Article

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Twist-1 Stimulates the Malignant Behaviors of Hydatidiform Mole via the PI3K/AKT Pathway

Chengling Zhang[1,*], Yan Song[1], Yanxue Yin[2], Hongchun Hou[1], Ziqi Ge[1,*]

[1]Department of Gynecology, Jinan Maternity and Child Care Hospital, 250001 Jinan, Shandong, China
[2]Department of Pathology, Jinan Maternity and Child Care Hospital, 250001 Jinan, Shandong, China
*Correspondence: jnfy1990@126.com (Chengling Zhang); 18053155019@163.com (Ziqi Ge)
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Background: Hydatidiform mole (HM) is a common pregnancy disease among women of gestational age. Twist-related protein 1 (Twist-1) is involved in the development of various tumors, but its role in HM is poorly defined. This study aimed to explore Twist-1 expression and its biological function in HM cells.

Methods: Twist-1 expression in HM was detected by immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR). The effects of silencing Twist-1 on choriocarcinoma (CCA) cell proliferation were detected by cell counting kit-8 (CCK-8) and clone formation assays. CCA cell migration and invasion were detected through transwell assay. Western blot was used to detect epithelial–mesenchymal transition (EMT) and the expression of phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway-related proteins.

Results: Twist-1 expression was upregulated in HM tissues ($p < 0.001$) and CCA cells ($p < 0.01$). Twist-1 silencing inhibited proliferation of BeWo and JAR cells ($p < 0.01$, $p < 0.05$) as shown by CCK-8 assay ($p < 0.01$) and clone formation assays ($p < 0.01$, $p < 0.05$). Twist-1 silencing inhibited the migration ($p < 0.01$) and invasion activity ($p < 0.01$, $p < 0.05$) of BeWo and JAR cells. Western blot results showed that Twist-1 silencing promoted E-cadherin ($p < 0.01$) expression, and inhibited N-cadherin ($p < 0.01$, $p < 0.05$) and vimentin ($p < 0.01$, $p < 0.05$) expression in BeWo and JAR cells. Twist-1 downregulation decreased protein levels of p-PI3K ($p < 0.01$) and p-AKT ($p < 0.01$, $p < 0.05$) in BeWo and JAR cells.

Conclusions: Silencing Twist-1 inhibits the malignant behavior of CCA cells, which may play a part by inhibiting the EMT process and the PI3K/AKT pathway.

Keywords: hydatidiform mole; Twist-1; EMT; PI3K/AKT pathway

Introduction

Gestational trophoblastic disease (GTD) is a group of pregnancy-related diseases originating from abnormal proliferation of placental trophoblastic epithelial cells [1], including hydatidiform mole (HM), invasive hydatidiform mole, choriocarcinoma (CCA), and epithelioid trophoblastic tumors [2–4]. HM, as the most common GTD, is characterized by placental villus edema and abnormal hyperplasia of trophoblast, and includes complete HMs (CHMs) and partial HMs (PHMs) based on the degree of trophoblastic proliferation of placental villi [5]. Approximately 1% to 5% of PHMs and 18% to 29% of CHMs will progress to gestational trophoblastic tumors (GTNs) [6]. Almost all invasive hydatidiform moles and 50% of CCAs involve malignant transformation from a HM [7]. HM patients with malignant transformation have poor prognosis, which poses a great threat to their physical and mental health. At present, the gold standard for the diagnosis of HM malignant transformation patients in clinical practice is pathological examination [8]; however, there is a lack of means to predict malignant transformation. Therefore, finding effective methods to predict the occurrence of malignant transformation in HM patients has important clinical significance for a comprehensive understanding of the patient's condition and the formulation of treatment plans.

Twist-related protein 1 (Twist-1) is widely expressed in mesodermal tissues and cells, and regulates cell migration during embryonic growth and development [9]. Twist-1 is involved in many biological processes, including cell differentiation, embryonic development as well as tumorigenesis, and is significantly expressed in carcinosarcoma [10]. The expression of Twist-1 is significantly increased in common malignant tumors, and upregulation of Twist-1 leads to poor prognosis of cancer patients [11–14]. Twist-1 is also involved in epithelial–mesenchymal transition (EMT) processes such as gastrula formation, neural crest migration, placenta formation, mesoderm formation, and trophoblastic differentiation [15]. The elevated Twist-1 expression in HM villous stromal cells is a sensitive and specific marker of CHMs/PHMs differentiation [16]. However, the underlying mechanisms of Twist-1 activity in HM remain unclear.

In this study, the expression of Twist-1 in HM and normal tissues was detected. The function experiments were introduced to explore the role of Twist-1 in HM development, thus providing a diagnostic index for early diagnosis of HM.

Materials and Methods

Sample Collection

Twenty HM tissues and thirty normal placental tissues were collected from Jinan Maternity and Child Care Hospital. All patients signed the informed consent. This study was approved by the ethics committees of Jinan Maternity and Child Care Hospital (No.2023-1-015), and was conducted according to the principles of the Declaration of Helsinki (2013) [17].

Immunohistochemistry (IHC)

The formalin-fixed, paraffin-embedded HM tissue was first dewaxed and rehydrated, then, antigen repair was performed by washing with phosphate-buffered saline (PBS) three times. The HM tissue was blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour. Rabbit anti-Twist-1 antibody (ab175430, dilution: 1:500, Abcam, Cambridge, UK) was incubated at 4 °C overnight. Tissues were incubated with anti-rabbit HRP-conjugated secondary antibodies (A-11011, 1:1000, Invitrogen, Los Angeles, CA, USA). The sections were stained using DAB (D8001, Sigma, Saint Louis, MO, USA) and hematoxylin (H3136, Sigma, Saint Louis, MO, USA). Samples were visualized under the microscope (CCD TP510, OPTEC, Chongqing, China). PBS instead of primary antibody was used as a negative control.

The results of each immunohistochemically stained tissue chip were evaluated by the double-blind method. The staining intensity was classified as follows: 3, brown cells; 2, brownish yellow cells; 1, light yellow cells; 0, unstained cells. The classification of proportion of stained cells is as follows: 4, $>$75%; 3, 50%–75%; 2, 25%–50%; 1, 10%–25%; 0, $<$10%. The score = stained cell proportion $\times$ staining intensity. $\geq$3 indicates positive expression, while $<$3 indicates negative expression.

Western Blotting Assay

Total protein was extracted from the collected cells using radioimmunoprecipitation assay (RIPA). Protein concentration was determined using bicinchoninic acid assay (BCA) kit (P0010S, Beyotime, Shanghai, China). 40 µg of protein was sampled, and transferred to PVDF membranes (IPVH00010, Millipore, MA, USA) after 10% SDS-PAGE electrophoresis. Then the membranes were blocked for 2 h in 5% skim milk powder, incubated in primary antibodies at 4 °C overnight. Next, the membranes were incubated with goat polyclonal anti-rabbit immunoglobulin G (IgG) H&L secondary antibody (31460, 1;10000, Invitrogen, Los Angeles, CA, USA) at 37 °C for 1 h. After washing, the proteins were developed using ECL reagent (E412-02, Vazyme, Jiangsu, China), and the images were captured under the protein imager (ChemiDocTM XRS+, Bio-Rad, Berkeley, CA, USA). The grayscale value of the blot was quantified with ImageJ software (V1.8.0, National Institutes of Health, Bethesda, MD, USA). The antibodies employed are as follows: anti-E-cadherin (1:1000, 20874-1-AP, Proteintech, Wuhan, China), anti-vimentin (1:500, 10366-1-AP, Proteintech, Wuhan, China), anti-N-Cadherin (1:1000, 22018-1-AP, Proteintech, Wuhan, China), anti-phosphatidylinositol 3-kinase (PI3K) (1:1000, 60225-1-Ig, Proteintech, Wuhan, China), anti-p-PI3K (1:1000, # 4228S, Cell Signaling Technology, Boston, MA, USA), anti-protein kinase B (AKT) (1:5000, ab38449, Abcam, Cambridge, UK), anti-p-AKT (1:5000, ab8933, Abcam, Cambridge, UK) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, 60004-1-Ig, Proteintech, Wuhan, China).

Cell Culture and Transfection

The human CCA cell line BeWo (XY-XB-1533), JAR (XY-XB-1301), and the normal extravillous trophoblast cell line (TEV-1; XY-XB-2471), were obtained from Xuanya Biotechnology Co., Ltd. (Shanghai, China). Both cell types were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; BC-SE-FBS08, Biochannel, Nanjing, China). All cells were tested for mycoplasma and confirmed to be uncontaminated. The cell lines used were identified by short tandem repeat (STR) identification.

Transfection was performed using si-Twist-1 and non-targeted siRNA (si-NC) at 10 nM by using Lipofectamine 2000 (11668019, Invitrogen, Los Angeles, CA, USA). All of the siRNAs were chemically synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of siRNA as following: si-Twist-1 forward, 5′-CCUCUGCAUUCUGAUAGAAdTdT-3′, reverse, 5′-UUCUAUCAGAAUGCAGAGG TdTd-3′; si-NC forward, 5′-CAUUCUCCUCUGGAUAGAAdTdT-3′, reverse, 5′-CAGAGUUCUAUCAGAAUGG TdTd-3′.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (15596-026, Invitrogen, Los Angeles, CA, USA). 0.5 µg of total RNA was used to synthesize cDNA along with a reverse transcription kit (TaKaRa, Tokyo, Japan). The PCR amplification reaction was conducted by Trans Start®Green qPCR Super Mix (TaKaRa, Tokyo, Japan) on an ABI 7500 fluorescence Quantitative PCR system (ABI 7500, Life Technologies, Carlsbad, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate mRNA expression levels. The primers are listed below: Twist-1-F: 5′-GGCTCAGCTACGCCTTCTC-3′, Twist-1-

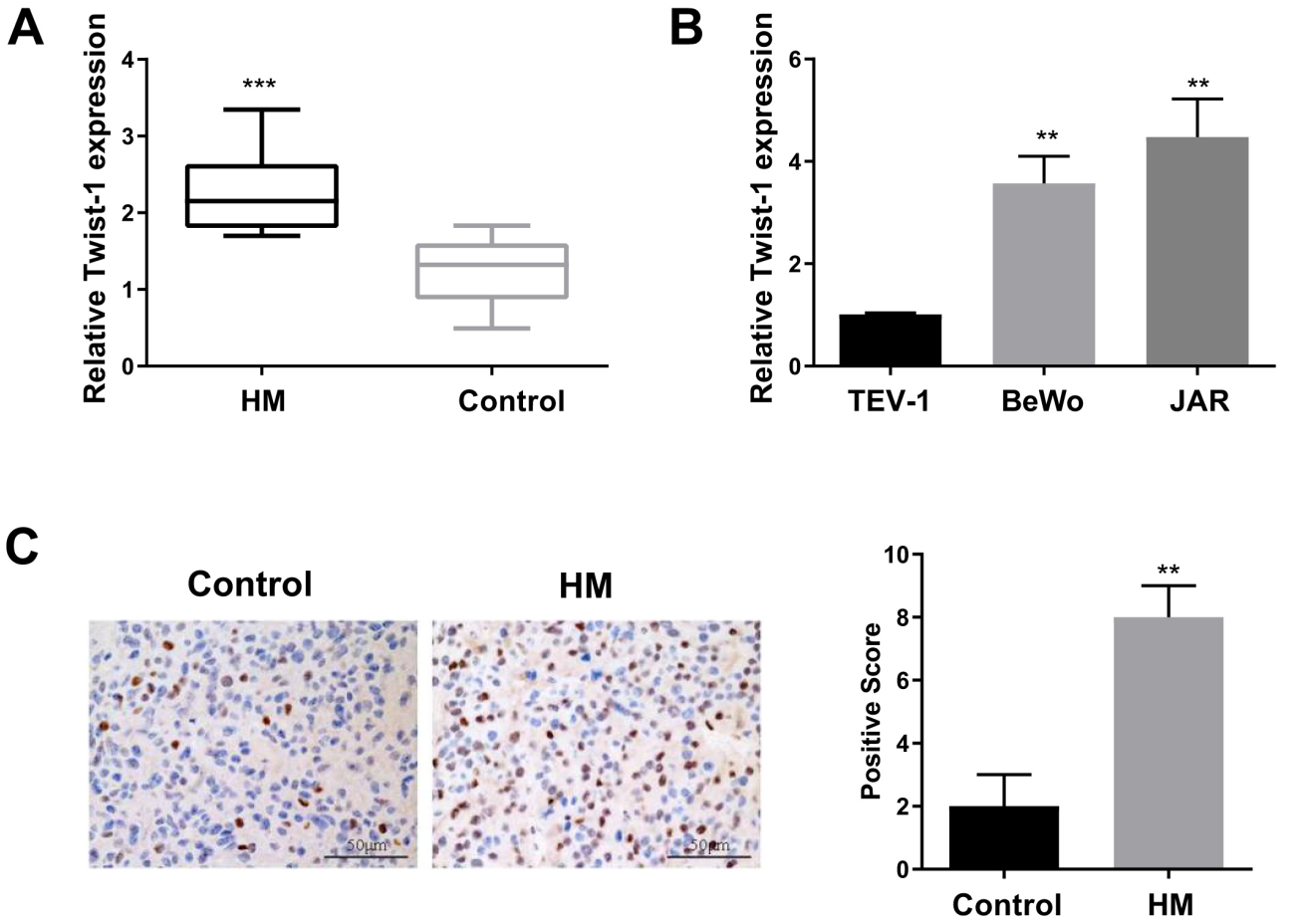

Fig. 1. Twist-1 was highly expressed in HM tissues and CCA cells. (A) The mRNA expression of Twist-1 was examined in fresh 20 HM tissues and 30 normal placental tissues. (B) The mRNA expression of Twist-1 was examined in HM cells (n = 3). (C) The expression of Twist-1 was examined by IHC (n = 3). ***$p < 0.001$, **$p < 0.01$. Twist-1, Twist-related protein 1; HM, hydatidiform mole; CCA, choriocarcinoma; IHC, immunohistochemistry.

R: 5′-TCCTTCTCTGGAAACAATGACA-3′; GAPDH-F: 5′-GGATTTGGTCGTATTGGG-3′, GAPDH-R: 5′-GGAAGATGGTGATGGGATT-3′.

Colony Formation Assay

The cells of each group were incubated in an incubator for 7–14 days. During the culture, the fluid was changed periodically as appropriate. Cultures were terminated when single-cell clonal colonies had grown to approximately 50 cells/colony or the size of a macroscopically small rice grain. Cells were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China). After staining with 1% crystal violet (C0121, Beyotime, Shanghai, China) for 10 min, the size of individual colonies in well plates was observed under a microscope (MODEL ECLIPSE Ts2, Nikon, Tokyo, Japan) and counted.

CCK-8 Assay

Cell viability was measured every 24 hours with cell counting kit-8 (CCK-8) reagent. After incubation, optical density was estimated at 450 nm using a microplate reader (IX31; Olympus, Tokyo, Japan).

Transwell Assay

After transfection for 48 h, 8-μm pore size culture inserts were used for the migration assay. Chambers with Matrigel were used for the invasion assay. 3×10^4 BeWo and JAR cells were added in the upper chamber. A total of 600 μL of medium was added to the lower chambers. After 24 h, cells that had transgressed to the bottom were stained with 0.1% crystal violet. The cells were photographed under a light microscope.

Statistical Methods

The data were expressed as mean ± SD. SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used to analyze data, differences between two groups were analyzed using *t* tests

and differences between >2 groups were tested with one-way analysis of variance. $p < 0.05$ were considered statistically significant.

Results

Upregulation of Twist-1 in HM Tissues and CCA Cells

The expression of Twist-1 in HM was estimated by quantitative real-time polymerase chain reaction (qRT-PCR). Twist-1 had significantly higher expression in the fresh 20 HM tissues than in the 30 normal placental tissues (Fig. 1A, $p < 0.001$). Meanwhile, the expression of Twist-1 in BeWo and JAR cells was elevated compared to TEV-1 cells (Fig. 1B, $p < 0.01$). To further visualize Twist-1 expression in trophoblastic tissues, immunohistochemistry (IHC) was performed in 20 HM tissues and 30 normal placental tissues. The above findings were confirmed by IHC, which indicate that Twist-1 is highly expressed compared to normal placental tissue (Fig. 1C, $p < 0.01$).

Silencing of Twist-1 Regulated CCA Cell Proliferation

To determine how Twist-1 affected proliferation of HM, changes in cell viability and colony formation after inhibition of Twist-1 expression were observed. First, the transfection efficiency of Twist-1 siRNA was checked using qRT-PCR, which validated the expected Twist-1 expression (Fig. 2A, $p < 0.01$, $p < 0.05$). The data from the CCK-8 assay indicated that proliferation of CCA cells in the si-Twist-1 group decreased versus the si-NC group (Fig. 2B,C, $p < 0.01$). Plate cloning found that the colony formation ability of CCA cells in the si-Twist-1 group also decreased versus the si-NC group (Fig. 2D,E, $p < 0.01$, $p < 0.05$).

The Downregulation of Twist-1 Inhibited CCA Cell Migration and Invasion

Next, the effect of Twist-1 on cell metastasis was investigated in CCA. Knockdown of Twist-1 restrained migration of BeWo and JAR cells ($p < 0.01$, Fig. 3A). Moreover, Twist-1 downregulation suppressed CCA cell invasion ($p < 0.05$, $p < 0.01$, Fig. 3B). Additionally, protein levels of EMT markers were compared in BeWo and JAR cells containing Twist-1 siRNA. Downregulation of Twist-1 lowered vimentin ($p < 0.01$, $p < 0.05$) and N-cadherin ($p < 0.01$, $p < 0.05$) expression and increased E-cadherin expression ($p < 0.01$, Fig. 3C). Overall, these findings imply that Twist-1 silencing could impair the metastatic behavior of CCA cells.

The Downregulation of Twist-1 Regulated the PI3K/AKT Pathway in CCA Cells

First, si-NC or si-Twist-1 was transfected into CCA cells. Western blot assay suggested that downregulation of Twist-1 restrained p-PI3K ($p < 0.01$) and p-AKT ($p < 0.01$, $p < 0.05$) expressions in BeWo and JAR cells (Fig. 4A,B). Thus, Twist-1 downregulation blocks the PI3K/AKT pathway.

Discussion

Although HM is a benign trophoblastic disease, it has a high probability of malignant transformation, especially CHMs, which have a 15% probability of malignant transformation, posing a great threat to the life of patients [18]. Currently, the clinical treatment of GTD is carried out by surgery and chemotherapy. However, the prognosis of patients varies greatly relying on the severity of disease diagnosis and on treatment strategy. The early diagnosis of GTD is the key to clinical treatment. The determination of β-human chorionic gonadotropin (hCG) levels is the main basis for clinical diagnosis of whether HM has transformed into invasive HM [1,19]. Therefore, searching for tumor markers that are more closely related to the malignant transformation of HM is the key to early diagnosis of trophoblastic tumors.

Twist-1, shown to be a carcinogenic agent, has been highly expressed in malignant tumors. For example, Twist-1 is upregulated in pancreatic cancer and promotes the aerobic glycolysis of tumor cells through transcriptional regulation [20]. Additionally, the highly expressed Twist1 is an active regulator of proliferation and EMT in breast cancer [21,22]. Here, we demonstrated the diverse oncogenic properties of Twist-1 and its expression in HM. In this study, Twist-1 has higher expression in the HM tissues than the normal placental tissues, which is consistent with previous literature reports [16]. Malignant proliferation, invasion and migration of tumor cells are important causes of tumor metastasis, and EMT is an early indicator of tumor metastasis [23]. Twist-1, as a significant regulator of cell metastasis and EMT of tumors, has attracted extensive attention in recent years [24,25]. The overexpression of Twist-1 is sufficient to enable cancer cells to obtain a mesenchymal phenotype with enhanced migration and invasion characteristics [26,27]. For the first time, this study reported that Twist-1 silencing could inhibit the EMT process, which was consistent with the results involving cell invasion and migration, and suggested that Twist-1 silencing could inhibit CCA cell metastasis. Moreover, interference with Twist-1 in BeWo and JAR cells inhibited cell proliferation *in vitro*. Consistent with the results of other studies, our data reveal the vital roles of Twist-1 in the EMT and metastasis of HM, and indicate that Twist-1 and PI3K/AKT pathway may be potential molecular targets for exploring better treatment against HM.

The PI3K/AKT pathway plays critical roles in cell biological processes, including angiogenesis, proliferation, and apoptosis progression, by influencing the activation states of multiple downstream effectors [28–30]. A previous study implied that Twist-1 plays a role in drug resis-

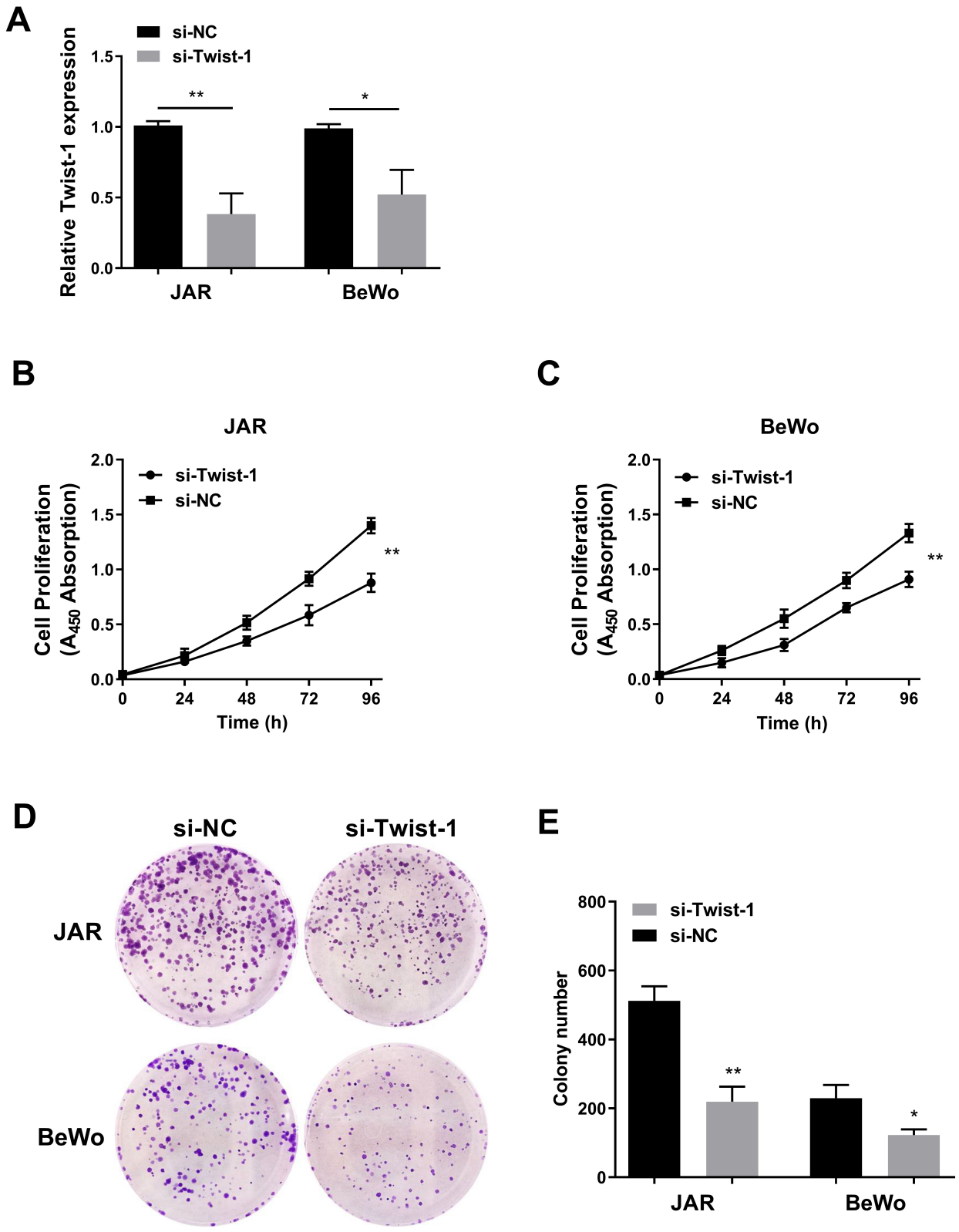

Fig. 2. Twist-1 silencing restrained CCA cell proliferation *in vitro*. (A) Twist-1 expression was observed in BeWo and JAR cells (n = 3). (B–E) The CCA cell proliferation was investigated by CCK-8 and Colony formation assay (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$. CCK-8, cell counting kit-8; si-NC, non-targeted siRNA.

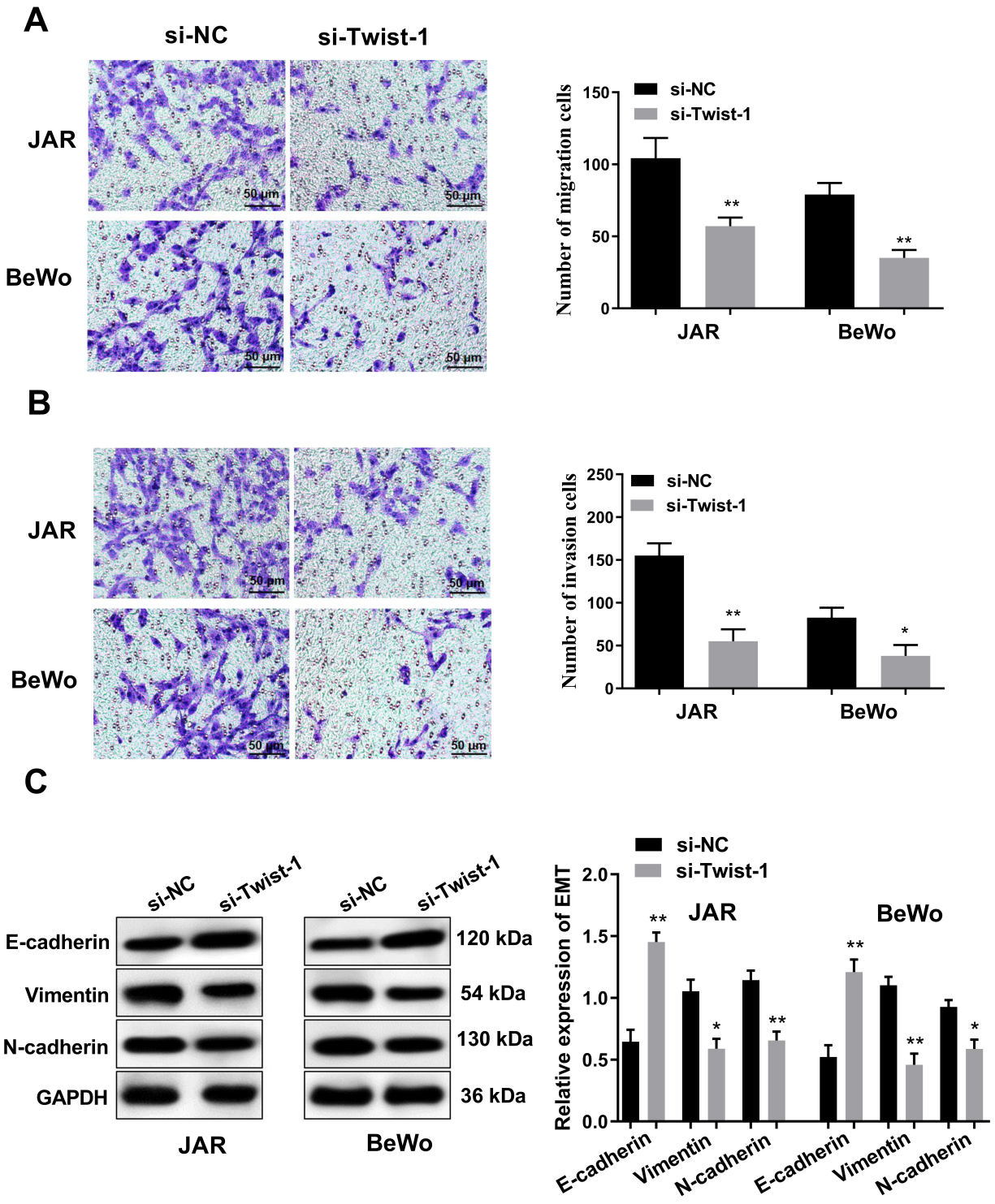

Fig. 3. Twist-1 silencing suppressed CCA cell migration and invasion. (A,B) The invasion and migration of CCA cells were assessed by transwell assay (n = 3). (C) The EMT was detected by Western blot (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$. EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tance in chronic myelogenous leukemia by acting on the PI3K/AKT pathway [31]. Recently, a large number of studies have confirmed the role of the PI3K/AKT signaling pathway in the infiltration and proliferation of trophoblast cells [32,33]. Similarly, we found the phosphorylation level of PI3K and AKT decreased when Twist-1 was downreg-

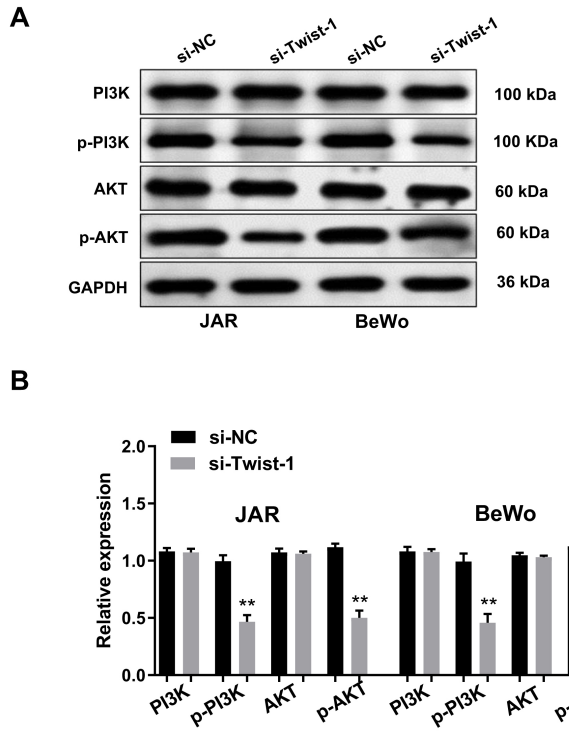

Fig. 4. Twist-1 silencing regulated the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway. (A,B) The protein levels were determined in BeWo and JAR cells by Western blot assay (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$.

ulated. Twist-1 might affect HM progression by inducing EMT and PI3K/AKT pathways, but the specific mechanism of its participation and regulation in the process needs further study.

Conclusions

In summary, Twist-1 expression is up-regulated in HM tissues and CCA cells, interfering with Twist-1 expression inhibits the malignant behavior of CCA cells. In addition, interfering with Twist-1 expression inhibits EMT and the PI3K/AKT pathway in HM. This may provide new research hotspots for the occurrence and development mechanism of HM. However, the occurrence and development mechanism of HM are relatively complex and cannot be fully verified through a single *in vitro* cell experiment, thus requiring further support through analysis of additional experimental data.

Availability of Data and Materials

The data used to support these findings of this study are available from the corresponding authors upon request.

Author Contributions

CZ made substantial contributions to conception and design. YS made substantial contributions to the data analysis and study preparation. YY and HH made substantial contributions to the interpretation of data and wrote the study. ZG made substantial contributions to perform the analysis with constructive discussions. All authors contributed to editorial changes in the manuscript. All authors have read and approved the final study. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was in line with the principles of the Declaration of Helsinki (2013) and approved by the ethics committee of Jinan Maternity and Child Care Hospital (No.2023-1-015). Patients and their families knew the purpose, significance, content and confidentiality of the study and signed informed consent.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Soper JT. Gestational Trophoblastic Disease: Current Evaluation and Management. Obstetrics and Gynecology. 2021; 137: 355–370.
[2] Kaur B. Pathology of gestational trophoblastic disease (GTD). Best Practice & Research. Clinical Obstetrics & Gynaecology. 2021; 74: 3–28.
[3] Lok C, Frijstein M, van Trommel N. Clinical presentation and diagnosis of Gestational Trophoblastic Disease. Best Practice & Research. Clinical Obstetrics & Gynaecology. 2021; 74: 42–52.
[4] Elias KM, Braga A, Horowitz NS, Berkowitz RS. A novel alternative to cytotoxic chemotherapy for gestational trophoblastic disease. The Lancet. Oncology. 2021; 22: 1490–1491.
[5] Hui P, Buza N, Murphy KM, Ronnett BM. Hydatidiform Moles: Genetic Basis and Precision Diagnosis. Annual Review of Pathology. 2017; 12: 449–485.
[6] Schoolmeester JK, Erickson LA. Gestational Trophoblastic Disease. Mayo Clinic Proceedings. 2017; 92: 1739–1740.
[7] Ranade M, Aguilera-Barrantes I, Quiroz FA. Gestational Trophoblastic Disease and Choriocarcinoma. Ultrasound Quarterly. 2015; 31: 221–223.
[8] Ray A, Kumari S. A Case Report of Twin Pregnancy with Hy-

datidiform Mole and Co-existing Live Fetus. Saudi Journal of Medicine & Medical Sciences. 2020; 8: 213–216.

[9] Tang H, Massi D, Hemmings BA, Mandalà M, Hu Z, Wicki A, *et al*. AKT-ions with a TWIST between EMT and MET. Oncotarget. 2016; 7: 62767–62777.

[10] Zhao Z, Rahman MA, Chen ZG, Shin DM. Multiple biological functions of Twist1 in various cancers. Oncotarget. 2017; 8: 20380–20393.

[11] Xu Y, Hu B, Qin L, Zhao L, Wang Q, Wang Q, *et al*. SRC-1 and Twist1 expression positively correlates with a poor prognosis in human breast cancer. International Journal of Biological Sciences. 2014; 10: 396–403.

[12] Zhu DY, Guo QS, Li YL, Cui B, Guo J, Liu JX, *et al*. Twist1 correlates with poor differentiation and progression in gastric adenocarcinoma via elevation of FGFR2 expression. World Journal of Gastroenterology. 2014; 20: 18306–18315.

[13] Oh BY, Kim SY, Lee YS, Hong HK, Kim TW, Kim SH, *et al*. Twist1-induced epithelial-mesenchymal transition according to microsatellite instability status in colon cancer cells. Oncotarget. 2016; 7: 57066–57076.

[14] Croset M, Goehrig D, Frackowiak A, Bonnelye E, Ansieau S, Puisieux A, *et al*. TWIST1 expression in breast cancer cells facilitates bone metastasis formation. Journal of Bone and Mineral Research. 2014; 29: 1886–1899.

[15] Luchini C, Parcesepe P, Mafficini A, Nottegar A, Parolini C, Veronese N, *et al*. Specific expression patterns of epithelial to mesenchymal transition factors in gestational molar disease. Placenta. 2015; 36: 1318–1324.

[16] Moussa RA, Eesa AN, Abdallah ZF, Abdelmeged A, Mahran A, Bahaa H. Diagnostic Utility of Twist1, Ki-67, and E-Cadherin in Diagnosing Molar Gestations and Hydropic Abortions. American Journal of Clinical Pathology. 2018; 149: 442–455.

[17] World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013; 310: 2191–2194.

[18] López CL, Figueira Gouvêa AL, Rodrigues FR, Braga A, Valente Machado MD, Lopes VS. Human epidermal growth factor receptor 2 fluorescence in situ hybridization and P57KIP2 immunohistochemistry using tissue microarray: Improving histopathological subtyping of hydatidiform mole. Placenta. 2020; 99: 166–172.

[19] Ngan HYS, Seckl MJ, Berkowitz RS, Xiang Y, Golfier F, Sekharan PK, *et al*. Diagnosis and management of gestational trophoblastic disease: 2021 update. International Journal of Gynaecology and Obstetrics. 2021; 155: 86–93.

[20] Wang XX, Yin GQ, Zhang ZH, Rong ZH, Wang ZY, Du DD, *et al*. TWIST1 transcriptionally regulates glycolytic genes to promote the Warburg metabolism in pancreatic cancer. Experimental Cell Research. 2020; 386: 111713.

[21] Xu Y, Qin L, Sun T, Wu H, He T, Yang Z, *et al*. Twist1 promotes breast cancer invasion and metastasis by silencing Foxa1 expression. Oncogene. 2017; 36: 1157–1166.

[22] Liu YQ, Cong YZ, Jiang J, Sheng JZ, Li XH, Zhao M, *et al*. MiR-526b suppresses cell proliferation, cell invasion and epithelial-mesenchymal transition in breast cancer by targeting Twist1. European Review for Medical and Pharmacological Sciences. 2020; 24: 3113–3121.

[23] Trivanović D, Krstić J, Jauković A, Bugarski D, Santibanez JF. Mesenchymal stromal cell engagement in cancer cell epithelial to mesenchymal transition. Developmental Dynamics. 2018; 247: 359–367.

[24] Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, *et al*. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004; 117: 927–939.

[25] Jin L, Zhang J, Fu HQ, Zhang X, Pan YL. FOXO3a inhibits the EMT and metastasis of breast cancer by regulating TWIST-1 mediated miR-10b/CADM2 axis. Translational Oncology. 2021; 14: 101096.

[26] Li J, Alvero AB, Nuti S, Tedja R, Roberts CM, Pitruzzello M, *et al*. CBX7 binds the E-box to inhibit TWIST-1 function and inhibit tumorigenicity and metastatic potential. Oncogene. 2020; 39: 3965–3979.

[27] Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ, *et al*. Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nature Cell Biology. 2008; 10: 295–305.

[28] Wu X, Tong R, Chen X, Jiang X, He X, Ma L. The miR-302s/367 Cluster Inhibits the Proliferation and Apoptosis in Sheep Fetal Fibroblasts via the Cell Cycle and PI3K-Akt Pathways. Mammalian Genome. 2021; 32: 183–194.

[29] Chen Q, Wang M, Shen C. Bauerane Induces S-Phase Cell Cycle Arrest, Apoptosis, and Inhibition of Proliferation of A549 Human Lung Cancer Cells Through the Phosphoinositide 3-Kinase (PI3K)/AKT and Signal Transducer and Activator of Transcription 3 (STAT3) Signaling Pathway. Medical Science Monitor. 2020; 26: e919558.

[30] Gao H, Peng C, Wu L, Gao S, Wang Z, Dai L, *et al*. Yiqi-Huoxue granule promotes angiogenesis of ischemic myocardium through miR-126/PI3K/Akt axis in endothelial cells. Phytomedicine. 2021; 92: 153713.

[31] Yuan R, Chang J, He J. Effects of Twist1 on drug resistance of chronic myeloid leukemia cells through the PI3K/AKT signaling pathway. Cellular and Molecular Biology (Noisy-le-Grand, France). 2020; 66: 81–85.

[32] Xu Y, Sui L, Qiu B, Yin X, Liu J, Zhang X. ANXA4 promotes trophoblast invasion via the PI3K/Akt/eNOS pathway in preeclampsia. American Journal of Physiology. Cell Physiology. 2019; 316: C481–C491.

[33] Zhang L, Deng X, Shi X, Dong X. Silencing H19 regulated proliferation, invasion, and autophagy in the placenta by targeting miR-18a-5p. Journal of Cellular Biochemistry. 2019; 120: 9006–9015.