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TNNT1 accelerates migration, invasion and EMT progression in lung cancer cells

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Abstract

Background: Clinically, most patients with lung cancer (LC) die from tumor spread and metastasis. Specific metastasis-related molecules can provide reference for clinical prediction of efficacy, evaluation of prognosis, and search for the best treatment plan. Troponin T1 (TNNT1) is highly expressed in various cancer tissues, which affects malignant behavior of tumor cells and is related to patients' survival and prognosis. However, the role and molecular mechanism of TNNT1 in LC invasion and metastasis have not yet been investigated.

Methods: Gene expression profiling interactive analysis (GEPIA) online analysis was used to analyze TNNT1 expression in LC tissues. Quantitative real-time-polymerase chain reaction (qRT-PCR) or western blot were performed to measure TNNT1 or epithelial-to-mesenchymal transition (EMT)-related and Wnt/β-catenin pathway-related protein expression in LC cells. After TNNT1 knockdown, cell scratch healing and transwell assays were introduced to assess cell migration and invasion, respectively.

Results: TNNT1 expression in LC tissues and cells was increased. TNNT1 knockdown notably impaired LC cell migration, invasion and EMT. TNNT1 knockdown inhibited Wnt/β-catenin pathway of LC cells. Lithium chloride (LiCl) addition partially restored the inhibition of TNNT1 knockdown on migration, invasion, EMT and Wnt/β-catenin of LC cells.

Conclusion: TNNT1 knockdown attenuated LC migration, invasion and EMT, possibly through Wnt/β-catenin signaling.

KEYWORDS

EMT, invasion and migration, lung cancer, TNNT1, Wnt/β-catenin

INTRODUCTION

Lung cancer (LC) is a common malignant tumor of the respiratory system, which is divided into small cell LC (SCLC) and non-SCLC (NSCLC) according to histomorphology, and the incidence of NSCLC is 80%–85%.[1,2] Early symptoms of LC patients are atypical and insidious, which can easily lead to missed and misdiagnosis, thereby delaying the best treatment time and increasing the risk of death.[3] The metastasis and recurrence of LC bring difficulties to clinical treatment.[4] Although the treatment of LC has developed from traditional chemotherapy and radiotherapy to the current stage of targeted therapy and immunotherapy, its efficacy is not ideal and can easily affect the life quality of patients.[5,6] Therefore, further elucidation of the molecular mechanism of LC metastasis, searching for new metastasis-related factors and developing metastasis-related targeted drugs to improve its prognosis have important clinical significance.

Epithelial-derived tumor cells first acquire migration and invasion ability by spreading from the primary tumor into the blood circulation, while some surviving circulating tumor cells form distant metastases through blood dissemination during metastasis.[7,8] Colonizing tumor cells self-adjust to the new microenvironment and change from a migration mode to a proliferative mode, thus forming

metastasis.[9] Epithelial-to-mesenchymal transition (EMT) is a dynamic and reversible molecular biological process that plays a crucial role in the initial stage of tumor metastasis.[10] EMT can occur in various tissue types and developmental stages.[11] In addition to being involved in embryogenesis, organ development and tissue formation, EMT also participates in genesis and metastasis of tumors and promotes invasion and movement of tumor cells.[12] In cancer, mesenchymal features mediated by EMT enable cancer cells to complete multiple steps of metastasis, including local invasion, vascular invasion, metastasis through the circulatory system, and distant metastasis.[13] The study of the process and mechanism of EMT has become an important direction for the study of tumor metastasis behavior. Therefore, exploring the mechanism of EMT in LC is beneficial for the development of new effective clinical treatment strategies.

Troponin T1 (TNNT1), a subunit of troponin T, is a regulatory complex located on sarcomere filaments that affects cell invasion and migration by regulating the interaction between myosin and actin.[14,15] TNNT1 mRNA is generally upregulated in immortalized RPE cells, a type of immortalized cell with self-renewal, replication and differentiation potential.[16] Immortalization is a sign of the transformation of cells from benign to malignant and is also a common feature of cancer cells.[17] Previous studies have confirmed that TNNT1 is upregulated to varying degrees in colorectal cancer, breast cancer, thyroid carcinoma, hepatocellular carcinoma and ovarian cancer, suggesting that TNNT1 may also be an oncogene.[18–22] In NSCLC, high expression of TNNT1 is related to clinical stage and lymph node metastasis, and is an independent risk factor affecting the survival of patients.[23] At present, it is not clear how TNNT1 affects LC cell invasion, migration and EMT. Here, exploring the effect of TNNT1 on LC malignant metastasis provides a theoretical basis and ideas for finding an effective target gene therapy for LC.

METHODS

Cell culture and transfection

LC cell lines (A427, A549, H1299, and SKLU1) and human bronchial epithelial cells (BEAS-2B) were purchased from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle medium (DMEM: Gibco, USA) and supplemented with 10% fetal bovine serum (FBS: Gibco, USA) in a 37°C incubator.

Targeted interference RNA sequence TNNT1 (si-TNNT1) and corresponding control (si-NC) were chemically synthesized from GenePharma (China). All cells required for the experiments were in a logarithmic growth phase. Lipofectamine 2000 (Invitrogen, USA), A549 and A427 cells were transfected with targeted interference RNA and divided into si-TNNT1 and si-NC groups, according to the manufacturer's instructions. The transfection efficiency was measured after culture at 48 h.

Scratch healing assay

Cells were seeded in a 12-well plate with 4×10^5 cells/well, and were evenly spread into monolayers overnight. The center of the well was gently scratched with a 200 μL pipette tip. After cells were rinsed three times with phosphate buffered saline (PBS) to remove floating cells, serum-free medium was added to continue the culture. Images were taken at 0 h and 24 h, and the scratch distance (SD) was measured. The cell scratch healing rate was (%) = $(SD_{0h}\text{-}SD_{24h})/SD_{0h} \times 100$.

Transwell chamber assay

The transfected cells were resuspended in serum-free medium with 1×10^5 cells/mL. A total of 500 μL of DMEM with 10% FBS was added to the lower transwell chamber, and 100 μL of cell suspension was added to the upper chamber which was coated with Matrigel (Becton Dickson, USA). After 24 h incubation, cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in the upper chamber of the transwell were erased with a cotton swab, and cells were observed under a microscope and counted.

qRT-PCR

Total RNA from cells was isolated using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized using the Prime Script reverse transcription kit (Invitrogen, USA), followed by quantitative real-time-polymerase chain reaction (qRT-PCR) using SYBR green PCR Master Mix (TaKaRa, Japan). Relative expression was calculated using the $2^{-\triangle\triangle Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Western blot

Proteins were extracted from LC cells using radioimmunoprecipitation assay (RIPA) lysates (Beyotime, China) and protein concentration was detected using a bicinchoninic acid (BCA) kit (Beyotime, China). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Subsequently, membrane was blocked with 5% skim milk for 2 h. Primary antibodies (Abcam, USA) were added and incubated overnight at 4°C. Membranes were incubated for another 1 h at room temperature by adding secondary antibody (Abcam, USA). Finally, proteins were visualized on a Tanon imaging system using enhanced chemiluminescence (ECL) reagent, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal parameter.

Statistical analysis

GraphPad Prism 6.0 software was used to analyze the results, and the experimental data are expressed as mean ± standard deviation. The *t* test was used to compare measurement data between the two groups. *p*-values < 0.05 indicated a statistically significant difference.

RESULTS

Expression of TNNT1 in LC

To understand whether TNNT1 expression changes are involved in human LC, we first analyzed TNNT1 mRNA expression data in 483 lung adenocarcinoma (LUAD) and

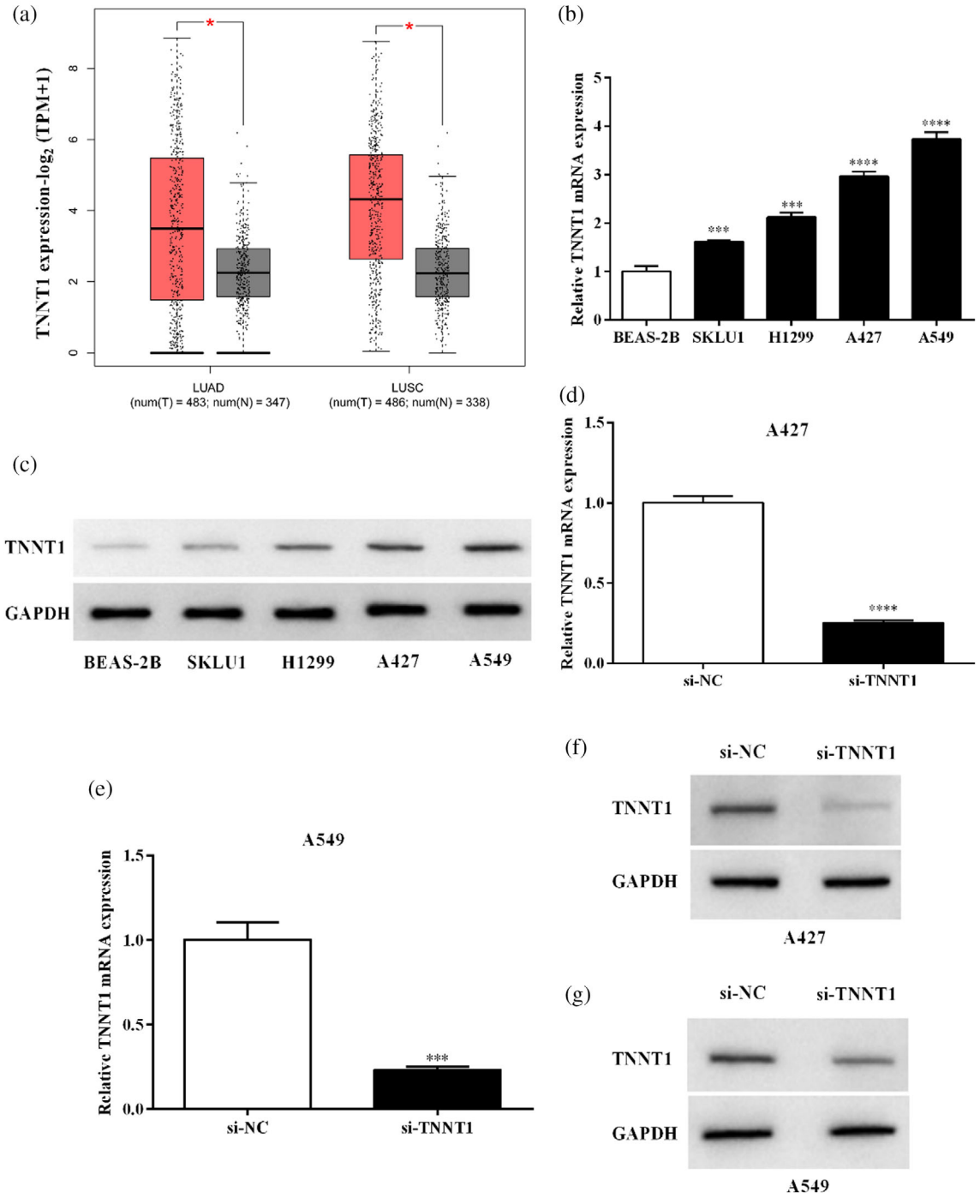

FIGURE 1 TNNT1 was upregulated in lung cancer (LC). (a) TNNT1 expression patterns in LC (lung adenocarcinoma [LUAD] and lung squamous cell carcinoma [LUSC]) tissue and normal tissue were analyzed using the GEPIA databank. (b, c) TNNT1 expression in LC cells was measured by quantitative real-time-polymerase chain reaction (qRT-PCR) and western blot assays. (d–g) Analysis of TNNT1 expression in LC cells after si-TNNT1 transfection. *$p <$ 0.05, ***$p < 0.001$, ****$p < 0.0001$, compared with normal tissues, BEAS-2B or si-NC group.

486 lung squamous cell carcinoma (LUSC) tumor samples and corresponding normal tissue samples from the online GEPIA database. GEPIA database analysis showed that TNNT1 expression was notably increased in LC (Figure 1a). TNNT1 mRNA (Figure 1b) and protein (Figure 1c) expressions were also increased in LC cells versus BEAS-2B cells. These results indicated that TNNT1 expression is upregulated in LC tissues and cells. Among LC cells, TNNT1 expression was the highest in A427 and A549 cells. Thus, A427 and A549 cells were selected for subsequent study.

The TNNT1 gene was interfered by siRNA to further verify the role of TNNT1 in LC development. TNNT1 mRNA (Figure 1d,e) and protein (Figure 1f,g) expression were markedly decreased after si-TNNT1 transfection, suggesting si-TNNT1 effectively reduced TNNT1 expression.

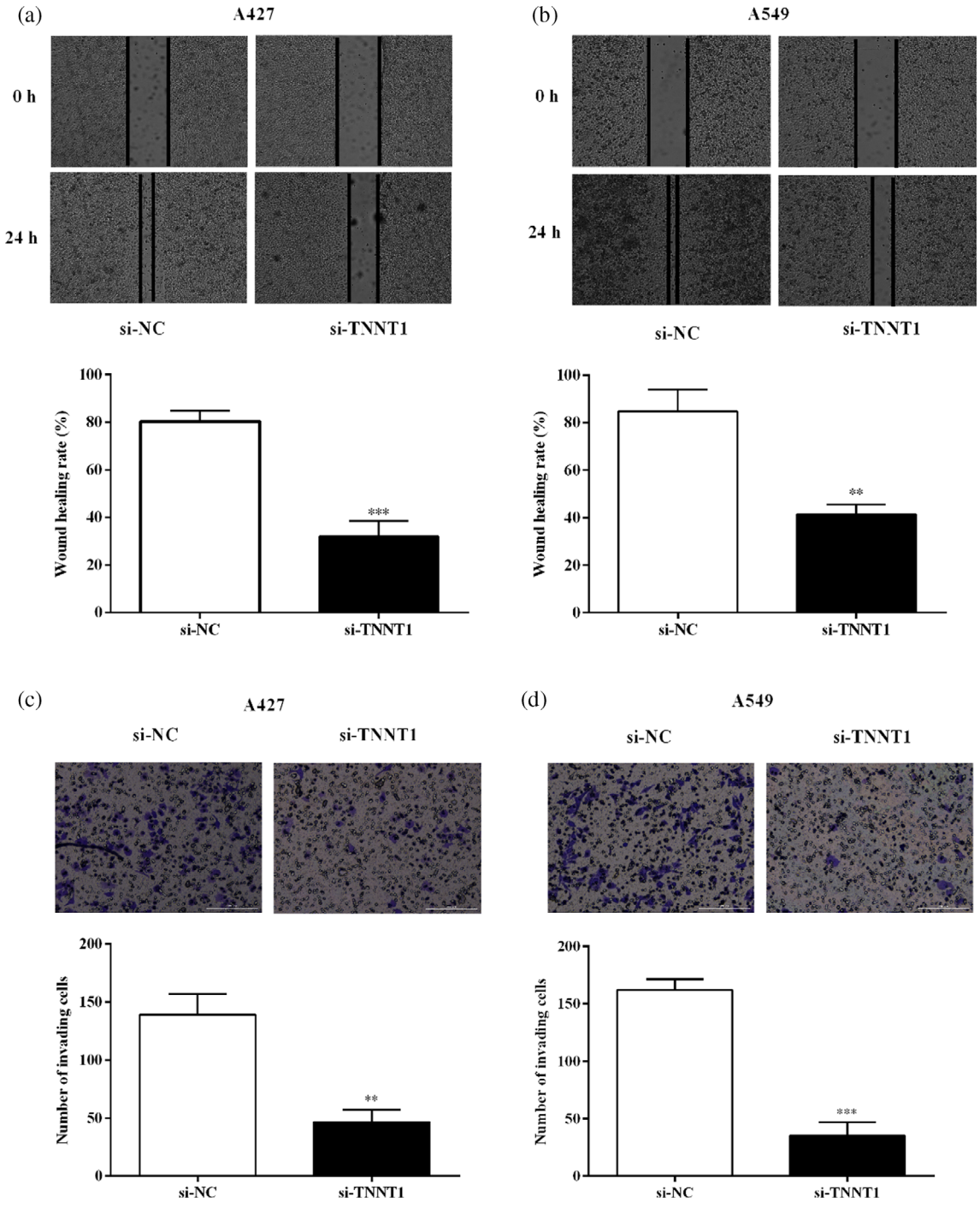

FIGURE 2 TNNT1 knockdown suppresses migration and invasion of lung cancer (LC) cells. (a, b) Cell migration of LC cells with TNNT1 knockdown. (c, d) Analysis of cell invasion of LC cells with TNNT1 knockdown. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the si-NC group.

Effect of TNNT1 knockdown on LC cell migration and invasion

In order to explore whether TNNT1 affect LC invasion and migration, transwell and scratch healing tests were performed on transfected cells to analyze changes in the invasion and migration ability after TNNT1 knockdown. The healing ability was weaker after 24 h in the si-TNNT1 group versus the si-NC group (Figure 2a,b). Cell invasion was decreased in the si-TNNT1 group versus the si-NC group (Figure 2c,d). These results suggest that TNNT1 knockdown inhibited LC cell migration and invasion.

Effect of TNNT1 knockdown on LC cell EMT and the Wnt/β-catenin pathway

After determining that downregulation of TNNT1 inhibited the invasion and migration of LC cells, EMT-related protein expression was detected to explore the functional relationship between TNNT1 and EMT. Snail, Slug, N-cadherin and vimentin proteins expression were decreased, while E-cadherin protein expression was increased after TNNT1 knockdown (Figure 3a,b). These results indicated that TNNT1 knockdown inhibited EMT of LC cells.

The Wnt/β-catenin pathway plays a crucial role in various cancers. In order to determine whether TNNT1 mediates the Wnt/β-catenin signal in LC, cMyc and β-catenin levels were detected by western blot. TNNT1 knockdown inhibited cMyc and β-catenin expression (Figure 3c,d), suggesting TNNT1 regulated the activity of the Wnt/β-catenin pathway.

Effect of TNNT1 on LC cells migration, invasion and EMT by the Wnt/β-catenin pathway

LC cells with si-TNNT1 were treated with lithium chloride (LiCl) to confirm whether TNNT1 regulates LC migration, invasion and EMT by the Wnt/β-catenin pathway. After transfection of si-TNNT1 and treatment with 20 mmol/L LiCl for 24 h, it was found that LiCl could partially restore cell migration, invasion and EMT after TNNT1 knockdown (Figures 4 and 5). In addition, β-catenin and cMyc levels were also increased (Figure 5c,d). These results indicate that

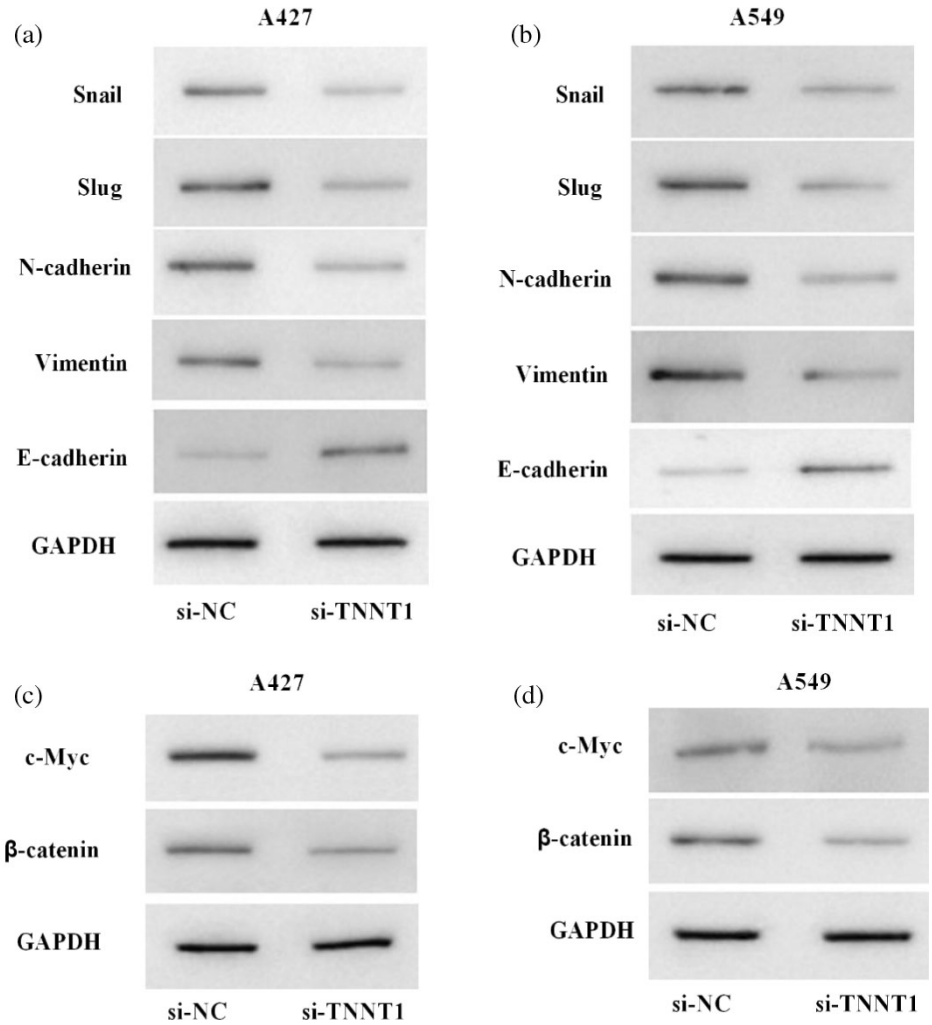

FIGURE 3 TNNT1 knockdown inhibited epithelial-to-mesenchymal transition (EMT) and the Wnt/β-catenin pathway of lung cancer (LC) cells. (a, b) EMT-related proteins expression in LC cells. (c, d) Western blot was used to detect the effect of TNNT1 knockdown on the expression of cMyc and β-catenin in LC cells.

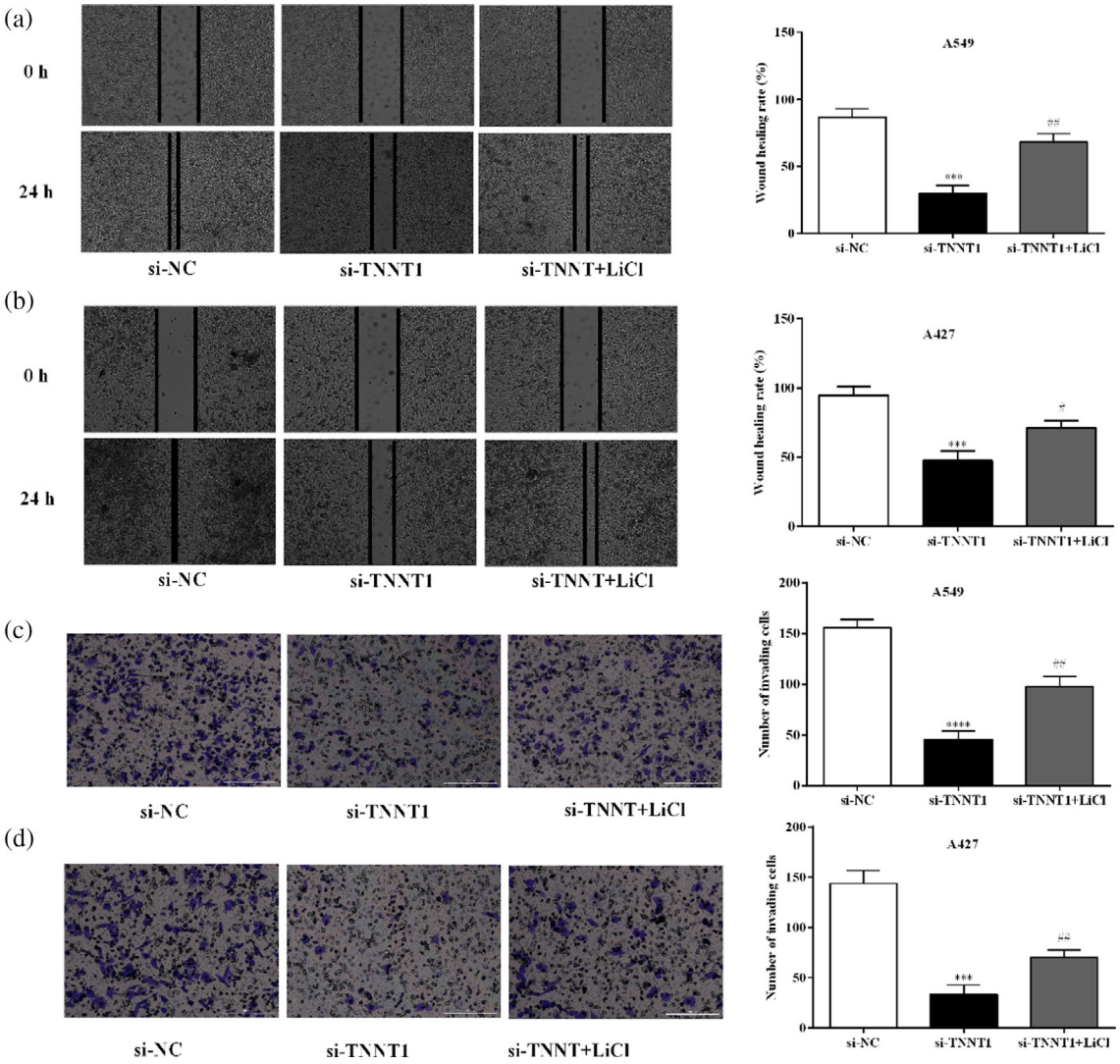

FIGURE 4 TNNT1 drives the migration and invasion of lung cancer (LC) through Wnt/β-catenin signaling. (a) Lithium chloride (LiCl) restored the migration of TNNT1 knockdown cells. (b) LiCl partially restored the invasion ability of TNNT1 silenced LC cells. ***$p < 0.001$, ****$p < 0.0001$, compared with si-NC group. #$p < 0.05$, ##$p < 0.01$, compared with si-TNNT1 + LiCl group.

TNNT1 induced LC cell migration, invasion and EMT by the Wnt/β-catenin pathway.

DISCUSSION

Tumor metastasis is the leading cause of tumor death, accounting for about 90% of tumor deaths.[24] Tumor metastasis is a series of complex processes that involves multiple steps, stages, pathways, and genetic changes.[25] Therefore, the human understanding of the mechanism and process of tumor metastasis is limited at present, and the study of tumor metastasis has important clinical and scientific significance. LC is prone to metastasis, which is also the main factor of poor prognosis in LC patients.[26,27] At present, the clinical treatment of tumor metastasis is limited and has little effect. Therefore, the regulatory mechanism of LC metastasis should be urgently explored in order to find new reliable biomarkers for LC prevention and treatment.

The ability of migration and invasion represents the ability of tumor cells to spread and metastasize to distant locations, and is also a key ability of tumor metastasis and further loss of tissue homeostasis in human tissues.[7] It has been reported that TNNT1 expression is increased in various tumor cells, promoting tumor cell proliferation, migration, invasion and EMT.[18–22] TNNT1 upregulation induced CRC cell migration and invasion.[18] In PTC cells, TNNT1 promoted migration, invasion and EMT process.[21] TNNT1 inhibition hampered hepatoma cells proliferation, invasion and EMT.[22] In this study, TNNT1 was found to be highly expressed in LUAD and LUSC in the TCGA/GTEx database by GEPIA analysis. TNNT1 expression was also increased in LC cells. The results of the loss-of-function assay showed that TNNT1 knockdown attenuated migration and invasion of LC cells. Metastasis of cancer is usually

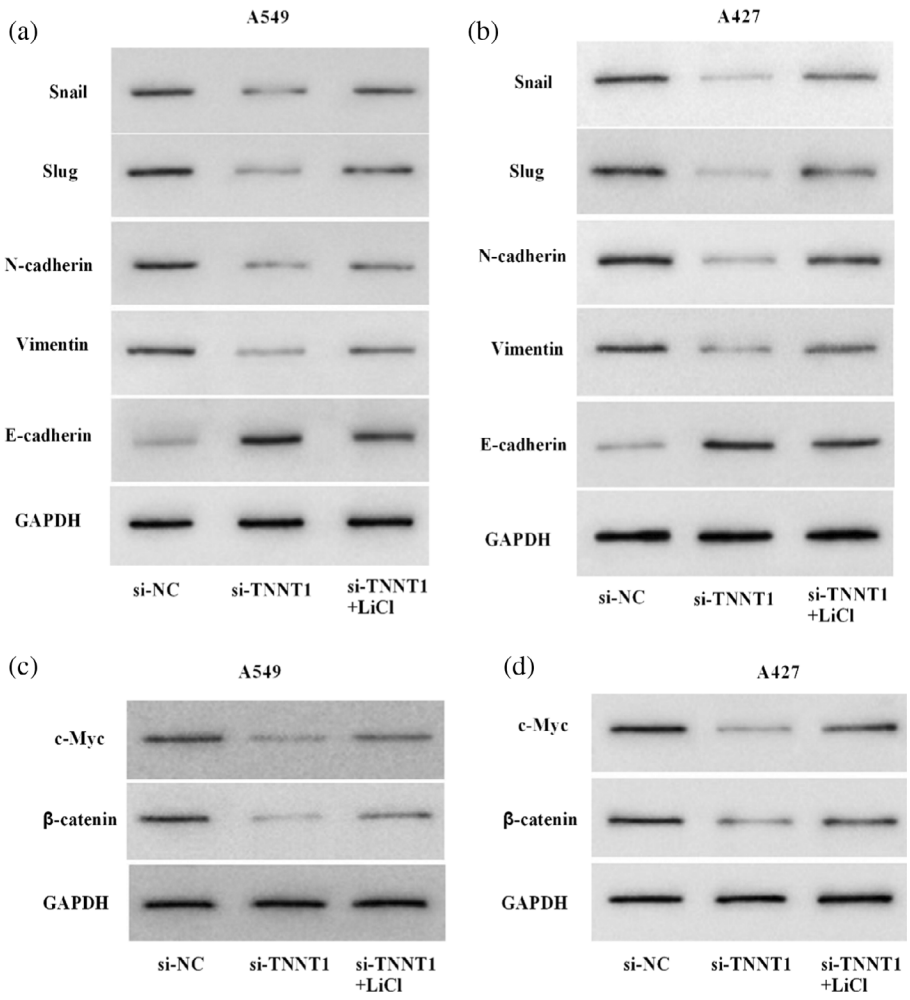

FIGURE 5 TNNT1 drives epithelial-to-mesenchymal transition (EMT) of lung cancer (LC) through Wnt/β-catenin signaling. (a–d) Lithium chloride (LiCl) partially restored the EMT-related and Wnt/β-catenin pathway-related protein expression in LC cells with knockdown of TNNT1.

accompanied by EMT, which is the loss of intercellular adhesion ability and the acquisition of migratory and invasive features.[28] The adhesion ability of tumor cells undergoing EMT is significantly weakened, with N-cadherin and vimentin upregulation, and E-cadherin downregulation being a key factor in the occurrence of EMT.[29] At the same time, Snail, Slug, ZEB1, ZEB2, or Twist (E-cadherin transcriptional repressors) is also activated.[30] Our results showed that TNNT1 knockdown reduced Snail, Slug, vimentin and N-cadherin expression, while increasing E-cadherin expression. These results suggest that TNNT1 may play a vital role in promoting LC metastasis.

Dysregulation of the Wnt/β-catenin pathway relates to occurrence and development of tumor invasion and metastasis.[31] Similarly, the Wnt/β-catenin pathway also plays an important role in the development of EMT in cancers.[32] β-catenin is considered as a key protein in the Wnt pathway, which can further initiate transcription of EMT-related genes, thereby promoting the metastasis of EMT primary tumors to other sites.[33,34] Decreased levels of β-catenin and cMyc were observed in TNNT1 knockdown cells, suggesting TNNT1 downregulation could inhibit the Wnt/β-catenin pathway. Adding LiCl to TNNT1 knockdown LC cells could partially restore migration, invasion and EMT, indicating that TNNT1-mediated Wnt/β-catenin activation promoted LC metastasis to a certain extent. However, the effect of TNNT1 on LC metastasis in vivo has not been further verified by animal experiments and needs to be further improved.

In conclusion, NNT1 expression was increased in LC tissues and cells. TNNT1 knockdown could attenuate LC cell migration, invasion and EMT, which might be related to the Wnt/β-catenin pathway. Hence, TNNT1 may be a potential molecular target for LC treatment, which also provides new idea for LC clinical treatment.

AUTHOR CONTRIBUTIONS

Xiaobin Ge: Writing-original draft and funding acquisition. Guangzhong Du: Data curation, formal analysis, investigation, methodology and validation. Qingchen Zhou: Investigation, methodology and validation. Bing Yan and Gonglei Yue: Conceptualization, writing-original draft, review and editing, project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets during the current study are available from the corresponding author on reasonable request.

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