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Downregulation of long non-coding RNA XIST inhibits cell proliferation, migration, invasion and EMT by regulating miR-212-3p/ CBLL1 axis in non-small cell lung cancer cells

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Abstract. – OBJECTIVE: Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors in the world and its 5-year survival rate is very low. Long non-coding RNA X-inactive specific transcript (lncRNA XIST) has been demonstrated to play vital roles in NSCLC, but the exact molecular mechanisms underlying NSCLC still need to be further explored.

PATIENTS AND METHODS: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed to detect the expression of XIST, miR-212-3p and Casitas B-lineage proto-oncogene like 1 (CBLL1). Dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay were conducted to analyze the relationship among XIST, miR-212-3p and CBLL1. Cell Counting Kit-8 (CCK-8) assay and transwell invasion assay were carried out to evaluate cell proliferation, migration and invasion, respectively. Western blot analysis was conducted to examine the protein expression of CBLL1, E-cadherin, N-cadherin and Vimentin. Murine xenograft assay was conducted to explore the role of XIST *in vivo*.

RESULTS: Expression levels of XIST and CBLL1 were markedly upregulated, while the miR-212-3p level was markedly downregulated in NSCLC tissues and cells. MiR-212-3p was identified as a direct target of XIST, and miR-212-3p was predicted to target CBLL1. XIST knockdown repressed NSCLC cell proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) *in vitro*, and suppressed tumor growth *in vivo*, while miR-212-3p inhibition restored the effects. Furthermore, CBLL1 overexpression could abolish the effects of miR-212-3p overexpression on NSCLC cell proliferation, migration, invasion and EMT *in vitro*.

CONCLUSIONS: XIST was significantly decreased in NSCLC tissues and cells, and XIST knockdown suppressed the proliferation, migration, invasion and EMT of NSCLC cells by miR-212-3p/CBLL1 axis. These findings facilitated our understanding of lncRNA regulation in NSCLC.

Key Words:
NSCLC, LncRNA XIST, MiR-212-3p, CBLL1, EMT.

Introduction

Lung cancer is one of the most serious malignant tumors with the highest morbidity and mortality rate seriously threating people's health and life[1,2]. Studies have shown that long-term smoking and air pollution are closely related to lung cancer, but the exact pathogenesis is not clear[3]. Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer. Although early diagnosis and treatment methods have been improved, the prognosis of patients is still unsatisfactory, and the five-year survival rate of NSCLC is only 15%[4,5]. Therefore, exploring new treatment strategies is especially important.

Long non-coding RNAs (lncRNAs) are a class of RNAs that have more than 200 nucleotides in length but without protein coding ability[6]. Emerging evidence has revealed that lncRNAs are associated with human cancers progression, including NSCLC[7]. For example, small nucleolar RNA host gene 1 (SNHG1) was highly expressed in NSCLC and promoted NSCLC progression[8]. The study of Li et al[9] showed that MALAT1 could promote NSCLC cell proliferation and colony formation. Scholars[10,13] have indicated that lncRNA X-inactive specific transcript (XIST) was aberrantly expressed and acted as an oncogene in NSCLC. Nevertheless, our knowingness about the exact roles of XIST in NSCLC is insufficient.

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MicroRNAs (miRNAs) are a group of non-coding RNAs with about 22 nucleotides and have been demonstrated to act as oncogenes or tumor suppressors by many studies[14,15]. It has been revealed that miR-212-3p acted as a tumor suppressor in many cancers, such as osteosarcoma[16], glioblastoma[17] and intrahepatic cholangiocarcinoma[18]. Tang et al[19] observed that miR-212 expression was decreased in NSCLC cells and its overexpression inhibited NSCLC cell metastasis. However, Li et al[20] indicated that miR-212 overexpression promoted cell proliferation, migration and invasion in NSCLC cells. Hence, the role of miR-212-3p in NSCLC still needs to be further investigated.

Casitas B-lineage proto-oncogene like 1 (CBLL1), also named HAKAI, is a recently discovered E3 ubiquitin ligase containing the RING-finger domain, which has been proved as a potential therapeutic target for human cancers[21-23]. Hui et al[24] demonstrated that CBLL1 was upregulated in NSCLC cells and CBLL1 promoted NSCLC cell proliferation and migration. MiRNA could bind to the 3'-untranslated region (3'-UTR) of mRNA to regulate gene expression at the post-transcriptional level[25]. Thus, we explored whether miR-212-3p could bind to CBLL1 in NSCLC.

In this study, we investigated the expression levels and function roles of XIST, miR-212-3p and CBLL1 in NSCLC. We found that XIST and CBLL1 were highly expressed, but miR-212-3p was lowly expressed in NSCLC tissues and cells. Furthermore, we found that XIST knockdown suppressed proliferation, migration, invasion and (EMT) of NSCLC cells by miR-212-3p/CBLL1 axis, indicating that XIST might be a novel target for NSCLC treatment.

Patients and Methods

Patient Samples

Thirty-three NSCLC tissue specimens and adjacent normal tissue specimens were obtained from patients who receive surgical resection in Xiangyang Central Hospital before this study. All samples were put into liquid nitrogen immediately and stored in a –80°C refrigerator until use. Patients did not receive any radiotherapy or chemotherapy and written informed consent was obtained from all patients prior to surgery. The study was approved by the Ethics Committee of Xiangyang Central Hospital.

Cell Culture and Transfection

Human NSCLC cell lines H522 and Calu3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and normal human bronchial epithelial cell line 16HBE was purchased from CHI Scientific, Inc., (Shanghai, China). All cells were incubated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, South-Logan, UT, USA), 100 U/mL penicillin (Gibco, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA), and cells were grown in a humidified atmosphere of 37°C and 5% CO_2.

MiR-212-3p mimic (miR-212-3p) and mimic scrambled control (miR-NC), miR-212-3p inhibitor (in-miR-212-3p) and its control (in-miR-NC), small interfering RNA against XIST (si-XIST) and its control (si-NC), XIST overexpression vector pcDNA3.1-XIST (XIST), CBLL1 overexpression vector pcDNA3.1-CBLL1 (CBLL1) and pcDNA3.1 empty vector (pcDNA), short hairpin RNA targeting XIST (sh-XIST) and empty vector (sh-NC) were purchased from Genepharma (Shanghai, China). Cell transfection was carried out by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) on the basis of manufacturer's instructions.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from NSCLC tissues, H522 cells and Calu3 cells using RNAiso Plus (TaKaRa Bio, Dalian, China). Then, RNA was quantified and reversely transcribed into cDNAs by using Prime Script RT reagent kit (Promega, Madison, WI, USA) or MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). QRT-PCR was carried out by using SYBR Premix PrimerEraser kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) on an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control of lncRNA and mRNA, U6 was used as an internal control of miRNA. The primers used in this study were: XIST: (forward) 5'-CTCTCCATTGGGTTCAC-3' and (reverse) 5'-GCGGCAGGTCTTAAGAGATGAG-3'; miR-212-3p: (forward) 5'- CGGCGGTAACAGTCTCCAGTC-3' and (reverse) 5'- GTGCAGGGTCCGAGGT-3'; CBLL1: (forward) 5'-GGAGTTGGATAGTAGAGGCGAGAG-3' and (reverse) 5'-AACATCAAGACCAC-

CCAAGGA-3'; GAPDH: (forward) 5'-GGAGCGAGATCCCTCCAAAAT-3' and (reverse) 5'-GGCTGTTGTCATACTTCTCATGG-3'; U6: (forward) 5'-ATTGGAACGATACAGAG-3' and (reverse) 5'-GGAACGCTTCACGAATTT-3'.

Dual Luciferase Reporter Assay

The sequences of wild type XIST containing the predicted binding sites of miR-212-3p or its mutant were introduced into the pmirGLO luciferase vector (Promega, Madison, WI, USA) to establish XIST WT or XIST MUT. Likewise, the 3'-UTR of CBLL1 containing the putative wild type miR-212-3p binding sites or its mutant were introduced into pmirGLO luciferase vectors (Promega, Madison, WI, USA) to construct CBLL1 3'-UTR-WT or CBLL1 3'-UTR-MUT. Then these luciferase reporter vectors were transfected into H522 and Calu3 cells with miR-212-3p or miR-NC by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activities were examined by using the dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) after 48-h co-transfection.

RNA Immunoprecipitation (RIP) Assay

EZMagna RIP kit (EMD Millipore, Billerica, MA, USA) was used to detect the XIST enrichment according to the protocols of manufacturer. In brief, H522 and Calu3 cells were lysed in RIP lysis buffer and then cells lysate was incubated with magnetic beads conjugated with anti-Ago2 (Sigma-Aldrich, St. Louis, MO, USA) or anti-IgG (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the samples were incubated with Proteinase K (Sigma-Aldrich, St. Louis, MO, USA) and qRT-PCR was conducted to detect the expression of XIST after RNA was extracted from immunoprecipitation complex.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was measured by CCK-8 assay. In short, transfected H522 and Calu3 cells were plated into 96-well plates and cultured in a humidified incubator containing 5% CO_2 at 37°C. Then, 10 μL CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into the plates at 0 h, 24 h, 48 h and 72 h, and the plates were incubated for additional 2 h. Finally, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

Cell migration and invasion abilities were evaluated by a 24-well transwell chamber with 8 μm pore size (Coaster, Corning, NY, USA). For cell migration assay, transfected H522 and Calu3 cells were seeded into the upper chamber and cultured in serum-free Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA), the lower chamber was added RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; HyClone, South-Logan, UT, USA). After culture for 24 h, cells that on the upper surface were removed and cells migrated to the lower surface were fixed with 95% methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) at room temperature. 10 min later, cells were counted under a light microscope (Olympus Corporation, Tokyo, Japan). For cell invasion assay, the upper chamber was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and subsequent procedures of cell invasion assay were same as cell migration assay.

Western Blot Analysis

Total proteins were isolated from H522 and Calu3 cells by lysing cells in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and measured by BCA Protein Assay Kit (Beyotime, Shanghai, China). The equal amount of proteins was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Then the membranes were blocked with 5% skim milk for 2 h and incubated with primary antibodies (anti-CBLL1, anti-E-cadherin, anti-N-cadherin or anti-Vimentin) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h. The proteins were visualized by an enhanced chemiluminescence detection kit (Amersham, GE Healthcare, Chicago, IL, USA). All antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA) and β-actin was used as control.

Murine Xenograft Assay

Six-week-old female nude mice were purchased from the National Laboratory Animal Center (Beijing, China) and divided into four groups (6 mice/group). Calu3 cells (1.0×10^6 cells) treated with sh-SNHG1, sh-NC, sh-XIST+in-miR-212-3p or sh-XIST+in-miR-NC were subcutaneously injected into the right dorsal flanks of the nude mice. Tumor size was detected weekly and tumor volume was calculated by the formula: $(\text{length}\times\text{width}^2)/2$. The mice were sacrificed after four weeks and tumors were collected for follow-

ing experiments. The animal experiments were approved by Xiangyang Central Hospital.

Statistical Analysis

The data were analyzed by GraphPad Prism 7 (GraphPad, San Diego, CA, USA) and showed as means ± standard deviation (SD) from at least three independent experiments. The differences were analyzed via Student's *t*-test or one-way analysis of variance (ANOVA). Tukey's test was used to validate ANOVA. The correlation among XIST, miR-212-3p and CBLL1 was evaluated by Pearson's correlation analysis. Kaplan-Meier method and log-rank test were used to evaluate the survival rate. *p*-value less than 0.05 was considered statistically significant.

Results

Expression Levels of XIST and miR-212-3p in NSCLC Tissues and Cells, and Prognostic Values of XIST and miR-212-3p for NSCLC Patients

To determine the potential roles of XIST and miR-212-3p in NSCLC, we detected the expression levels of XIST and miR-212-3p in 33 NSCLC tissues, two cell lines (H522 and Calu3) and healthy controls by qRT-PCR. We found that XIST was highly expressed in NSCLC tissues compared to normal tissues and higher expression of XIST was observed in H522 and Calu3 cells relative to 16HBE cells (Figure 1A and B). However, the expression of miR-212-3p was markedly downregulated in NSCLC tissues and cells compared to normal controls (Figure 1D and E). In addition, we divided these 33 NSCLC tissues into two groups according to the expression levels of XIST and miR-212-5p. Then, overall survival rate in these two groups was analyzed by Kaplan-Meier analysis. The data indicated that there was a shorter overall survival rate in high XIST expression group/low miR-212-3p expression group (n=18) compared to low XIST expression group/high miR-212-3p expression group (n=15) (Figure 1C and F). These results suggested that the aberrant expression of XIST and miR-212-3p might involve in NSCLC occurrence and could be potential biomarkers to predict the prognosis of patients with NSCLC.

XIST Directly Interacted with miR-212-3p and Negatively Regulated miR-212-3p Expression

Emerging evidence has demonstrated that lncRNAs might exert their functions by sponging miRNAs[26]. In view of the above results, we predicted the potential interaction between XIST

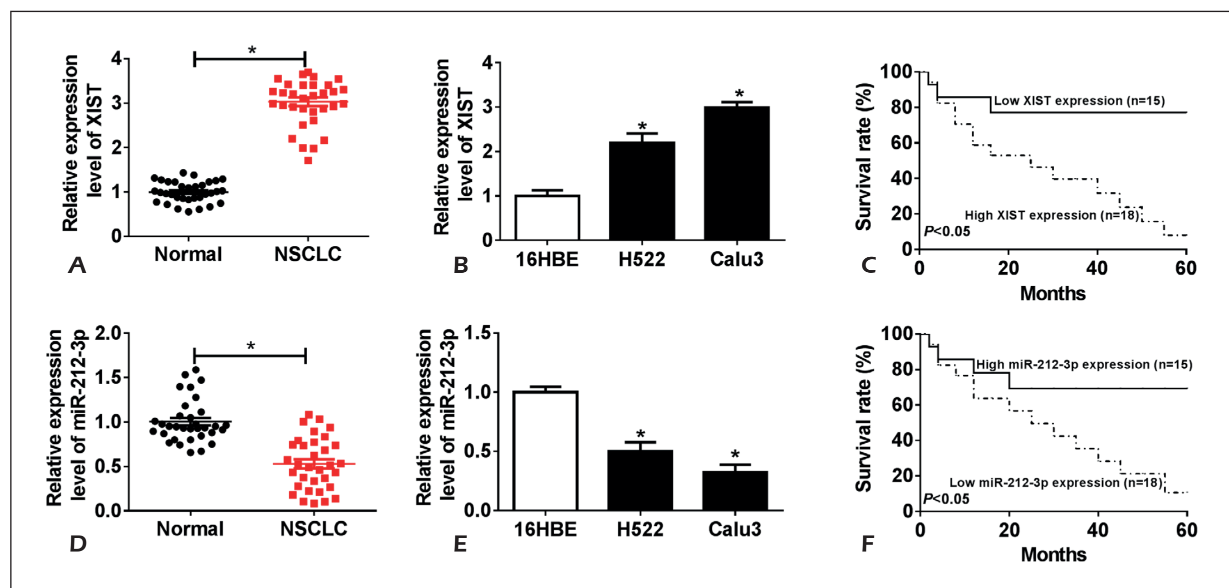

Figure 1. The expression of XIST and miR-212-3p, and the prognostic values of XIST and miR-212-3p for NSCLC. , The expression level of XIST in NSCLC tissues and normal tissues was detected by qRT-PCR. **B**, XIST expression in H522, Calu3 and 16HBE cells was detected via qRT-PCR. **C**, Overall survival rate of patients with low XIST expression or high XIST expression was monitored. **D**, The expression of miR-212-3p in NSCLC tissues and normal tissues was detected utilizing qRT-PCR. **E**, The level of miR-212-3p expression in H522, Calu3 and 16HBE cells was detected by qRT-PCR. **F**, Overall survival rate of NSCLC patients with high miR-212-3p expression or low miR-212-3p expression was recorded. *$p<0.05$.

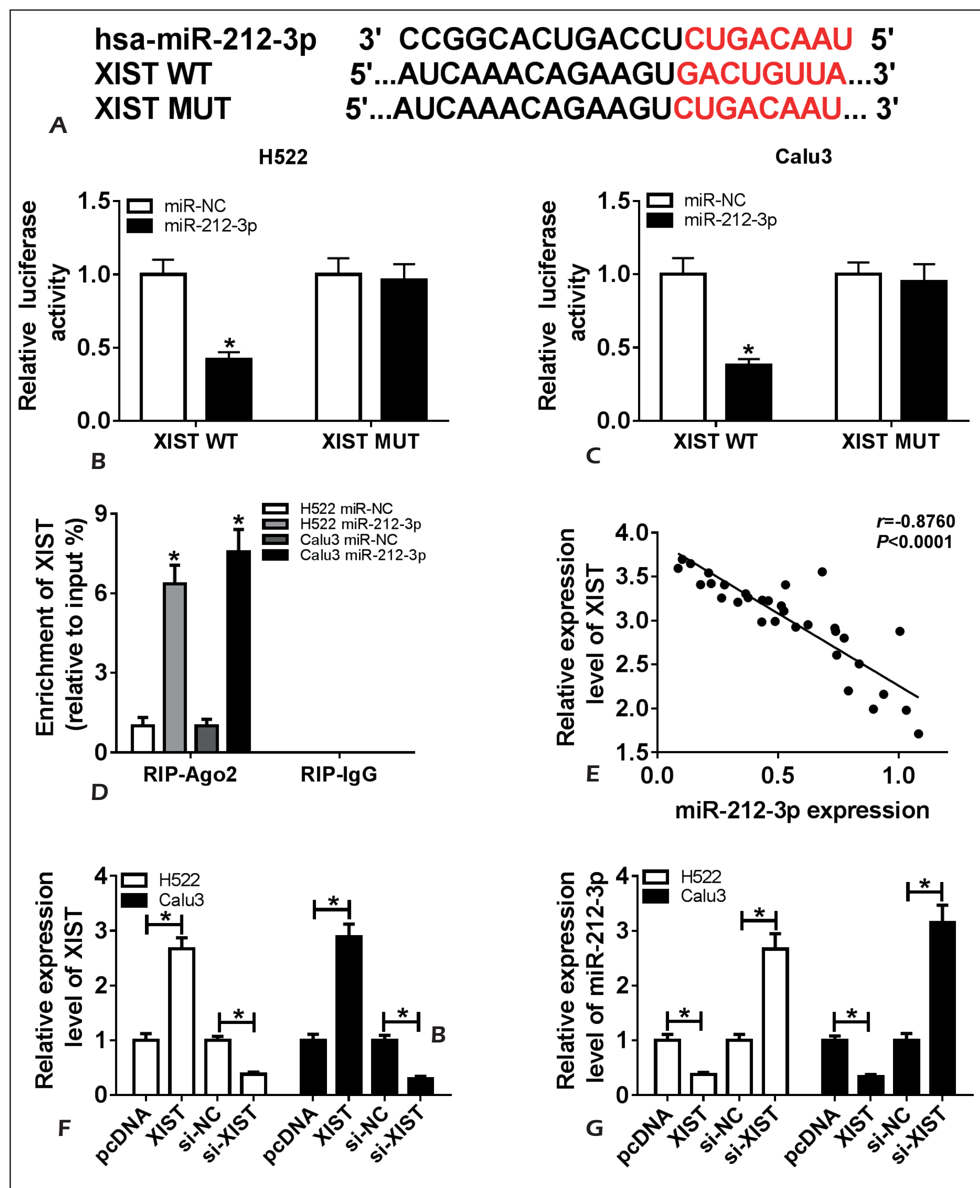

Figure 2. XIST directly targeting miR-212-3p to suppress. **A**, The potential binding sites of XIST and miR-212-3p were shown. **B-C**, Dual luciferase reporter assay was conducted to evaluate the luciferase activities of XIST WT or XIST MUT and miR-212-3p or miR-NC co-transfected H522 and Calu3 cells. **D**, RIP and qRT-PCR were performed to detect the enrichment of XIST. **E**, Pearson's correlation analysis was performed to evaluate the correlation of XIST and miR-212-3p. **F-G**, QRT-PCR was performed to detect the expression of XIST and miR-212-3p in XIST or si-XIST transfected H522 and Calu3 cells. $*p<0.05$.

and miR-212-3p in NSCLC cells by online software DIANA TOOLS. The result revealed that XIST contained the complementary binding sites of miR-212-3p (Figure 2A). Then dual luciferase reporter assay and RIP assay were carried out to verify this prediction. Dual luciferase reporter assay results showed that co-transfection of XIST WT and miR-212-3p led to significant suppression of the luciferase activities compared to XIST WT and miR-NC co-transfected groups in both H522 and Calu3 cells, but the luciferase activities were not changed in XIST MUT groups (Figure 2B and C). RIP assay data indicated that the enrichment of XIST was increased in Ago2 immunoprecipitation complex by miR-212-3p overexpression compared to miR-NC transfection groups in H522 and Calu3 cells (Figure 2D), which further verified the direct interaction of XIST and miR-212-3p. Furthermore, we found that XIST expression was negatively correlated with miR-212-3p expression in NSCLC tissues (Figure 2E). Subsequently, we examined the expression of XIST and miR-212-3p by qRT-PCR assay. The results exhibited that XIST expression was greatly increased while miR-212-3p expression was greatly decreased in H522 and Calu3 cells with XIST up-regulation, whereas silenced XIST promoted miR-212-3p expression (Figure 2F and G). Taken together, the data demonstrated that XIST interacted with miR-212-3p and negatively regulated miR-212-3p expression in NSCLC cells.

XIST Knockdown Suppressed Cell Proliferation, Migration, Invasion and EMT by miR-212-3p in NSCLC

In order to explore the biological functions of XIST and miR-212-3p in cell proliferation, migration and invasion of NSCLC cells, we transfected si-XIST, si-NC, si-XIST+in-miR-212-3p or si-XIST+in-miR-NC into H522 and Calu3 cells. Then CCK-8 assay and transwell assay were carried out to examine proliferation, migration and invasion of H522 and Calu3 cells, respectively. The results indicated that cell proliferation, migration and invasion were all notably inhibited by XIST knockdown in H522 and Calu3 cells compared to control groups, while these inhibitory effects were all abolished by miR-212-3p inhibition (Figure 3A-D). Meanwhile, the protein levels of EMT associated markers (including E-cadherin, N-cadherin and Vimentin) in H522 and Calu3 cells were measured by Western blot analysis. The data revealed that si-XIST led to a significant increase of E-cadherin and a significant decrease of N-cadherin and Vimentin, which were all reversed by the introduction of in-miR-212-3p

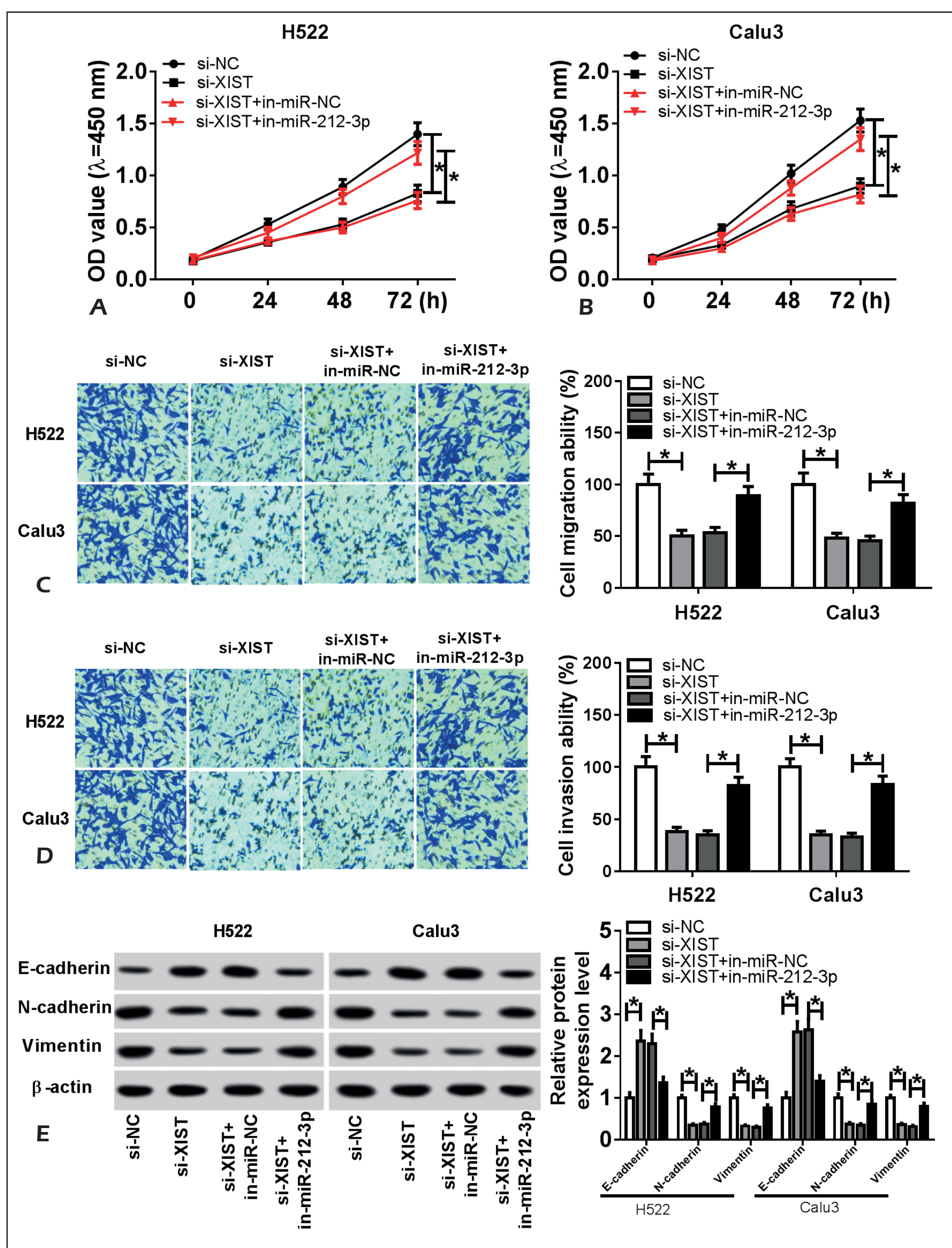

Figure 3. MiR-212-3p inhibition reversed the effects caused by XIST knockdown on proliferation, migration, invasion and EMT of NSCLC cells. H522 and Calu3 cells were transfected with si-XIST, si-NC, si-XIST+in-miR-212-3p or si-XIST+in-miR-NC. **A-B**, Cell proliferation was evaluated by CCK-8 assay. **C-D**, Cell migration and invasion were examined by transwell assay. **E**, The protein levels of EMT-associated proteins (E-cadherin, N-cadherin and Vimentin) were detected by western blot analysis. *$p<0.05$.

(Figure 3E). Collectively, these data demonstrated that XIST knockdown repressed NSCLC cells proliferation, migration, invasion and EMT by regulating miR-212-3p expression.

Knockdown of XIST Repressed Tumor Growth In Vivo

To further investigate the role of XIST in NSCLC *in vivo*, a xenograft tumor mouse model was established by injecting sh-SNHG1, sh-NC, sh-XIST+in-miR-212-3p or sh-XIST+in-miR-NC transfected Calu3 cells into the right flank of nude mice. Tumor volume was measured every week. Four weeks later, the mice were killed, and tumors were collected and weighed. The data showed that XIST knockdown decreased tumor volume and weight compared to sh-NC groups, while in-miR-212-3p reversed the effects on tumor volume and weight mediated by XIST knockdown (Figure 4A and B). Moreover, the expression levels of miR-212-3p and EMT-associated proteins were measured by qRT-PCR and Western blot analysis, respectively. QRT-PCR data showed that miR-212-3p expression was elevated in sh-XIST group; however, miR-212-3p inhibition decreased miR-212-3p expression in sh-XIST transfected mice (Figure 4C). Western blot analysis displayed that E-cadherin was upregulated, whereas N-cadherin and Vimentin were downregulated in sh-XIST group, while miR-212-3p inhibition restored all these effects caused by si-XIST (Figure 4D). These results indicated that XIST knockdown suppressed tumor growth *in vivo*.

XIST Positively Regulated CBLL1 Expression by Sponging miR-212-3p in NSCLC Cells

In order to further explore the molecular mechanism of XIST/miR-212-3p axis in NSCLC, bioinformatics software TargetScan was used to predict the potential target of miR-212-3p. CBLL1 was identified as a target gene of miR-212-3p and the putative binding sites were shown in Figure 5A. This prediction was confirmed by luciferase reporter assay. The luciferase activities were markedly inhibited by co-transfection with CBLL1

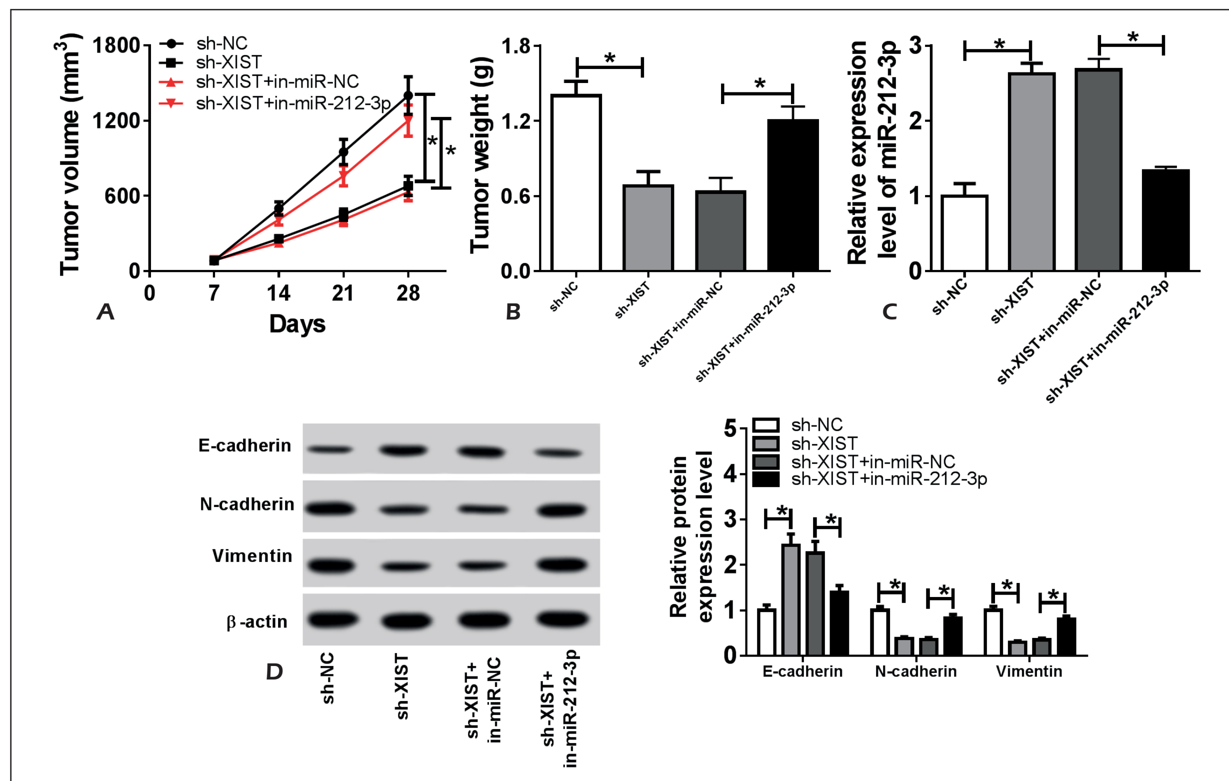

Figure 4. XIST knockdown suppressed tumors growth in vivo. **A**, Tumors volume was measured every week for four weeks. **B**, Tumors weight was measured after four weeks. **C**, The expression of miR-212-3p in collected xenograft tumors was detected by qRT-PCR. **D**, The expression levels of E-cadherin, N-cadherin and Vimentin in collected xenograft tumors were detected by Western blot analysis. *p<0.05.

3'-UTR-WT and miR-212-3p compared to miR-NC groups in H522 and Calu3 cells, while the luciferase activities were not changed in CBLL1 3'-UTR-MUT groups (Figure 5B and C). Next, the mRNA expression of CBLL1 in NSCLC tissues and cells was determined by qRT-PCR and the data exhibited that the mRNA expression of CBLL1 was greatly elevated in NSCLC tissues and cells (H522 and Calu3 cells) (Figure 5D and E). Moreover, there was an inverse correlation between CBLL1 expression and miR-212-3p expression in NSCLC tissues (Figure 5F). The protein expression of CBLL1 was further examined by Western blot analysis and the data revealed that the protein level of CBLL1 in H522 and Calu3 cells transfected with miR-212-3p was obviously downregulated compared to miR-NC groups (Figure 5G). We also found that XIST expression was positively correlated with CBLL1 in NSCLC tissues (Figure 5H). To determine whether XIST could regulate the expression of CBLL1 by sponging miR-212-3p, si-XIST, si-NC, si-XIST+in-miR-212-3p or si-XIST+in-miR-NC was transfected into H522 and Calu3 cells and the protein level of CBLL1 was measured. The results suggested that si-XIST notably decreased CBLL1 protein expression, while in-miR-212-3p abolished the effect in both H522 and Calu3 cells (Figure 5I). All these data demonstrated that XIST could regulate CBLL1 expression via miR-212-3p in NSCLC cells.

Overexpression of MiR-212-3p Suppressed Proliferation, Migration, Invasion and EMT in NSCLC Cells by Targeting CBLL1

To explore the biological functions of miR-212-3p/CBLL1 in NSCLC, miR-212-3p, miR-NC, miR-212-3p+CBLL1 or miR-212-3p+pcDNA was transfected into H522 and Calu3 cells for detection of cell proliferation, migration and invasion. CCK-8 assay and transwell assays implied that proliferation, migration and invasion abilities of H522 and Calu3 cells were all effectively suppressed by miR-212-3p compared to miR-NC groups, but the suppressive effects were overturned by CBLL1 overexpression (Figure 6A-D). In addition, the protein levels of E-cadherin, N-cadherin and Vimentin were detected by western blot analysis. As a result, miR-212-3p overexpression elevated E-cadherin expression and reduced N-cadherin expression and Vimentin expression, whereas these effects were abolished by CBLL1 overexpression (Figure 6E). These data suggested that overexpression of MiR-212-3p suppressed proliferation, migration, invasion and EMT in NSCLC cells by targeting CBLL1.

Discussion

In recent years, more and more studies have shown that lncRNAs play important roles in the development of many human cancers, including NSCLC[27,28]. In the current study, we explored the function roles and molecular mechanisms of lncRNA XIST in NSCLC progression. We found that XIST was upregulated and exerted a carcinogenic role in NSCLC through miR-212-3p/CBLL1 axis.

Literature has revealed that XIST is abnormally expressed and involved in development of multiple human cancers. For instance, Shen et al[29] demonstrated that XIST was elevated in pancreatic cancer (PC) and XIST knockdown suppressed migration, invasion and EMT in PC cells. Yao et al[30] also showed that in glioblastoma XIST expression was apparently increased, and knockdown of XIST suppressed cell proliferation, migration and invasion *in vitro*, and suppressed tumor growth *in vivo*. Furthermore, Wang et al[13] observed that XIST was highly expressed in NSCLC tissues and cells, and XIST knockdown repressed cell proliferation and invasion *in vitro* and repressed the growth of tumors *in vivo*. The research of Jiang et al[31] also showed carcinogenesis function of XIST by promoting viability and invasion ability of NSCLC cells. Consistently with these findings, we observed that XIST was distinctly increased in NSCLC tissues and cell lines (H522 and Calu3) and there was a shorter overall survival in high XIST expression group. Downregulation of XIST greatly decreased proliferation, migration and invasion of NSCLC cells. Meanwhile, EMT associated markers levels were determined by Western blot analysis and the results showed N-cadherin and Vimentin were decreased and E-cadherin was increased by si-XIST, indicating XIST knockdown suppressed the EMT of NSCLC cells. Moreover, we found that XIST knockdown significantly inhibited tumor growth. All these results revealed that XIST could function as an oncogene in NSCLC.

Given that lncRNAs could act as miRNAs sponges and regulate their functions[26], we investigated whether XIST could function as a miRNA sponge by online software DIANA TOOLS, dual luciferase reporter assay and RIP assay. The results showed that XIST contained the binding sites of miR-212-3p. MiR-212-3p was aberrantly expressed and involved in different human cancers development[16-18]. In our current study, miR-212-3p was distinctly decreased in NSCLC tissues and cells and there was a longer overall survival in high miR-212-3p expression group.

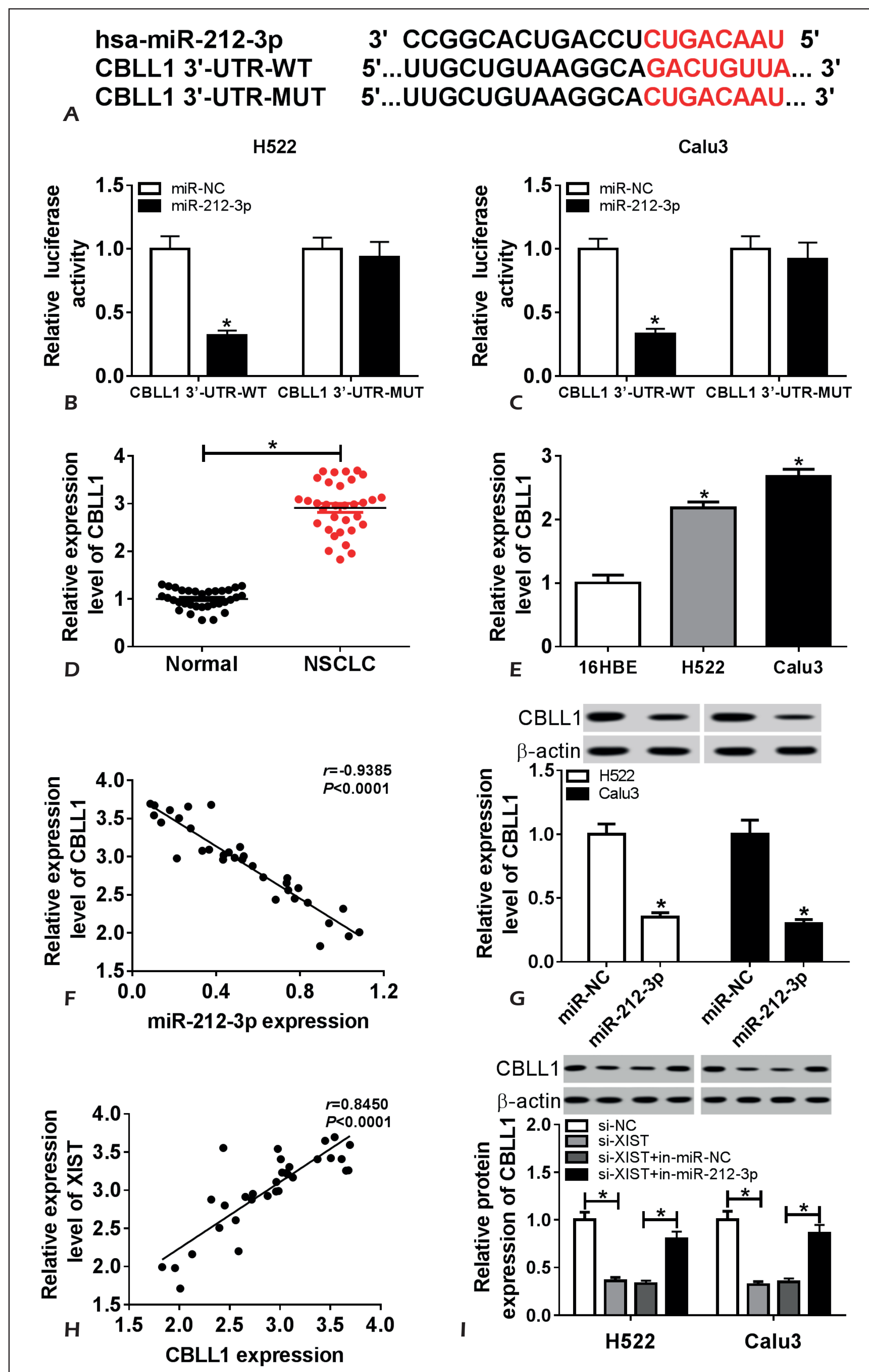

Figure 5. XIST regulated CBLL1 expression via miR-212-3p in NSCLC cells. **A**, The putative binding sites of miR-212-3p and CBLL1 3'-UTR were exhibited. **B-C**, Luciferase reporter assay was performed to evaluate the luciferase activities of CBLL1 3'-UTR-WT or CBLL1 3'-UTR-MUT and miR-212-3p or miR-NC co-transfected H522 and Calu3 cells. **D-E**, QRT-PCR was performed to detect the mRNA expressions of CBLL1 in NSCLC tissues, H522 cells and Calu3 cells. **F**, Pearson's correlation analysis was performed to evaluate the correlation of CBLL1 mRNA level and miR-212-3p level. **G**, Western blot analysis was carried out to evaluate the protein levels of CBLL1 in miR-212-3p or miR-NC transfected H522 cells and Calu3 cells. **H**, Pearson's correlation analysis was performed to evaluate the correlation of XIST level and CBLL1 mRNA level. **I**, Western blot analysis was carried out to evaluate the protein levels of CBLL1 in si-XIST, si-NC, si-XIST+in-miR-212-3p or si-XIST+in-miR-NC transfected H522 cells and Calu3 cells. *$p<0.05$.

Furthermore, miR-212-3p level was negatively correlated with XIST level and miR-212-3p inhibition reversed the effects of XIST knockdown on proliferation, migration, invasion and EMT of NSCLC cells, as well as tumor growth. These results indicated that miR-212-3p could function as a tumor suppressor in NSCLC, which was consistent with previous reports[19,32].

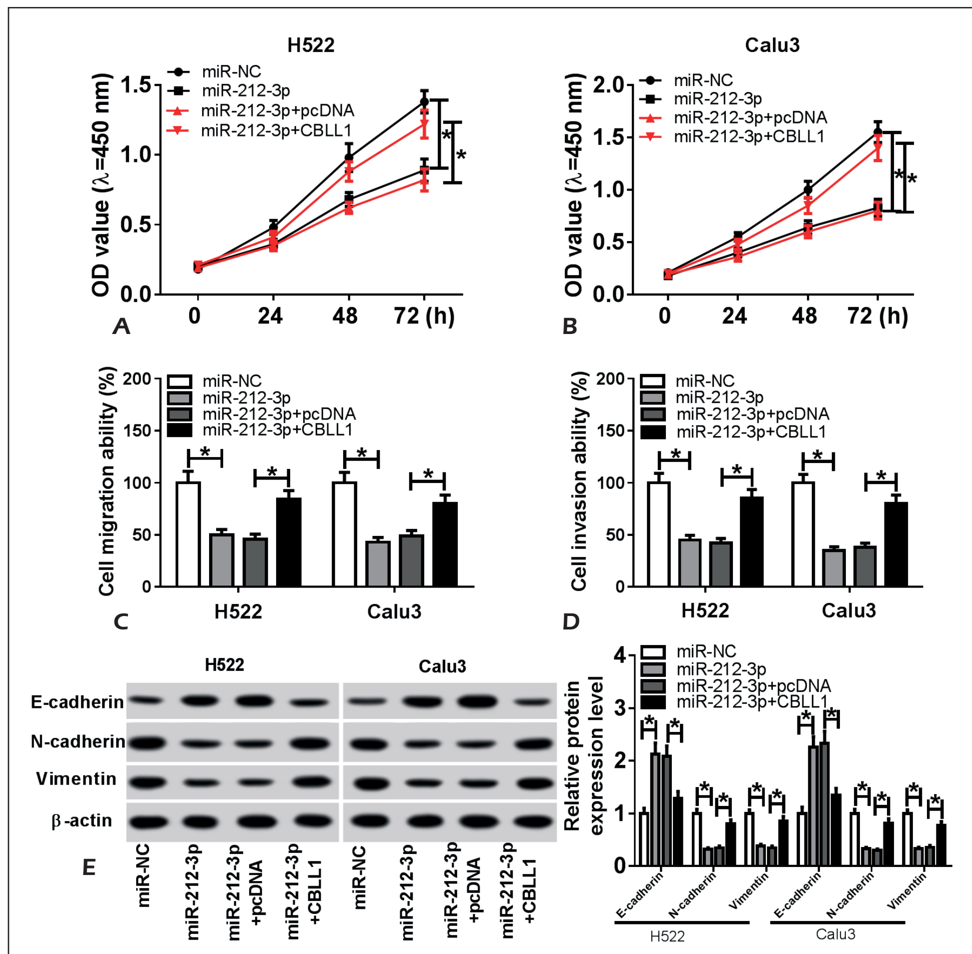

Figure 6. CBLL1 overexpression reversed the effects mediated by miR-212-3p overexpression on the proliferation, migration, invasion and EMT of NSCLC cells. H522 and Calu3 cells were transfected with miR-212-3p, miR-NC, miR-212-3p+CBLL1 or miR-212-3p+pcDNA. **A-B**, Cell proliferation was evaluated by CCK-8 assay. **C-D**, Cell migration and invasion were examined by transwell assays. **E**, The protein levels of E-cadherin, N-cadherin and Vimentin were detected by Western blot analysis. *$p<0.05$.

CBLL1 was identified as a target of miR-212-3p, and CBLL1 expression was significantly elevated in NSCLC tissues and cells. CBLL1 expression was positively correlated with XIST expression and negatively correlated with miR-212-3p expression. Upregulation of CBLL1 reversed the inhibitory effects of miR-212-3p on cell proliferation, migration, invasion and EMT in NSCLC. A previous study also showed higher expression of CBLL1 and the promotion effects of CBLL1 on cell growth and invasion in NSCLC[24], and our data revealed similar results.

Conclusions

We determined that XIST and CBLL1 were upregulated and miR-212-3p was downregulated in NSCLC tissues and cells. The expression of both XIST and miR-212-3p was associated with the survival rates of NSCLC patients. Moreover, we found that XIST downregulation inhibited proliferation, migration, invasion and EMT of NSCLC cells *in vitro* and inhibited tumor growth *in vivo* by regulating CBLL1 expression via miR-212-3p. This study revealed that XIST might be a novel target for treatment and prognosis of NSCLC patients.

Conflict of Interests

The authors report that they have no conflicts of interest in this study.

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