservation. These samples were stored in liquid nitrogen for up to 12 months. In addition, we regularly added liquid nitrogen (once a week) and regularly checked the storage environment to ensure optimal conditions for sample preservation. Our collection obtained informed consent from all patients, and our operations strictly follow the guidelines of the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (KY2022-048-01).

Cell culture

We purchased human BC cells MDA-MB-468 (468), MDA-MB-231 (231), MDA-MB-453 (453), MCF7, BT474, HS578T, human normal mammary epithelial cells MCF10A from Cell Resource Center, Institute of Medical Basic sciences, Chinese Academy of Medical Sciences (Beijing, China). Human brain microvascular endothelial cells hCMEC/D3 were obtained from Beijing tiantan Hospital, Capital Medical University. Mouse breast cancer cells 4T1 cells were obtained from ATCC (ATCC, catalog CRL-2539), and cultured in RPMI 1640 (corning, USA) complete culture medium. MCF7, 231, 453, 468, BT474, HS578T, MCF10A and hCMEC/D3 cells were cultured in DMEM (corning, USA) complete culture medium. The complete culture medium contains 90% 1640 or DMEM, 10% fetal bovine serum (FBS, Lonsera, USA), and 1% penicillin/streptomycin (Solarbio, Beijing, China). All cells were cultured in a 5% CO_2 incubator.

Cell transfection

The siRNA sequence (seq) of CoL10A1 was as follows: siRNA-1 (1828): SS Seq: 5′-GCAACAGCAUUAUGACCCATT-3′,

AS Seq: 5′-UGGGUCAUAAUGCUGUUGCTT-3′; siRNA-2 (228): SS Seq: 5′-CCUACACCAUAAAGAGUAATT-3′, AS Seq: 5′-UUACUCUUUAUGGUGUAGGTT-3′; siRNA-3 (2013): SS Seq: 5′-CCAUCAUCGAUCUCACAGATT-3′,

AS Seq: 5′-UCUGUGAGAUCCAUGAUGGTT-3′) were synthesized by GenePharma (Shanghai, China). Then, siRNA-expressing were transfected into 468 cells using Lip3000 (Thermo Fisher, USA) with a fusion rate of 80%. After 72 h, the transfection efficiency was determined by qRT-PCR and Western Blot.

Quantitative real-time PCR (qRT-PCR)

Total RNA were extracted from the BC cells or tissues using RNAiso Plus (Takara, Beijing, China) reagent. RNA was reverse transcription into cDNA with the-DNA Synthesis kit (Novoprotein, Suzhou, China). Quantitative real-time PCR system was used to amplify all transcripts using the SYBR q-PCR kit (Novoprotein, Suzhou, China). Primer sequences are as follows:

CoL10A1: F 5′-TTTGGGGTTTCGCCCTATCC-3′, R 5′- CTGGGGGTGAGGGGACTAAA-3′;
Actin: F 5′-CCTTCCTTCCTGGGCATGG-3′, R 5′- GATCTTCATTGTGCTGGGTGC-3′.
The specific reaction conditions are as follows:

Temperature	Time	Number cycles
95℃	1 min	1
95℃	$\left.\frac{20\text{ s}}{1\text{ min}}\right\}$	40
60℃		

Western blot

Total protein lysates (30 μg) were separated by 10% SDS-PAGE gel electrophoresis, 80v, 30 min, then switch to 100v, 1 h for constant voltage electrophoresis. Then, transferred to PVDF membranes, 200 mA, 90 min on ice for transfer, and blocked for two hours in 5% nonfat milk, and incubated at 4 ℃ overnight with primary antibody. The primary antibody was used at dilutions of 1:800 (anti-CoL10A1 Rabbit, Affinity, Jiangsu, China); 1:5000 (anti-PCNA Rabbit, proteintech, Wuhan, China); 1:2000 (anti-c-MYC Rabbit, proteintech, Wuhan, China); 1:1000 (anti-ki-67 Rabbit, abcam, UK); 1:5000 (anti-β-Catenin Rabbit, proteintech, Wuhan, China); 1:800 (anti-N-cadherin Rabbit, Wanleibio, Shenyang, China); 1:1200 (anti-SLUG Rabbit, Wanleibio, Shenyang, China); 1:1200 (anti-SNAIL Rabbit, Wanleibio, Shenyang, China); 1:800 (anti-E-cadherin Rabbit, Wanleibio, Shenyang, China); 1:500 (anti-Vimentin Rabbit, Wanleibio, Shenyang, China); 1:800 (anti-ZO-1 Rabbit, Wanleibio, Shenyang, China); 1:300 (anti-ZEB1 Rabbit, Wanleibio, Shenyang, China); 1:10,000 (anti-β-actin, proteinteh, Wuhan, China). An HRP-conjugated secondary antibody (Goat anti Rabbit, proteinteh, China) at dilutions 1:10,000 was applied for 1 h at 37 ℃. ECL luminescence liquid was used to detect the intensity of each protein, and quantitative analysis with Image J. In addition, β-actin was used as a loading control and that its expression was consistent across all samples.

Cell proliferation

Cells (5×10^3) were inoculated in a 96-well plate and placed in culture incubator for future cultivation. Divided into 3 groups, 468 group (blank control), siRNA-1 and siRNA-2 groups, we detected two at 0, 24 h, 48 h and 72 h. Respectively, removed the corresponding culture plates, and added 10 μl of CCK-8 to each well for another 1 h at incubator. And we measured the absorbance at 450 nm using a microplate reader (Epoch2, BioTek, USA).

Colony formation assay

500 cells were inoculated in 6-well plate and cultured in DMEM complete medium for the colony formation assay. After 2 weeks cultured, discard the supernatant and washed with PBS. Fixed 30 min with 4% paraformaldehyde (Solarbio, Beijing, China), and stained 30 min with 0.1% crystal violet (Beyotime, Shanghai, China), and photographed under a light microscope (Nikon, Japan).

Wound healing assay

Cells (5×10^5 /well) were inoculated into 6-well plate and cultured to approximately 90% confluence. We used a 10 μl plastic tip to form a wound at cell surface. Then, we washed the floating cells with PBS, and photographed the cell images after make wound 24 h using a microscope (Nikon, Japan).

Cell migration and invasion

Transwell assays were used to detect cell migration and invasion (Constar, 3422, USA). For cell migration, Cells (5×10^4/100 μl) were inoculated into the upper chamber, then added 600 μl of complete culture medium into the lower chamber, and incubated in an incubator for 24 h. We wiped gently the free cells in the Transwell upper chamber with a cotton swab, fixed and stained with crtstal violet. Taken photos and counted to calculate the migration ability of 468 cells under a microscope.

For cell invasion, Transwell assays were employed to examine the effect of different treatment methods on endothelial cells (ECs) barrier function. hCMEC/D3 (1×10^5) cells were seeded inn the transwell upper chamber, while added 600 μl of complete culture medium into the lower chamber and placed in the incubator for cultivation overnight. 468 cells (5×10^4) were labeled with CM-DIL (red, Invitrogen, USA) and inoculated onto the h-CMEC/D3 cells. After 2 days, we wiped gently off the free cells in the upper chamber with a cotton swab, fixed and then stained with DAPI (Beyotime, Shanghai, China). Taken photos and counted cells under fluorescence microscope to calculate the invasion ability of 468 cells. The migration and invasion experiments were independently repeated three times.

Internal carotid artery injection

Female C57BL/6 N mice (6–8 weeks) were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China). We used a total of 6 mice, the mice were randomly divided into 2 groups, 4T1 cells (shNC), and 4T1 cells with Col10A1 knock-down (shCoL10A), with 3 mice in each group. Every mouse was anesthetized and

fixed on a rubber plate. Shaved off the hair and expose the skin on the neck. Find a suitable area for dissection until exposed the common carotid artery. Firstly, tighten the common carotid artery's proximal end and release its distal end using a 5-0 silk suture material. Made a little common carotid artery incision and slowly injected 100 μl of cell suspension into the arterial lumen, than tied the distal ligature tightly, and sutured the skin. After the mouse waked up, placed them back in original place to continue feeding and monitor them status.

IVIS imaging

Every mouse was intraperitoneal injection of 150 mg/kg D-luciferin (MedChemExpress, Beijing, China). After 5 min, the mice were anesthetized, and an IVIS200 camera (Waltham, MA, USA) was used for bioluminescence imaging. The exposure time was set to 20 s and the light intensity to 80%. Utilizing luciferase and substrates to generate light signals in vivo, the idensity of these light signals was detected by an IVIS system to quantitatively analyze biological processes. The data were analyzed with the IVIS software (Living Imaging Software for IVIS). After the experiment, all animals were euthanized using a mixed method. Firstly, the animals were anesthetized with 2% isoflurane and then euthanized using physical methods. All methods were performed in accordance with the American Veterinary Association's (AVMA) Animal Euthanasia Guidelines.

Statistical analysis

All data were performed statistical analysis using GraphPad prism 8. Using repeated analysis of variance, Bonferroni test, and the Student's t-test to examine how the experimental and control groups differed from one another, and display with mean ± SD, $p < 0.05$ was considered to have a difference, and the difference was statistically significant.

Results

The overexpression of CoL10A1 in BMBC

To investigate CoL10A1 expression in BC, we analyzed CoL10A1 expression in TCGA database. The results indicated that CoL10A1 in BC tissues was highly expressed, and low expressed in normal tissues (Fig. 1A,B). The expression of CoL10A1 in human tissues and organs was analyzed using the consensus dataset of the Human Protein Atlas (HPA), and the results were shown in Fig S1.We further validate this result using clinical samples. The results showed that CoL10A1 expression in breast cancer tissues was higher than that in adjacent tissues (Fig. S2A,B). In addition, we detected CoL10A1 expression in 19 BMBC samples and 5 non-tumor samples at RNA and protein levels, respectively. The results showed that CoL10A1 was highly expressed at mRNA and protein level in BMBC tissues (Fig. 1C,D). We also provided the clinical basic data of BMBC patients (Table S1). These results indicated that CoL10A1 in BMBC tissues was highly expressed.

The expression of CoL10A1 in breast cancer cell lines

First, we analyzed CoL10A1 expression in BC cell lines, MCF7, 231, 453, 468, BT474, HS578T and human normal mammary epithelial cells MCF10A. Our results showed that except BT474 cells and 231 cells, the mRNA expression of CoL10A1 (Fig. 2A) in other BC cell line was higher than that in MCF10A, in addition, the protein expression of CoL10A1 (Fig. 2B) in BC cell lines was higher than that in MCF10A. And the 468 cells were selected for knocking down CoL10A1. Hence, we transfer 468 cells with 3 different siRNA to knockdown CoL10A1, all of which significantly reduced the expression of CoL10A1 at the mRNA (Fig. 2C) and protein (Fig. 2D) level in 468 cells, and select siRNA-2 and siRNA-3 for cell function. In addition, the MCF7 cells was selected for overexpression CoL10A1. The results showed that the transfection efficiency was detected by q-PCR and Western blot (Fig. S4A,B).

CoL10A1 promotes breast cancer cells proliferation

CCK8 assays was used to investigate the cell proliferation by CoL10A1 knockdown, the results indicated that CoL10A1 knocking down inhibited 468 cells proliferation ($p < 0.001$) compared with that in the siNC group (Fig. 3A). In addition, Overexpression CoL10A1 promoted cell proliferation (Fig. S4C). We also analyzed proliferation related protein expression, such as PCNA, c-MYC and Ki67. Our results showed that the proliferation protein expression were significantly lower expression in CoL10A1 knocking down group (Fig. 3B,C). The clone forming ability of 468 cells were detected using the colony formation assay, which showed that the clone formation ability was inhibited in CoL10A1 knocking down group (Fig. 3D). We found that knocking down CoL10A1 inhibited the cloning ability of 468. According to the studies above, CoL10A1 plays a significant part in BC cells proliferation.

CoL10A1 promotes breast cancer cell migration and invasion

Transwell assays were used to detect how CoL10A1 affected on 468 cells migration and invasion. We analyzed the migration rate of 468 cells using wound healing at 2 days, which showed that the migration rate of 468 cells was 1.0, which was significantly reduced after transfection with siRNA (siRNA-2 was 0.62, siRNA-3 was 0.18) compared to siNC group, (Fig. 4A,D, $^{**}p < 0.01$; $^{***}p < 0.001$). We also used Transwell assay to detect the migration rate of 468 cells at 24 h, which showed that the migration rate in transfected 468 cells with siRNA groups were significantly reduced (0.76 for siRNA-2 and 0.30 for siRNA-3) when compared with 1.0 in negative control group, (Fig. 4B,E, $^{*}p < 0.05$; $^{***}p < 0.001$). In addition, overexpression of CoL10A1 promoted the migration of MCF7 cells (Fig. S4D). These results showed that CoL10A1 regulate breast cancer cell migration and invasion.

CoL10A1 influences the permeability of the BBB and tumor cell invasion

Transwell assays were used to detect the ability of CoL10A1 to penetrate the BBB in 468 cells. To further detect the penetration of CoL10A1 transfected 468 cells into hCMEC/D3 cells. Incubated 468 cells stained with CM-

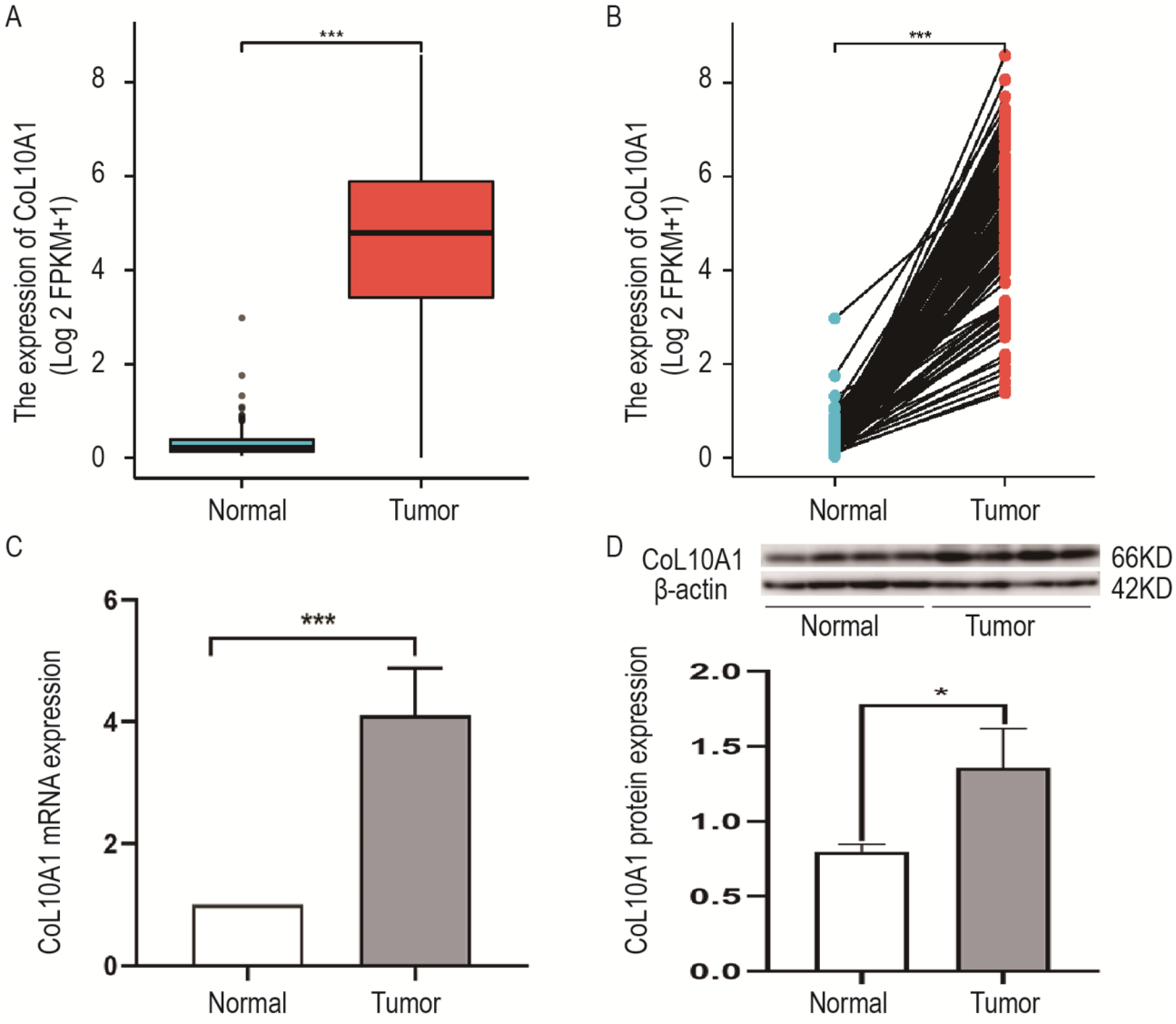

Fig. 1. CoL10A1 is highly expressed in BC tissues. (**A**,**B**) CoL10A1 was highly expressed in BC tissues ($n = 1085$) compared to normal tissues ($n = 291$) in the TCGA database. (**C**) qRT-PCR detected the mRNA expression of CoL10A1in clinical BMBC samples ($n = 19$) and normal tissues ($n = 5$). (**D**) Western Blotting detected the protein expression of CoL10A1 (66KD) in clinical BMBC samples ($n = 19$) and normal tissues ($n = 5$). $^{*}p < 0.05$, $^{***}p < 0.001$.

DIL onto hCEMC/D3 cell monolayers and cultured in incubator. Then counted the 468 cells passed through under a fluorescence microscope. The results showed that there had fewer 468 cells 468 cells passed through in the knockdown CoL10A1 group compared with that in the siNC group (Fig. 4C,F; $^{*}p < 0.05$; $^{***}p < 0.001$). The bar chart indicated that the percentage of invasion rate 1.0 in 468 group, which was significantly reduced after transfection with siRNA (siRNA-2 group was 0.76, siRNA-3 group was 0.23). In addition, overexpression of CoL10A1 promoted the ability of MCF7 cells to cross hCEMC/D3 cells (Fig. S4E). These results indicated that CoL10A1 regulated 468 cells migration and invasion.

CoL10A1 regulates the cell migration and invasion via the EMT

One way of cancer metastasis is through EMT. Previous studies showed that the EMT pathway was involved in regulate cell migration and invasion in various types of cancer, such as glioblastoma[23], lung cancer[24], and bladder cancer[25]. GSEA was used to evaluate pathway related to CoL10A1 in the TCGA database. The results showed that CoL10A1 expression was associated with ECM receptor interaction signing pathway (Fig. 5A). Ander Levchenko's research found that changes in ECM like cell adhesion matrix could regulate the process of EMT[26]. Therefore, we speculate that CoL10A1 may regulate BC cells migration and invasion via the EMT pathway. We evaluate whether EMT pathway could regulate migration and invasion of CoL10A1 regulated BC cells. The expression of EMT related proteins, such as E-cadherin, β-catenin, N-cadherin, Vimentin, Slug, Snail, ZEB1 was detected (Fig. 5B,C). The results indicated that the expression of β-catenin and E-Cadherin was elevated, and

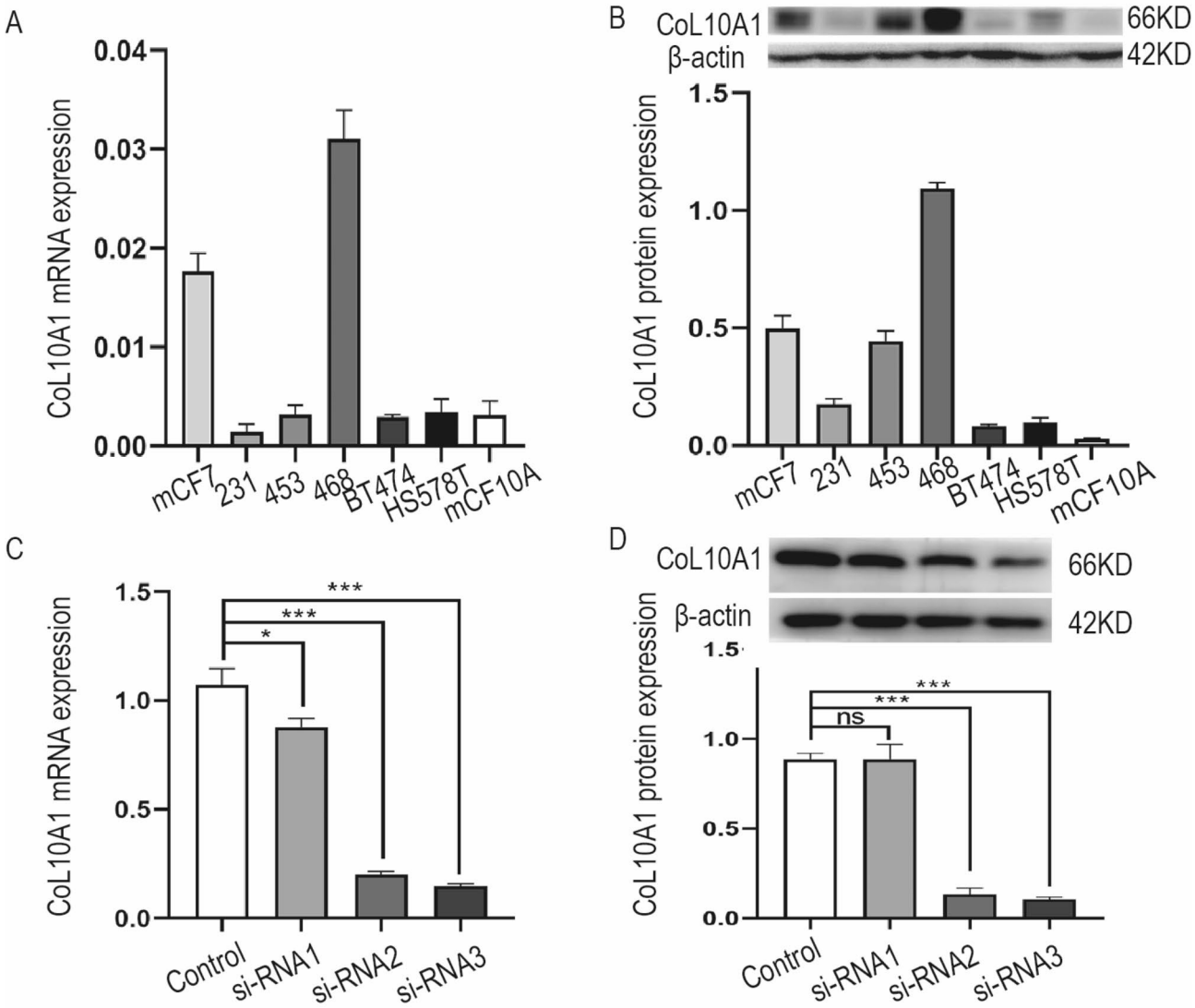

Fig. 2. CoL10A1 expressed in BC cell lines. (**A**) CoL10A1 mRNA expression in BC cell lines MCF7, 231, 468, BT474, HS578T compared to MCF10A. (**B**) Western Blotting detected CoL10A1 (66KD) expression in BC cell lines. (**C**) q-PCR detected the efficiency of 468 cells knocking down CoL10A1 groups (siNC, siRNA-1, siRNA-2, siRNA-3) $^{*}p < 0.05$, $^{***}p < 0.001$. (**D**) Western Blotting detected knockdown efficiency of 468 cells, $^{*}p < 0.05$, $^{***}p < 0.001$ compared to siNC group.

the expression of N-cadherin, Vimentin, Slug, Snail and ZEB1 was reduced. These results suggest that CoL10A1 regulated cell migration and invasion via the EMT pathway.

Down-regulation of CoL10A1 suppresses tumorigenicity in vivo

We have demonstrated that CoL10A1 could promote BC cells proliferation, migration and invasion. Furthermore, we detected whether CoL10A1 has a tumor-promoting effect in animal models. Construction of brain metastasis models of breast cancer by injecting cells into internal carotid artery. The stable CoL10A1 knockdown in 4T1 cells were injected into nude mice, displayed the location of the tumor in the brain, and significantly slowly growth in CoL10A1 knockdown group (Fig. 6A,B). In addition, histopathology images of the brain sections to prove the metastatic lesions, the results showed that knocked down CoL10A1 could inhibit the lesions of brain metastases (Fig. S5A,B). There results suggested that knocked down CoL10A1 could inhibit the tumor growth in nude mice models.

Discussion

BC is the tumor with highest incidence rate among woman in the world, ranking first in the global female malignant tumor, which endangers women's physical and mental health and quality of life[1–4]. BC is recurrence and metastasis, which is still a difficult point in clinical treatment[27]. However, there is a lack effective prognostic

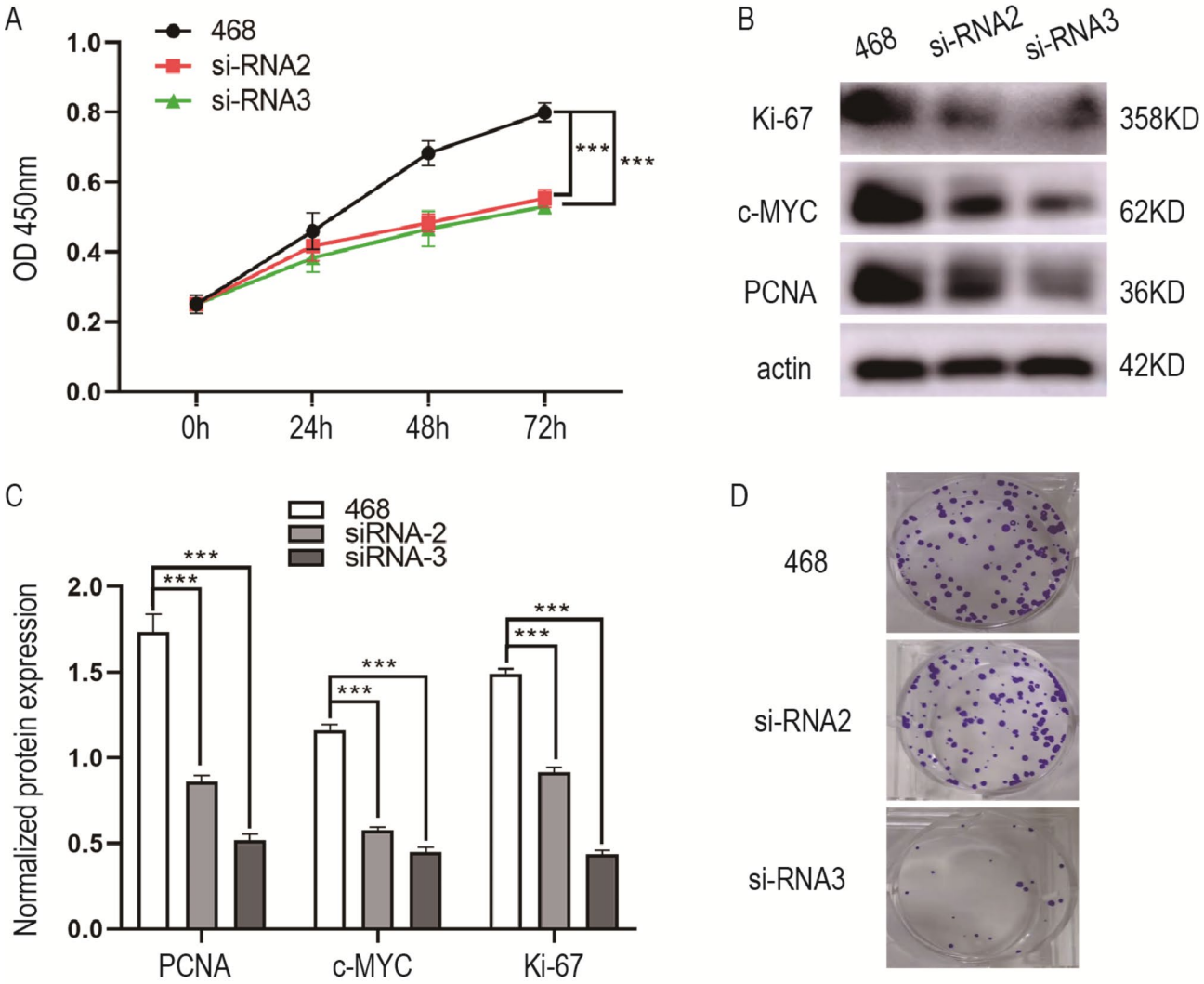

Fig. 3. CoL10A1 inhibited 468 cells proliferation. (**A**) CCK-8 assays detected cells proliferation, $^{***}p < 0.001$ compared to siNC group. (**B**) Western Blotting detected the proliferation related proteins (PCNA (36KD), c-MYC (62KD), and Ki67 (358KD)) expression. (**C**) Quantitative analysis of proliferation related proteins, $^{***}p < 0.001$. (**D**) The image of the colony formation assay.

target in clinic, so it is urgent to find new prognostic target. New prognostic target not only predict the risk of recurrence, but also help guide individualized clinical treatment.

In our study, we used the TCGA database to identify the differential expression of CoL10A1 in breast cancer and normal tissues. Then, we evaluated the prognosis value of CoL10A1 in cancer patients to analysis its relationship with clinical outcomes such as overall survival and Disease Specific Survival (Fig S3). In addition, we reviewed the current literature, including the study by Weijian Zhou et al.[28], which reported that high expression of CoL10A1 promotes breast cancer progression and predicts poor prognosis, particularly for DMFS. This study supports the focus on CoL10A1 in BM. In conclusion, through a systematic approach involving bioinformatics analysis, prognostic value assessment, we have identified CoL10A1 as a potential candidate gene for BMBC. While CoL10A1 may have roles in other types of metastases, our date suggest that its involvement is particularly significant in BM. Further research is warranted to explore the mechanisms underlying this specificity and to develop targeted therapies based on these findings.

CoL10A1 is a secreted protein with 4 transcripts, consisting of three homologous trimmers forming the a1 (X) chain[29]. In numerous studies, the NC1 domain of collagen X has been proven to be crucial for subunit interactions and trimerization of collagen X molecules[30]. CoL10A1 was high expression in many tumor tissues[31]. CoL10A1 was involved in cell proliferation, invasion and metastasis of various malignant tumors, which may regulate the prognosis of malignant tumor by affecting cells proliferation, apoptosis, invasion and metastasis[32,33]. However, there are few studies on the role of CoL10A1 in BC[34]. Our results indicated that CoL10A1 was up-regulated in human BMBC tissues, and promoted proliferation, migration and invasion of 468 cells. The nude mouse models of brain metastasis indicated that knocking down CoL10A1 inhibited the formation of BMBC.

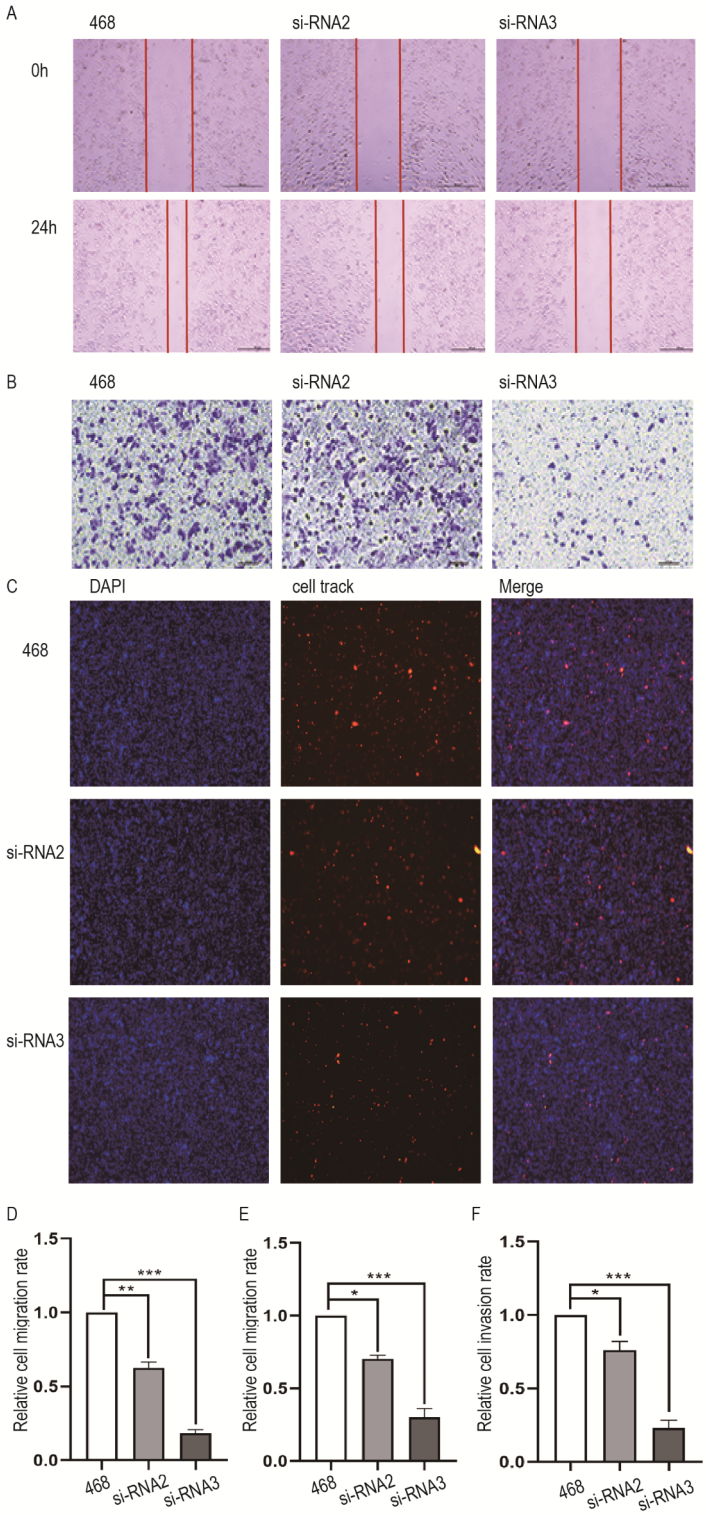

Fig. 4. CoL10A1 inhibited migration and invasion of 468 cells. (**A**) Wound healing assay detected the migration (×100). (**B**) Transwell assay detected the migration cells (×100). (**C**) Transwell assay detected the invaded cells through the hCMEC/D3 cell monolayer (×100), a number of 468 cells stained in red, and nucleus stained in blue with DAPI. (**D**) Quantitative analysis of cells migration rate, $^{**}p < 0.01$, $^{***}p < 0.001$. (**E**) Quantitative analysis of relative cells migration rate, $^{*}p < 0.05$, $^{***}p < 0.001$. (**F**) Quantitative analysis invaded hCMEC/D3 cell monolayer, $^{*}p < 0.05$, $^{***}p < 0.001$.

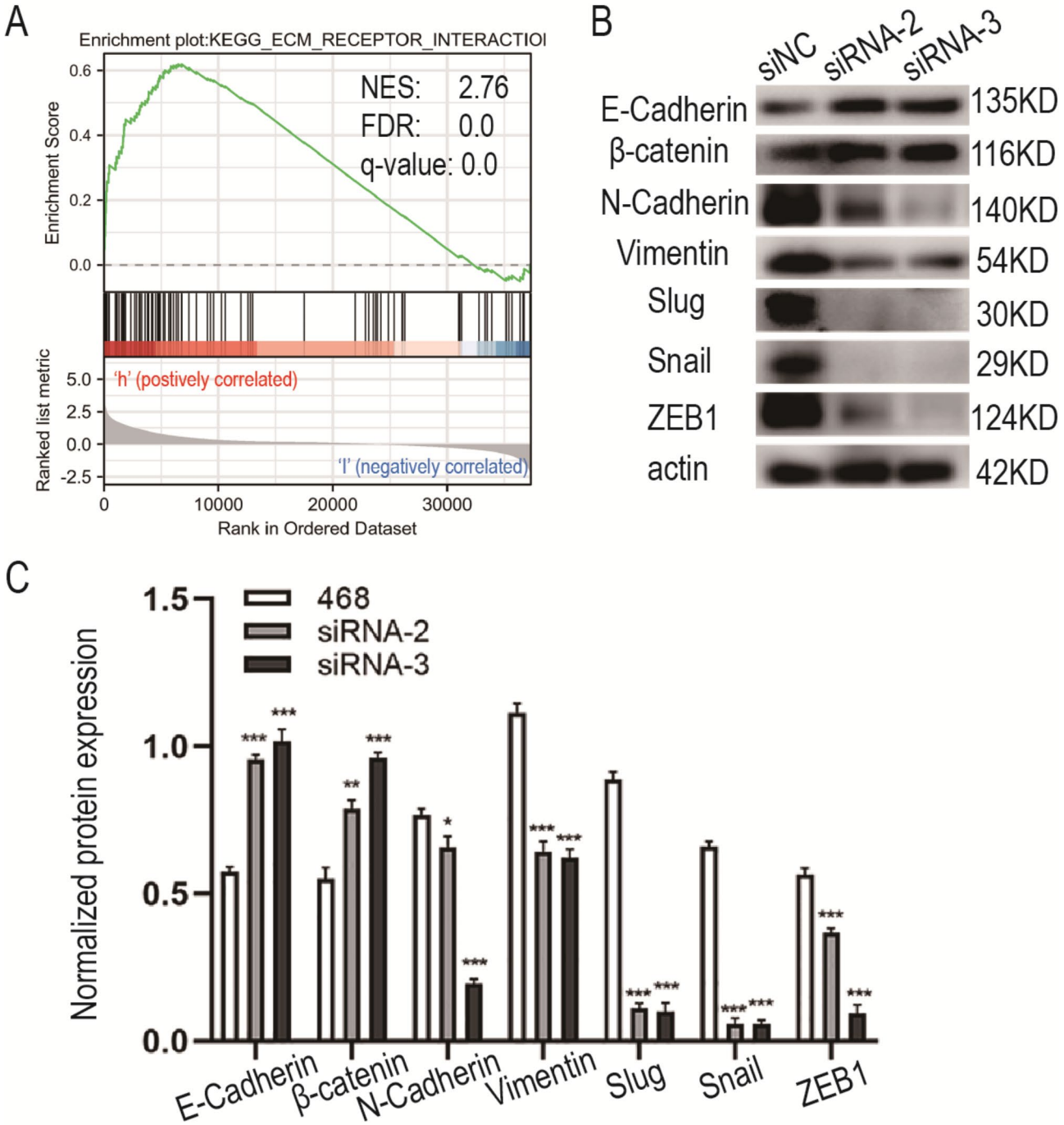

Fig. 5. EMT signaling pathway was involved in CoL10A1 induced 468 cells migration and invasion. (**A**) GSEA results showed that CoL10A1 exerts regulatory effects via ECM receptor interaction pathway activation. (**B**) Western Blotting detected the EMT pathway related proteins (E-cadherin (135KD), β-catenin (116KD), N-cadherin (140KD), Vimentin (54KD), Slug (30KD), Snail (29KD), ZEB1 (124KD)). (**C**) Quantitative analysis of EMT related proteins. $^{**}p < 0.01$, $^{***}p < 0.001$.

The regulatory mechanisms of CoL10A1 in cancers, particularly in BMBC, are complex and multifaceted. CoL10A1 plays a significant role in the ECM[35] and has been found to be associated with cancer progression, including BC[36]. In BMBC, the specific mechanisms of CoL10A1 regulation may differ due to the unique characteristic of the brain microenvironment and the blood-brain barrier. The elevated expression of CoL10A1 in BC can enhance their ability to invasion and migration, potentially facilitating BM. However, the exact mechanisms by which CoL10A1 contributes to BMBC may involve different signaling pathways or interactions with other proteins that are specific to the brain environment. Understanding the specific regulatory mechanisms of CoL10A1 in BMBC is crucial for developing targeted therapies.

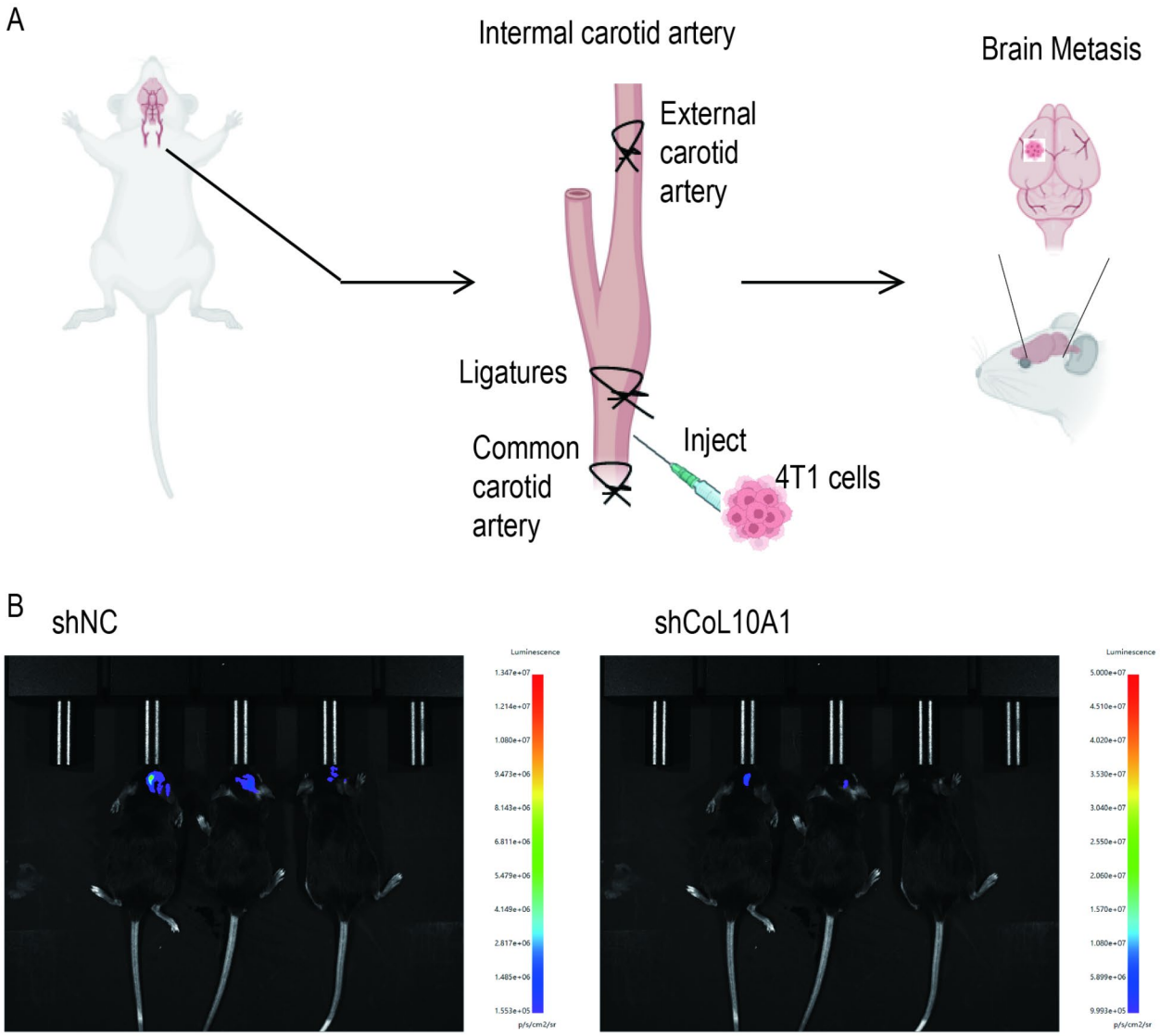

Fig. 6. CoL10A1 promotes BMBC tumor growth in mice models. (**A**) Schematic diagram of internal carotid artery injection. (**B**) In vivo epiffuorescence images of representative brains of tumor-bearing mice.

In addition, The EMT signaling pathway was involved in the regulatory effect, we identified some several candidate downstream molecules (E-Cadherin, β-catenin, N-Cadherin, Vimentin, Slug, Snail, ZEB1) that were regulated by CoL10A1. E-Cadherin is a type of calcium binding protein that provides cell adhesion between epithelial cells and maintains the interity of the epithelial cell layer. During the EMT process, the expression of E-cadherin is down-regulated, leading to reduce intercellular adhesion and emabing cells to acquire the ability to migrate and invade. β-catenin is an important component of intercellular adhersion connections, and together with E-Cadherin, maintains the stability of the epithelial cell layer. Unlike E-cadherin, N-cadherin is expressed in mesenchymal cells and promotes cell migration and invasion. Vimentin is an intermediate silk protein highly expressed in mesenchymal cells. It provides structural support for cells and participates in cell migration. Slug is a transcription inhibitory factory that can suppress the expression of E-Cadherin and promote EMT. Snail is a key regulatory factory of EMT, which promotes tumor cell invasion and metastasis. ZEB1 is a transcription inhibitory factory that can inhibit the expression of epithelial cell markers and promote the expression of mesenchymal cell features. The changes in these proteins are not only markers of EMT, but also key factors in tumor invasion and metastasis. The above results suggested that CoL10A1 may act as a new target in human BC.

These studies showed that CoL10A1 was up-regulated in various solid tumor types[37], such as colorectal cancer[19], gastric cancer[20]; and regulated cell proliferation, tumor vasculature, migration, and invasion[21,38,39]. In our study, we found that knockdown CoL10A1 inhibited cell migration and invasion. Additionally, we used hCMEC/D3, a special type of vascular epithetlial cells, which simulated tumor cells passing through cerebral vascular epithelial cells in vitro. The results showed that knockdown CoL10A1 inhibited tumor cells from crossing cerebral vascular epithelial cells. We acknowledge that our research method has certain limitations. In

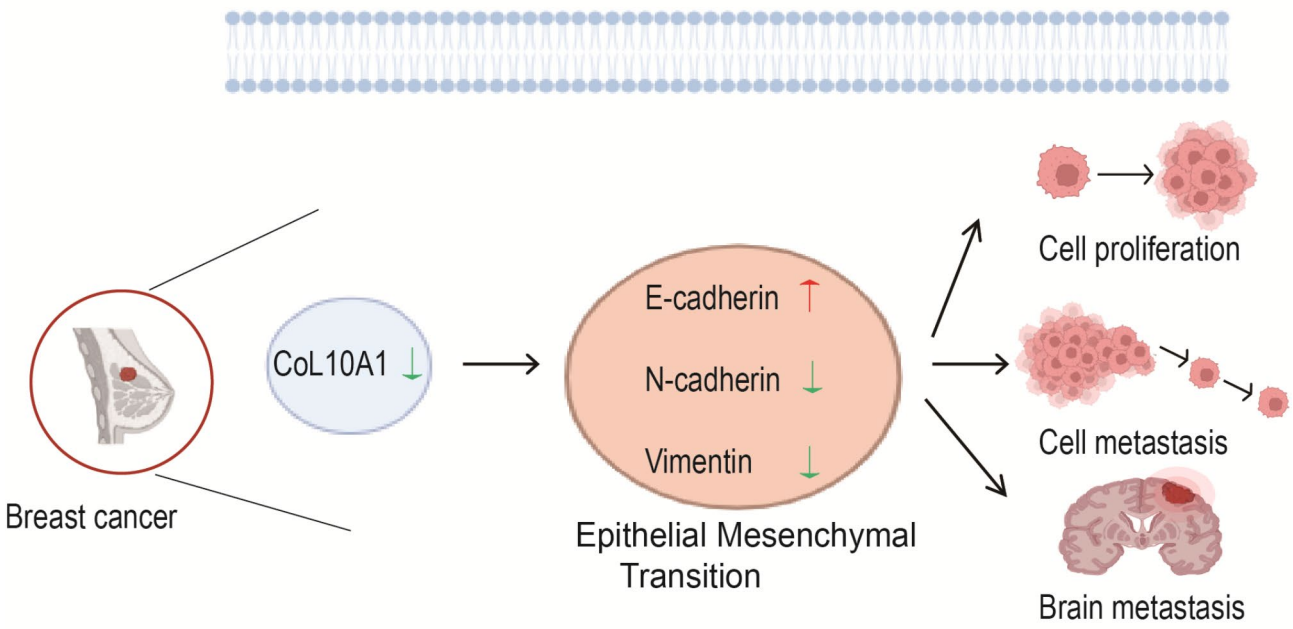

Fig. 7. CoL10A1 regulates the progression of breast cancer via EMT.

our study, we focused on the established role of hCMEC/D3 cells in simulating the blood-brain barrier. However, we recognize the importance of including endothelial, human astrocyte and pericytes to better simulate the in vivo environment of the cerebrovascular system. However, due to the three cell co-culture system had some issues, we only used hCMEC/D3 and madrigal as endothelial barriers for cerebral vascular invasion. In response to this limitation, we constructed an animal model of brain metastasis by injecting into the common carotid artery. These results suggest that CoL10A1 may regulate vascular invasion in vitro and in vivo.

Previous studies have demonstrated that the EMT pathway in modulating cell migration and invasion[40–42]. In breast cancer study revealed that the EMT pathway activation could enhance cell migration and invasion[43]. In bladder cancer, high expression of CoL10A1 was associated with EMT pathway using GSEA analysis[44]. Otherwise, in gastric cancer, CoL10A1 promoted cell invasion and metastasis via EMT pathway[45]. In our study, the expression of relative proteins was detected in the EMT pathway. E-cadherin and β-catenin was highly expressed in the 468 cells by knockdown CoL10A1 group. In addition, N-cadherin, Vimentin, Slug, Snail and ZEB1 were low expression in the knockdown CoL10A1 group. The expression of E-Cadherin is down-regulated, while the expression of mesenchymal cell markers such as N-Cadherin and Vimentin is up-regulated, which is usually associated with enhanced invasion and metastasis ability of tumor cells. Knockdown down CoL10A1 inhibited the migration and invasion of BC cells via EMT pathway. Our results suggested that CoL10A1 may affect BC migration and invasion via EMT pathway.

In the process of studying brain metastasis, mouse models and in vitro models are two important tools, but they both have some limitations. The limitations of mouse models include genetic differences, immune system differences and environmental factors. The main difference between in vitro models and actual human bodies is the lack of complexity and model stability. Despite these limitations, mouse models and in vitro models remain important tools for studying brain metastases. They can help scientists understand disease mechanism, test new treatment methods, and provided preliminary data before pushing new therapies into clinical practice.

In conclusion, our study found that CoL10A1 was highly expressed in BMBC, and knocking down CoL10A1 could inhibit cell proliferation, migration and invasion of BMBC (Fig. 7). Therefore, CoL10A1 may act as in BMBC progress, which could be study as a new potential treatment targets for BMBC treatment, including diagnostic and therapeutic, drug target, clinical studies, safety and efficacy, and combination therapy. However, we have not studied the mechanism, and will continue to delve deeper into it.

Data availability

All data underlying the study's findings are contained within the manuscript, supplementary materials, and can be obtained from the corresponding authors upon the reasonable request.

Received: 19 June 2024; Accepted: 20 January 2025
Published online: 01 April 2025

References

1. Yu, B. et al. KMT5A-methylated SNIP1 promotes triple-negative breast cancer metastasis by activating YAP signaling. *Nat. Commun.* **13** (1), 2192. https://doi.org/10.1038/s41467-022-29899-w (2022).

2. Giussani, M. et al. Extracellular matrix proteins as diagnostic markers of breast carcinoma. *J. Cell. Physiol.* **233** (8), 6280–6290. https://doi.org/10.1002/jcp.26513 (2018).
3. Hulvat, M. C. Cancer incidence and trends. *Surg. Clin. N. Am.* **100** (3), 469–481. https://doi.org/10.1016/j.suc.2020.01 (2020).
4. Murphy, L. C. & Leygue, E. The role of estrogen receptor-β in breast cancer. *Semin. Reprod. Med.* **30** (1), 5–13. https://doi.org/10.1055/s-0031-1299592 (2012).
5. Maughan, K. L., Lutterbie, M. A. & Ham, P. S. Treatment of breast cancer. *Am. Family Phys.* **81** (11), 1339–1346 (2010).
6. McDonald, E. S., Clark, A. S., Tchou, J., Zhang, P. & Freedman, G. M. Clinical diagnosis and management of breast cancer. *J. Nucl. Med.* **57** (Suppl 1), 9s–16s. https://doi.org/10.2967/jnumed.115.157834 (2016).
7. Lev, S. Targeted therapy and drug resistance in triple-negative breast cancer: the EGFR axis. *Biochem. Soc. Trans.* **48**(2), 657–665. https://doi.org/10.1042/BST20191055 (2020).
8. Harbeck, N., Gnant, M. Breast cancer. *Lancet*. **389**(10074), 1134–1150. https://doi.org/10.1016/S0140-6736(16)31891-8 (2017).
9. Srivastava, A. K. et al. Human genome meeting 2016: Houston, TX, USA. 28 February–2 March 2016. *Hum. Genom.* ;**10**(Suppl 1), 12. https://doi.org/10.1186/s40246-016-0063-5 (2016).
10. Schouten, L. J., Rutten, J., Huveneers, H. A. & Twijnstra, A. Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma. *Cancer* **94** (10), 2698–2705. https://doi.org/10.1002/cncr.10541 (2002).
11. Barnholtz-Sloan, J. S. et al. Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the Metropolitan Detroit Cancer Surveillance System. *J. Clin. Oncol.* **22** (14), 2865–2872. https://doi.org/10.1200/JCO.2004.12.149 (2004).
12. Gavrilovic, I. T. & Posner, J. B. Brain metastases: epidemiology and pathophysiology. *J. Neurooncol.* **75** (1), 5–14. https://doi.org/10.1007/s11060-004-8093-6 (2005).
13. Rostami, R., Mittal, S., Rostami, P., Tavassoli, F. & Jabbari, B. Brain metastasis in breast cancer: a comprehensive literature review. *J. Neurooncol.* **127** (3), 407–414. https://doi.org/10.1007/s11060-016-2075-3 (2016).
14. Bailleux, C., Eberst, L. & Bachelot, T. Treatment strategies for breast cancer brain metastases. *Br. J. Cancer.* **124**(1), 142–155. https://doi.org/10.1038/s41416-020-01175-y (2021).
15. Kaleem, M. et al. An insight into molecular targets of breast cancer brain metastasis. *Int. J. Mol. Sci.*. **23**(19). https://doi.org/10.3390/ijms231911687 (2022).
16. Wang, Y., Ye, F., Liang, Y. & Yang, Q. Breast cancer brain metastasis: insight into molecular mechanisms and therapeutic strategies. *Br. J. Cancer*. **125** (8), 1056–1067. https://doi.org/10.1038/s41416-021-01424-8 (2021).
17. Zhang, X. et al. A novel sequence variant in COL10A1 causing spondylometaphyseal dysplasia accompanied with coxa valga: a case report. *Med. (Baltim).* **98** (30), e16485. https://doi.org/10.1097/md.0000000000016485 (2019).
18. Necula, L. et al. High plasma levels of COL10A1 are associated with advanced tumor stage in gastric cancer patients. *World J. Gastroenterol.* **26** (22), 3024–3033. https://doi.org/10.3748/wjg.v26.i22.3024 (2020).
19. Huang, H. et al. High expression of COL10A1 is associated with poor prognosis in colorectal cancer. *Onco Targets Ther.* **11**, 1571–1581. https://doi.org/10.2147/ott.S160196 (2018).
20. Li, T. et al. TGF-β1-SOX9 axis-inducible COL10A1 promotes invasion and metastasis in gastric cancer via epithelial-to-mesenchymal transition. *Cell Death Dis.* **9** (9), 849. https://doi.org/10.1038/s41419-018-0877-2 (2018).
21. Lv, D. et al. COL10A1 is a novel factor in the development of choroidal neovascularization. *Microvasc. Res.* **139**, 104239. https://doi.org/10.1016/j.mvr.2021.104239 (2022).
22. Zhou, W. et al. High expression COL10A1 promotes breast cancer progression and predicts poor prognosis. *Heliyon* **8** (10), e11083. https://doi.org/10.1016/j.heliyon.2022.e11083 (2022).
23. Zhong, C. et al. Novel LncRNA OXCT1-AS1 indicates poor prognosis and contributes to tumorigenesis by regulating miR-195/CDC25A axis in glioblastoma. *J. Exp. Clin. Cancer Res.* **40** (1). https://doi.org/10.1186/s13046-021-01928-4 (2021).
24. Erturk, E. et al. Targeting the epithelial–mesenchymal transition (EMT) pathway with combination of wnt inhibitor and chalcone complexes in lung cancer cells. *J. Cell. Biochem.* **124** (8), 1203–1219. https://doi.org/10.1002/jcb.30442 (2023).
25. Wang, X. et al. Prognostic value of COL10A1 and its correlation with tumor-infiltrating immune cells in urothelial bladder cancer: a comprehensive study based on bioinformatics and clinical analysis validation. *Front. Immunol.* **14** https://doi.org/10.3389/fimmu.2023.955949 (2023).
26. Park, J. et al. Switch-like enhancement of epithelial-mesenchymal transition by YAP through feedback regulation of WT1 and rho-family GTPases. *Nat. Commun.* **10** (1). https://doi.org/10.1038/s41467-019-10729-5 (2019).
27. A MB, R VS, J ME, A AO. Breast cancer biomarkers: risk assessment, diagnosis, prognosis, prediction of treatment efficacy and toxicity, and recurrence. *Curr. Pharm. Des.* **20**(30), 4879–4898. https://doi.org/10.2174/1381612819666131125145517 (2014).
28. Zhou, W. et al. High expression COL10A1 promotes breast cancer progression and predicts poor prognosis. *Heliyon* **8** (10). https://doi.org/10.1016/j.heliyon.2022.e11083 (2022).
29. Rosati, R. et al. Normal long bone growth and development in type X collagen-null mice. *Nat. Genet.* **8** (2), 129–135. https://doi.org/10.1038/ng1094-129 (1994).
30. Tan, J. T. et al. Competency for nonsense-mediated reduction in collagen X mRNA is specified by the 3' UTR and corresponds to the position of mutations in Schmid metaphyseal chondrodysplasia. *Am. J. Hum. Genet.* **82** (3), 786–793 (2008).
31. Chapman, K. B. et al. COL10A1 expression is elevated in diverse solid tumor types and is associated with tumor vasculature. *Future Oncol.* **8** (8), 1031–1040. https://doi.org/10.2217/fon.12.79 (2012).
32. Zhang, M., Jin, M., Gao, Z., Yu, W. & Zhang, W. High COL10A1 expression potentially contributes to poor outcomes in gastric cancer with the help of LEF1 and Wnt2. *J. Clin. Lab. Anal.* **36** (9), e24612. https://doi.org/10.1002/jcla.24612 (2022).
33. Li, H. H., Wang, J. D., Wang, W., Wang, H. F. & Lv, J. Q. Effect of miR-26a-5p on gastric cancer cell proliferation, migration and invasion by targeting COL10A1. *Eur. Rev. Med. Pharmacol. Sci.* **24**(3), 1186–1194 (2020).
34. Brodsky, A. S. et al. Identification of stromal ColXα1 and tumor-infiltrating lymphocytes as putative predictive markers of neoadjuvant therapy in estrogen receptor-positive/HER2-positive breast cancer. *BMC Cancer*. **16**, 274. https://doi.org/10.1186/s12885-016-2302-5 (2016).
35. Kahlert, U. D. et al. COL10A1 allows stratification of invasiveness of colon cancer and associates to extracellular matrix and immune cell enrichment in the tumor parenchyma. *Front. Oncol.* **12**, 1007514. https://doi.org/10.3389/fonc.2022.1007514 (2022).
36. Yang, W., Wu, X., Zhou, F., Collagen Type, X. & Alpha 1 (COL10A1. Contributes to cell proliferation, migration, and invasion by targeting prolyl 4-hydroxylase beta polypeptide (P4HB) in breast Cancer. *Med. Sci. Monitor: Int. Med. J. Exp. Clin. Res.* **27**, e928919. https://doi.org/10.12659/msm.928919 (2021).
37. Xiao, Q. et al. MEF2A transcriptionally upregulates the expression of ZEB2 and CTNNB1 in colorectal cancer to promote tumor progression. *Oncogene* **40** (19), 3364–3377. https://doi.org/10.1038/s41388-021-01774-w (2021).
38. He, C. et al. VSNL1 promotes cell proliferation, Migration, and Invasion in Colorectal Cancer by binding with COL10A1. Annals of clinical and laboratory science. ;**52**(1):60–72. (2022).
39. Boudreau, N. & Bissell, M. J. Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr. Opin. Cell Biol.* **10** (5), 640–646. https://doi.org/10.1016/s0955-0674(98)80040-9 (1998).
40. Dongre, A. & Weinberg, R. A. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. *Nat. Rev. Mol. Cell. Biol.* **20** (2), 69–84. https://doi.org/10.1038/s41580-018-0080-4 (2019).
41. Gonzalez, D. M. & Medici, D. Signaling mechanisms of the epithelial-mesenchymal transition. *Sci. Signal.* **7** (344), re8. https://doi.org/10.1126/scisignal.2005189 (2014).
42. Hu, G. et al. Hypoxia-induced lncHILAR promotes renal cancer metastasis via ceRNA for the miR-613/206/ 1-1-3p/Jagged-1/Notch/CXCR4 signaling pathway. *Mol. Ther.* **29** (10), 2979–2994 (2021).

43. Alard, A. et al. Breast cancer cell mesenchymal transition and metastasis directed by DAP5/eIF3d-mediated selective mRNA translation. *Cell. Rep.* **42** (6), 112646. https://doi.org/10.1016/j.celrep.2023.112646 (2023).
44. Wang, X. et al. Prognostic value of COL10A1 and its correlation with tumor-infiltrating immune cells in urothelial bladder cancer: a comprehensive study based on bioinformatics and clinical analysis validation. *Front. Immunol.* **14**, 955949. https://doi.org/10.3389/fimmu.2023.955949 (2023).
45. Li, T. et al. TGF-beta1-SOX9 axis-inducible COL10A1 promotes invasion and metastasis in gastric cancer via epithelial-to-mesenchymal transition. *Cell. Death Dis.* **9** (9), 849. https://doi.org/10.1038/s41419-018-0877-2 (2018).

Author contributions
X.Y.W. conducted the cellular and animal experiment, S.M. conducted bioinformatics analysis and assisted in animal experiments, including daily feeding. S.L., W.J. and D.N. designed the project and completed the manuscript. All authors reviewed the manuscript.

Funding
This work was supported by Henan Medical Science and Technology Research Program Project (LHGJ20220622).

Declarations

Consent for publication
We obtained written informed consent from patients' tumor samples for publication. The researchers stated that the identification information had been removed.

Competing interests
The authors declare no competing interests.

Ethics apporval
This study was comply with ARRIVE guidelines, and approved by Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (EC-022-074) and the Experimental Animal Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (KY2022-048-01) on patient material, and the national research committee.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-87475-w.

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