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Original Article

Long noncoding RNA CASC15 predicts unfavorable prognosis and exerts oncogenic functions in non-small cell lung cancer

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Abstract: Background: Aberrant expression of long non-coding RNA cancer susceptibility 15 (lncRNA CASC15) has been documented in various human tumors, and upregulation of CASC15 is closely correlated with cancer progression. However, the expression profile and potential biological functions of lncRNA CASC15 in non-small cell lung cancer (NSCLC) have not been fully characterized. Methods: The expression levels of CASC15 were assessed by qRT-PCR in human NSCLC tissues and by *in situ* hybridization in NSCLC tissue microarray. The relationship between CASC15 expression and clinical parameters, as well as prognosis were analyzed and validated in TCGA NSCLC datasets. The biological functions of CASC15 were analyzed by CCK-8 assay, cell migration and invasion assay in NSCLC cell lines *in vitro*. In addition, a mouse xenograft model was established to evaluate the effect of CASC15 knockdown on NSCLC tumor growth *in vivo*. Epithelial-mesenchymal transition (EMT) related molecules were examined by western blot and immunohistochemistry staining. Results: We found that CASC15 was upregulated in NSCLC tissues and cell lines. High expression levels of CASC15 were correlated with malignancies and poor survival rate in NSCLC patients. Multivariate analysis revealed that CASC15 was an independent risk factor of prognosis. In addition, we demonstrated that CASC15 knockdown inhibited NSCLC cell proliferation, migration and invasion in vitro. Xenograft model showed CASC15 knockdown significantly suppressed NSCLC tumor growth. Mechanistically, we revealed that CASC15 regulated EMT-related molecules and promoted the NSCLC progression and metastasis. Conclusion: In summary, our findings suggest CASC15 exhibits an oncogenic role in promoting NSCLC tumorigenesis via regulating EMT.

Keywords: lncRNA, CASC15, prognosis, EMT, NSCLC

Introduction

Lung cancer is one of the most frequently neoplasm and has emerged as the leading cause of cancer-related mortality worldwide [1]. Non-small cell lung cancer (NSCLC), which includes adenocarcinoma (LUAD), squamous cell carcinoma (LUSC) and large cell carcinoma, accounts for most diagnosed lung cancer cases [2]. Despite significant progresses made in therapeutic strategies, the 5-year survival rates of NSCLC remain dismal due to the high metastasis rate of NSCLC [2]. Thus, it is of paramount importance to elucidate the mechanisms underlying NSCLC metastasis for developing potential diagnostic biomarkers and therapeutic targets of NSCLC.

Long noncoding RNAs (lncRNAs) account for the vast majority of transcripts and functional RNAs with > 200 nucleotides [3]. Mounting literatures have documented that lncRNAs are important factors involved in cancer progression and play crucial roles in regulating gene expression in multiple cancers, including NSCLC [4-6]. For example, Li et al. found lncRNA UCA1 was upregulated in NSCLC and was associated with lymph node metastasis and prognosis [7]. Zhang et al. reported that upregulated lncRNA SNHG1 predicted poor survival rate of NSCLC and promoted tumor progression through Wnt/β-catenin pathway [8]. In addition, lncRNA PVT1 was indicated as a novel prognostic biomarker and potential therapeutic target in patients with NSCLC [9].

LncRNA cancer susceptibility 15 (CASC15), a novel lncRNA mapped to chromosome 6p22.3, was originally indentified as a neuroblastoma susceptibility locus and revealed to be able to gain a genomic segment in melanoma [10]. Recently, several studies confirmed that CASC15 was commonly dysregulated and involved in progression of various cancers including gastric cancer, hepatocellular carcinoma, melanoma and colorectal cancer [11-14]. However, the expression profile of CASC15 in NSCLC remains unknown, and the mechanism through which CASC15 exerts its oncogenic role needs to be elucidated.

In present study, we found that CASC15 was significantly unregulated in NSCLC tissues and cell lines. High level of CASC15 was closely related with NSCLC metastasis and poor survival rate. Furthermore, we demonstrated that CASC15 knockdown inhibited NSCLC cell proliferation and invasion *in vitro* and suppressed NSCLC tumor growth *vivo* via inducing Epithelial-mesenchymal transition (EMT). Taking together, our findings suggest that upregulation of CASC15 promote NSCLC progression and metastasis via inducing EMT, which could serves as a prognostic marker and therapeutic target for NSCLC patients.

Materials and methods

Patients and tissue microarray construction

This study was performed in accordance with the Helsinki Declaration and approved by the Research Advisory Council of The Second Affiliated Hospital of Zhengzhou University (SAHZZU). We collected 95 pairs of NSCLC tumor and matched normal lung tissues from 2009 to 2012. The diagnoses were confirmed by histological examination. All patients signed informed consents and approved by SAHZZU. The NSCLC tissue microarray (TMA) was constructed with all the 95 pairs of tissues according to our previous study [15]. For each patient, a 0.75 mm diameter core of the tissue was punched from FFPE tissues and arranged into the TMA blocks. In addition, 30 pairs of tissues were selected from these 95 patients of NSCLC cohorts at random for further qRT-PCR analysis.

TCGA expression dataset analysis

TCGA dataset analysis was performed according to our previous study [15, 16]. Briefly, we downloaded and analyzed NSCLC gene expression and corresponding clinical data from the Cancer Genome Atlas Project (TCGA, https://cancergenome.nih.gov/) containing a total of 1145 NSCLC tumor samples (herein after referred to as the TCGA-LUAD cohort and TCGA-LUSC cohort). TCGA-LUAD cohort contains 59 normal tissues and 535 LUAD tissues while TCGA-LUSC cohort has 49 normal tissues and 502 LUSC tissues.

RNA extraction and quantitative real-time PCR

Total RNA from NSCLC cell lines or specimens was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Briefly, total RNA was reversely transcribed to cDNA using cDNA reverse transcription kits (Roche, Germany). RNA concentration of tissue specimens or cells was measured by Nanodrop (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was performed using FastStart Universal SYBR Green Master (Roche, Germany) and a BIO-RAD C1000 Thermal Cycler. The relative fold-change in expression with respect to a control sample was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with normalization to GAPDH.

Cell culture and reagents

Human NSCLC cell lines (H1703, A549, SPC-A1, NCI-H460 and NCI-1650) and normal bronchial epithelial cell line (HBE and NLECs) were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM or RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2. The following antibodies were used in the study: anti-GAPDH, anti-KI67, anti-E-cadherin, anti-N-cadherin, and anti-Vimentin were from Proteintech Technology (Wuhan, China).

Western blot analysis

Cell proteins lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose (NC) membranes (Sigma, USA). The membranes were blocked with 5% non-fat milk in PBS for 1 h at room temperature. Specific primary antibodies against indicated proteins were added for overnight incubation at 4°C, followed by HRP-labeled secondary antibodies incubation (Beyotime, China) and detected by

the odyssey infrared imaging system (LI-COR Inc., USA) Figure S1.

Cell transfection and generation of CASC15 stable knockdown cell lines

The lentivirus-sh-CASC15 (sh-CASC15) and negative control (NC) vectors were purchased from Hanbio (Shanghai, China). Cells (1×10^5) were cultured until 80% confluence before transfection. The vectors were transfected into NSCLC cell lines using lipofectamine RNAiMAX reagents (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Cells were cultured with 1.5-μg/mL puromycin (Invitrogen, USA) for 36 hours to select the stable knockdown cells.

CCK-8 assay

Cell proliferation was assessed by CCK-8 assay (Beyotime, China). Briefly, a total of approximately 5000 cells were seeded into 96-well plates in 100 μl medium per well. CCK-8 solution was added into each well 24 h after shRNA transfection. The absorbance at 490 nm was determined via Spectra Max 250 spectrophotometer (MD, USA). All experiments were performed in triplicate.

5-Ethynyl-20-deoxyuridine (EdU) assay

Cells were seeded into 24-well tissue culture plates. After incubation for 24 h, the DNA synthesis rate of NSCLC cell line was determined by the EdU assay kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. The number of EdU-positive cells was counted in three random images per well by microscopy using a 100 × objective (Olympus, Tokyo, Japan).

Colony formation assay

Transfected cells were seeded (1000 cells/well) in a dish and cultured for 10 days. Surviving colonies were fixed, stained with 5% gentian violet (Sigma-Aldrich, USA) and counted. The experiment was carried out in triplicate wells for three times.

Transwell migration and invasion assay

Cell migration and invasion ability were assessed using transwell chamber (Corning) covered with or without matrigel (BD Biosciences). A total of approximately 1×10^5 cells were added to the top chamber of 24-well plates, while 20% FBS was added into the bottom well. After incubation for 24 h, the migrated or invaded cells were fixed with 4% paraformaldehyde and then staining with Crystal violet. And the cells were counted and imaged under an inverted microscope (Olympus, Japan). Each experiment was repeated three times.

Immunohistochemistry (IHC) and in situ hybridization (ISH) staining

IHC and ISH was performed according to our previous studies [17, 18]. For IHC staining, paraffin-embedded tissue sections were stained with H&E or were processed for IHC staining using standard procedures. Briefly, section slides were incubated with primary antibody against KI67, E-cadherin, N-cadherin, or Vimentin at 4°C overnight and then probed with secondary antibodies. After staining, sections were dehydrated, cleared with xylene and mounted with resinene. For ISH staining, a digoxigenin-labeled CASC15 oligonucleotide probe was obtained from Boster Biological Technology (BOSTER, China). Section slides were deparaffinized and then pre-hybridized. Afterward, TMA was hybridized with CASC15 probe overnight, followed by incubation with antibody against digoxigenin. Section slides were incubated with anti-DIG reagent and the expression signal was determined by diaminobenzidine (DAB) solution (BOSTER, China). The ISH staining of CASC15 was assessed independently by two scientists (Y.C and Z.G) who had no knowledge of the clinical information.

In vivo tumor growth

All animal procedures were approved by Animal Care Committee of The Second Affiliated Hospital of Zhengzhou University. 12 BALB/c nude mice were purchased from Vital River Laboratory (Beijing, China). A549 cells (5×10^6) stably transfected with sh-CASC15 or negative control vector were subcutaneously injected into nude mice. Mice were photographed with an IVIS@ Lumina II system (Caliper Life Sciences, USA) every 7 days. Tumor volume was measured every 7 days and was calculated by the following formula: volume (mm^3) = (length × $width^2$)/2.

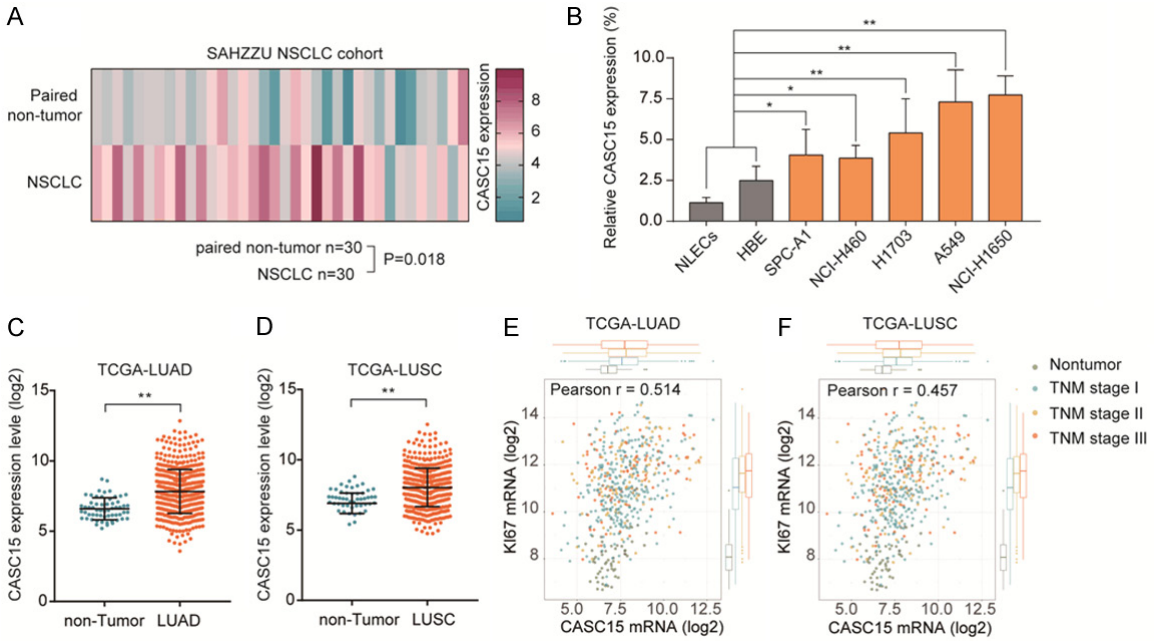

Figure 1. LncRNA CASC15 is highly expressed in human NSCLC tissues and cell lines. A. The expression levels of lncRNA CASC15 in 30 NSCLC samples and adjacent non-tumor lung tissues were analyzed by qRT-PCR. B. The expression levels of lncRNA CASC15 in lung cancer cell lines (SPC-A1, NCI-H460, H1703, A549 and NCI-H1650) and in normal lung epithelial cells (NLECs and HBE) were analyzed by qRT-PCR. $^{*}P < 0.05$ vs. NLECs and $^{\&}P < 0.05$ vs. HBE. C, D. The expression levels of lncRNA CASC15 in LUAD and LUSC tissues and the corresponding non-tumor tissues based on TCGA NSCLC dataset analysis. E, F. The Pearson correlation of CASC15 levels with KI67 expression in TCGA-LUAD and TCGA-LUSC cohort of different TNM stages was analyzed. Mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired or paired Student's *t*-test.

Statistical analysis

The data were presented as the mean ± SEM from at least three independent experiments. Student's *t*-test (two-tailed) and one-way analysis of variance were used to compare the differences between two groups and more than two groups, respectively. A chi-squared test was used to evaluate the association between CASC15 expression and clinicopathological parameters. The survival rates were determined using the Kaplan-Meier method (log-rank test). A *P* value of < 0.05 was considered significant. Statistical analysis was performed using SPSS (SPSS Inc., USA).

Results

LncRNA CASC15 is highly expressed in human NSCLC tissues and cell lines and CASC15 overexpression positively associates with poor prognosis of NSCLC patients

We first investigated the expression levels of CASC15 in 30 pairs of NSCLC tissues and paired normal lung tissues. As shown in **Figure 1A**, qRT-PCR results showed that CASC15 was significantly unregulated in NSCLC tissues compared with that in normal lung tissues. We further demonstrated that the expression levels of CASC15 in lung cancer cell lines (SPC-A1, NCI-H460, H1703, A549 and NCI-H1650) were markedly higher than that in normal lung epithelial cells (NLECs and HBE) (**Figure 1B**). Moreover, we downloaded and analyzed NSCLC gene expression and corresponding clinical data in two NSCLC cohorts (TCGA-LUAD and TCGA LUSC) from TCGA database containing a total of 1145 NSCLC tumor samples. The results showed that CASC15 were significantly overexpressed in NSCLC tumor tissues both in LUAD and LUSC cohorts (**Figure 1C** and **1D**). Intriguingly, we found that CASC15 expression was positively related to KI67 expression while higher CASC15 expression correlated with advanced TNM stages in both cohorts (**Figure 1E** and **1F**).

Subsequently, we explored the clinical significance of CASC15 in TCGA-LUAD and TCGA LU-

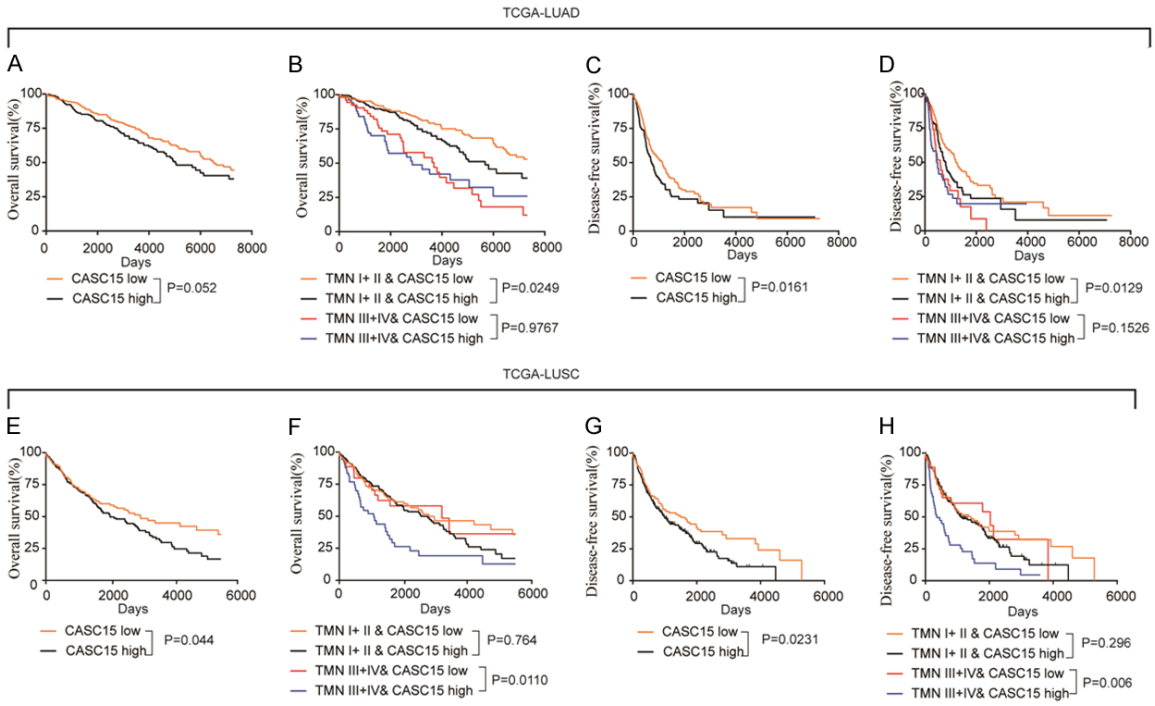

Figure 2. CASC15 overexpression positively associates with poor prognosis of NSCLC patients. A. Kaplan Meier analysis of the correlation between CASC15 expression and overall survival (OS) rate in TCGA LUAD cohorts; B. Kaplan Meier analysis of the correlation between CASC15 expression and OS rate of patient at different TMN stages in TCGA LUAD cohorts; C. Kaplan Meier analysis of the correlation between CASC15 expression and disease-free survival (DFS) rate in TCGA LUAD cohorts; D. Kaplan Meier analysis of the correlation between CASC15 expression and DFS rate of patients at different TMN stages in TCGA LUAD cohorts; E. Kaplan Meier analysis of the correlation between CASC15 expression and OS rate in TCGA LUSC cohorts; F. Kaplan Meier analysis of the correlation between CASC15 expression and OS rate of patient at different TMN stages in TCGA LUSC cohorts; G. Kaplan Meier analysis of the correlation between CASC15 expression and DFS rate in TCGA LUSC cohorts; H. Kaplan Meier analysis of the correlation between CASC15 expression and DFS rate of patients at different TMN stages in TCGA LUSC cohorts.

SC datasets. Kaplan-Meier analysis results suggested that high CASC15 expression was negatively correlated with NSCLC patients' overall survival and disease-free survival rates both in TCGA-LUAD and TCGA LUSC cohorts (**Figure 2A**, **2C**, **2E** and **2G**). However, subgroup analysis revealed that early-stage (stages I and II) NSCLC patients with high CASC15 expression had significant shorter OS and DFS rates than those with low CASC15 expression in TCGA-LUAD cohort (**Figure 2B** and **2D**), while late-stage (stages III and IV) NSCLC patients with high CASC15 expression had significant shorter OS and DFS rates than those with low CASC15 expression in TCGA-LUSC cohort (**Figure 2F** and **2H**). In summary, these results indicate that high expression of CASC15 might be a potential prognostic biomarker for NSCLC patients.

High CASC15 expression is associated with poor prognosis in NSCLC TMA cohort

To further verify the expression pattern and clinical significance of CASC15 in NSCLC, ISH was performed in TMA containing 95 NSCLC tissues (SFAZZU cohort, **Table 1**). Consistent with our previous findings, CASC15 was highly expressed in most NSCLC tissues compared with that in normal non-tumor lung tissues (**Figure 3A**). Based on CASC15 ISH staining intensity (**Figure 3B**), 40 patients were classified in the low CASC15 expression group (with score 1+ and 2+) and 55 patients were classified in the high CASC15 expression group (with score 3+ and 4+). Our results demonstrated that enhanced CASC15 expression was significantly related with tumor size (**Figure 3C**), lymph node metastasis (**Figure 3D**) and advanced TNM stages (**Figure 3E**). Moreover, NSCLC

Table 1. Correlation of clinico-pathological features with CASC15 expression in NSCLC TMA cohort

Clinicopathological Variables features		CASC15 expression		*P-value*
		Low expression (n = 40)	High expression (n = 55)	
Age (years)	≤ 65	20 (50.0)	33 (60.0)	0.332
	> 65	20 (50.0)	22 (40.0)	
Gender	Male	25 (62.5)	37 (67.2)	0.629
	Female	15 (37.5)	18 (32.7)	
Smoking history	Smokers	27 (67.5)	32 (58.2)	0.600
	Never smokers	13 (32.5)	23 (41.8)	
Histologic subtype	Squamous cell carcinoma	28 (70.0)	31 (56.3)	0.176
	Adenocarcinoma	12 (30.0)	24 (43.6)	
TNM stage	Stage I	20 (50.0)	12 (21.8)	0.014
	Stage II	9 (22.5)	16 (29.1)	
	Stage III	11 (27.5)	27 (49.1)	
Tumor size (cm)	≤ 5	26 (65)	24 (43.6)	0.039
	> 5	14 (35)	31 (56.3)	
Lymph metastasis	No	27 (67.5)	20 (36.3)	0.003
	Yes	13 (32.5)	35 (63.6)	

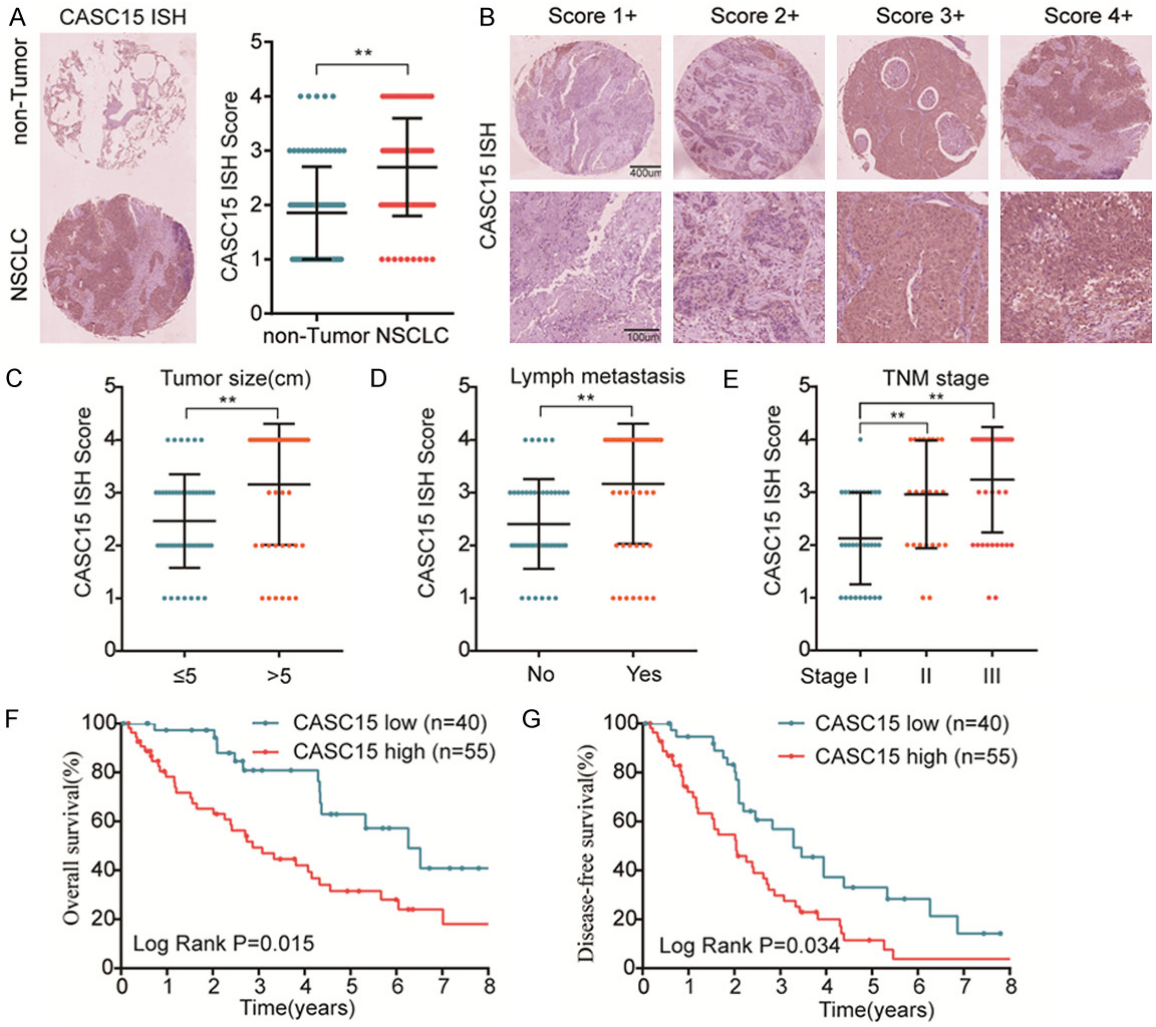

Figure 3. High CASC15 expression is associated with poor prognosis in NSCLC TMA cohort. (A) CASC15 expression in NSCLC tumor specimens and paired adjacent non-tumor tissues was analyzed by ISH from TMA cohort. (B) Representative photographs of CASC15 ISH staining patterns with different staining scores in NSCLC tissues. Scale bar, 400 μm (up panel) or 100 μm (lower panel). (C-E) Distribution of CASC ISH scores in NSCLC tissues with different tumor size (C), Lymph metastasis (D) and TNM classification (E). Kaplan-Meier analysis of the OS rate (F) and DFS rate (G) in NSCLC patients with different CASC15 expression levels. Mean ± SD, $^{**}P < 0.01$, Mann-Whitney rank test or Kaplan Meier analysis.

Table 2. Correlation of clinico-pathological features with CASC15 expression in NSCLC TMA cohort

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Univariate and multivariate analysis of overall survival in NSCLC patients (n = 95)						
Age	1.115	0.695-1.920	0.541			
Gender	0.754	0.546-1.365	0.417			
Smoking history	1.141	0.876-1.364	0.249			
Histologic subtype	0.543	0.3212-0.873	0.014	0.611	0.358-1.008	0.052
TNM stage	2.451	1.775-3.423	0.001	2.309	1.301-2.973	0.003
Tumor size (cm)	1.827	1.129-2.954	0.013	1.420	0.820-2.331	0.274
Lymph metastasis	2.032	1.259-3.291	0.003	1.609	0.907-2.702	0.045
CASC15 expression	2.039	1.259-3.346	0.005	1.901	1.704-2.319	0.015
Univariate and multivariate analysis of disease-free survival in NSCLC patients (n = 95)						
Age	1.106	0.634-1.960	0.724			
Gender	0.853	0.481-1.523	0.573			
Smoking history	0.689	0.389-1.271	0.241			
Histologic subtype	0.658	0.371-1.204	0.175			
TNM stage	3.534	2.317-5.426	0.002	2.412	1.745-4.214	0.002
Tumor size (cm)	2.023	1.152-3.603	0.021			
Lymph metastasis	3.128	1.490-5.938	< 0.001	1.936	1.729-2.835	0.036
CASC15 expression	5.210	2.540-10.487	< 0.001	2.803	1.804-6.319	0.002

patients with high CASC15 expression had remarkably lower OS and DFS rate than those with low CASC15 expression in SAHZZU cohort through Kaplan-Meier survival analysis (**Figure 3F** and **3G**). Moreover, Univariate analysis and Multivariate analysis revealed that CASC15 expression, lymph node metastasis and TNM stage were correlated with OS and DFS in NSCLC patients (**Table 2**, $P < 0.05$).

CASC15 knockdown suppresses NSCLC cell proliferation, migration and invasion in vitro

To further investigate the function of CASC15 in NSCLC cells, we evaluated three different shRNAs targeting CASC15 in NCI-H1650 and A549 cells, which had the highest levels of CASC15. ShRNA knockdown efficiency was confirmed by real-time PCR analysis (**Figure 4A**) and sh-CASC15-1 was used for further experiments (termed as sh-CASC15 later). CCK-8 assays showed that CASC15 silencing inhibited cell proliferation of NCI-H1650 and A549 cells (**Figure 4B**). Consistently, we observed a dramatically decrease in cell division and colony formation in NCI-H1650 and A549 cells transfected with sh-CASC15 in comparison with those in control cells, as demonstrated by EDU/DAPI staining and colony formation assays (**Figure 4C** and **4D**). Furthermore, transwell assays showed that the relative migration and invasion cells of NCI-H1650 and A549 cells were remarkably decreased after CASC15 knockdown (**Figure 4E** and **4F**). Taken together, these results suggest that CASC15 knockdown suppresses NSCLC cell proliferation, migration and invasion *in vitro*.

CASC15 knockdown inhibits NSCLC tumor growth in vivo

To further determine the effect of CASC15 on NSCLC tumorigenesis *in vivo*, A549 cells were transfected with sh-NC or sh-CASC15. Cells

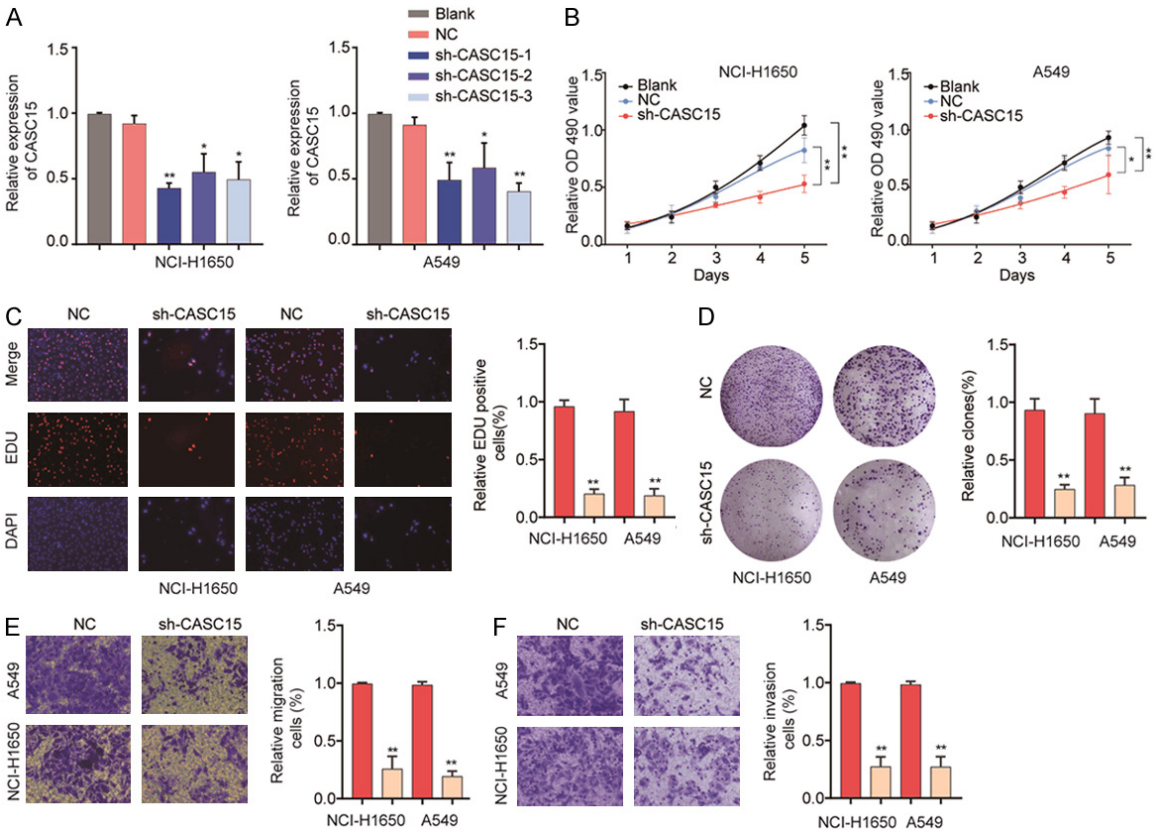

Figure 4. CASC15 knockdown suppresses NSCLC cell proliferation, migration and invasion *in vitro*. NSCLC NCI-H1650 or A549 cells were transfected with shRNAs targeting CASC15 (sh-CASC15-1/2/3) or negative control (NC). (A) ShRNA Knockdown efficiency was determined by qRT-PCR. (B) Cell proliferation of NCI-H1650 or A549 cells was assessed by CCK-8 assay. (C) Cell division of NCI-H1650 or A549 cells was assessed by EDU/DAPI staining. Scale bar, 100 μm. (D) The colony formation of NCI-H1650 or A549 cells transfected with sh-CASC15 or NC was analyzed. (E) Cell migration and (F) invasion of NCI-H1650 or A549 cells were analyzed by transwell assay. Results were shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired or paired Student's *t*-test.

with stably CASC15 knockdown or control cells were subcutaneously injected into nude mice and tumor growth were monitored. As shown in **Figure 5A**, CASC15 knockdown significantly inhibited the tumor growth in sh-CASC15 group compared with that in NC group. Consistently, bioluminescence imaging showed that CASC15 knockdown decreased mean luciferase signal in xenograft tumors (**Figure 5B** and **5C**). In addition, the weights of xenograft tumors in sh-CASC15 group were also markedly lower than that in NC group (**Figure 5D**). IHC analysis revealed that KI67 expression was significant downregulated in xenograft tumors derived from sh-CASC15 group compared with that in NC group (**Figure 5E** and **5F**). Taken together, these results indicate that CASC15 may function as an oncogene *in vivo*.

CASC15 promotes NSCLC progression and metastasis via regulating EMT

To investigate the underlying mechanisms by which CASC15 knockdown inhibits NSCLC tumorigenesis, we performed Gene Set Enrichment Analysis (GSEA) and found that the enhanced activity of Anastassioucancer mesenchymal transition signature and Jechlinger epithelial to mesenchymal transition up signature genes were enriched in patients with high levels of CASC15 (**Figure 6A** and **6B**). Further experiments were performed to determine the effect of CASC15 on expression of EMT-related molecules. As shown in **Figure 6C** and **6D**, western blot results revealed that knockdown CASC15 significantly upregulated the expression of E-cadherin, while downregulated the expression of EMT-related molecules such as

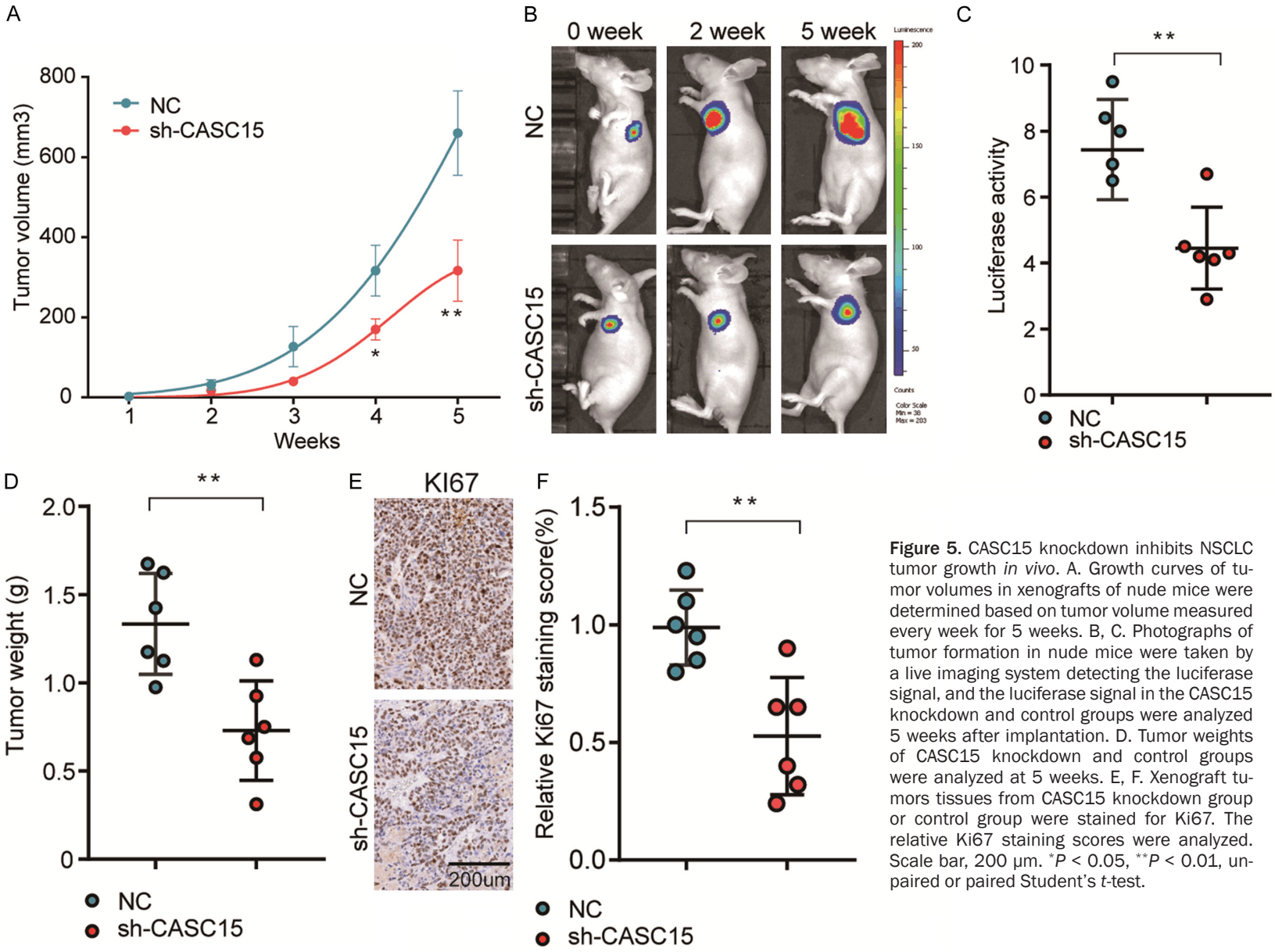

Figure 5. CASC15 knockdown inhibits NSCLC tumor growth *in vivo*. A. Growth curves of tumor volumes in xenografts of nude mice were determined based on tumor volume measured every week for 5 weeks. B, C. Photographs of tumor formation in nude mice were taken by a live imaging system detecting the luciferase signal, and the luciferase signal in the CASC15 knockdown and control groups were analyzed 5 weeks after implantation. D. Tumor weights of CASC15 knockdown and control groups were analyzed at 5 weeks. E, F. Xenograft tumors tissues from CASC15 knockdown group or control group were stained for Ki67. The relative Ki67 staining scores were analyzed. Scale bar, 200 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired or paired Student's *t*-test.

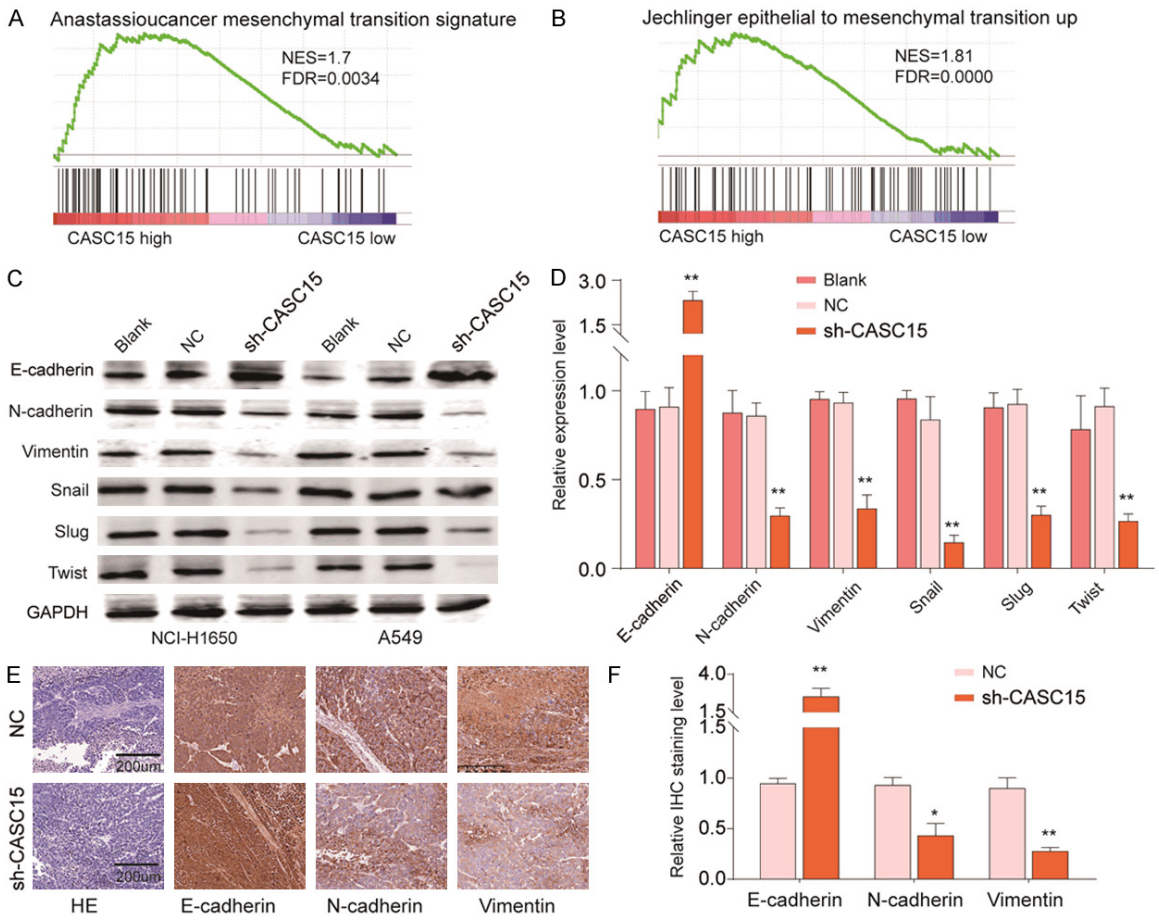

Figure 6. CASC15 promotes NSCLC progression and metastasis via regulating EMT. A, B. The Gene Set Enrichment Analysis (GSEA) of the relationship between the expression level of CASC15 and EMT-related gene signatures in TCGA cohort. C, D. Western blot analysis of E-cadherin, N-cadherin, Vimentin, Snail, Slug and Twist expression in NCI-H1650 or A549 cells transfected with sh-CASC15 or NC. E, F. IHC staining analysis of N-cadherin, Vimentin, and E-cadherin in xenograft tumor tissues from CASC15 knockdown or control groups. Scale bar, 200 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired or paired Student's *t*-test. Representative images and data are based on three independent experiments.

N-cadherin, Vimentin, Snail, Slug and Twist. We also examined the expression of EMT markers in xenograft tumor tissues from sh-CASC15 or NC group. CASC15 knockdown significantly decreased expression of N-cadherin and Vimentin, while enhanced expression of E-cadherin as demonstrated by IHC staining (**Figure 6E** and **6F**). Collectively, these data suggest that repression of CASC15 could inhibit the tumorigenesis of NSCLC, at least in part, via regulating EMT process.

Discussion

Developing effective therapeutic strategies for NSCLC remains a big challenging due to its high metastasis rate. Mounting evidences have revealed that lncRNAs have been involved in progression and metastasis of various types of cancers including lung cancer [19]. Recent studies have suggested that multiple lncRNAs are involved in the progression of lung cancer, such as MALAT1, CCAT1 and PVT1 [20-22].

Numerous lncRNAs are aberrantly expressed in NSCLC and play critical roles in the progression and metastasis of NSCLC by acting as either tumor suppressors or oncogenes. Increased expression of CASC15 was observed in gastric cancer and colorectal cancer, indicating that CASC15 might function as oncogene in these cancers [11-13]. In the present study, we investigated the role of CASC15 in NSCLC and explored the underlying mechanisms. We found

that CASC15 was highly overexpressed in the NSCLC specimens and cell lines in comparison to that in normal lung tissues as demonstrated by qRT-PCR and ISH analysis. Moreover, expression level of the CASC15 inversely associated with tumor invasion, lymph node metastasis and TNM stages in NSCLC. NSCLC patients with high level of CASC15 expression showed poorer prognosis than those with low CASC15 expression. This is in agreement with previous findings that CASC15 functions as oncogene in many other tumor tissues [10-14]. In summary, these findings indicate that CASC15 serves as an oncogene and plays a critical role in NSCLC progression and metastasis.

Limitless proliferation is the remarkable hallmark of malignancies [23]. We found high CASC15 expression was positively correlated with strong KI67 expression. In addition, loss-of-function experiment showed that CASC15 knockdown significantly inhibited NSCLC cell proliferation *in vitro*, and suppressed tumor growth in *vivo*. Similar to our findings, Wu et al. reported that CASC15 plays a critical role in cell proliferation via the induction of cell cycle arrest in gastric cancer [13]. In colon cancer, CASC15 could promote cancer growth and metastasis through the activation of the Wnt signaling pathway [12]. In addition, CASC15 knockdown also impaired hepatocellular carcinoma cell proliferation *in vitro* and impaired tumor growth *in vivo* [11]. Our results confirmed that silencing CASC15 in NSCLC cells significantly inhibited the cell proliferation, suggesting CASC15 might be a potential therapeutic target for NSCLC.

High metastasis rate is the major obstacle to the improvement of cancer patient prognosis [24]. In the current study, we found that high CASC15 expression was significantly associated with lymph node metastasis and TNM stages in NSCLC specimens. Consistently, CASC15 knockdown inhibited migration and invasion of lung cancer cells *in vitro*. Similar phenomenon was observed in other cancers [11, 13, 14]. These results indicated CASC15 was involved in the metastasis process of NSCLC. Extensive studies have indicated that EMT is associated with a high invasive behavior and driving the distant metastasis of cancer [25]. A multitude of lncRNAs has been identified as EMT regulator in NSCLC, including PVT1, OGFRP1 and LSINCT5 [9, 25-27], despite that diverse mechanisms were involved. Here, our results revealed that CASC15 knockdown led to a significant decrease in the protein levels of downstream effectors of the EMT (such as N-cadherin and Vimentin) wile a remarkably enhanced E-Cadherin expression. Our findings suggest that EMT may be involved in CASC15-mediated promotion of tumorigenesis.

In summary, we found lncRNA CASC15 was upregulated in NSCLC, and high expression of CASC15 was a negative prognostic marker for NSCLC patients. Functional experiments revealed that CASC15 knockdown suppressed tumor growth and tumorigenesis both *in vitro* and *in vivo*. Furthermore, CASC15 played an oncogenic role through promoting EMT of NSCLC cells. Our findings provide better knowledge of the pathogenesis of NSCLC, and suggest the potential of CASC15 as a therapeutic target for lncRNA-based cancer therapy.

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Disclosure of conflict of interest

None.

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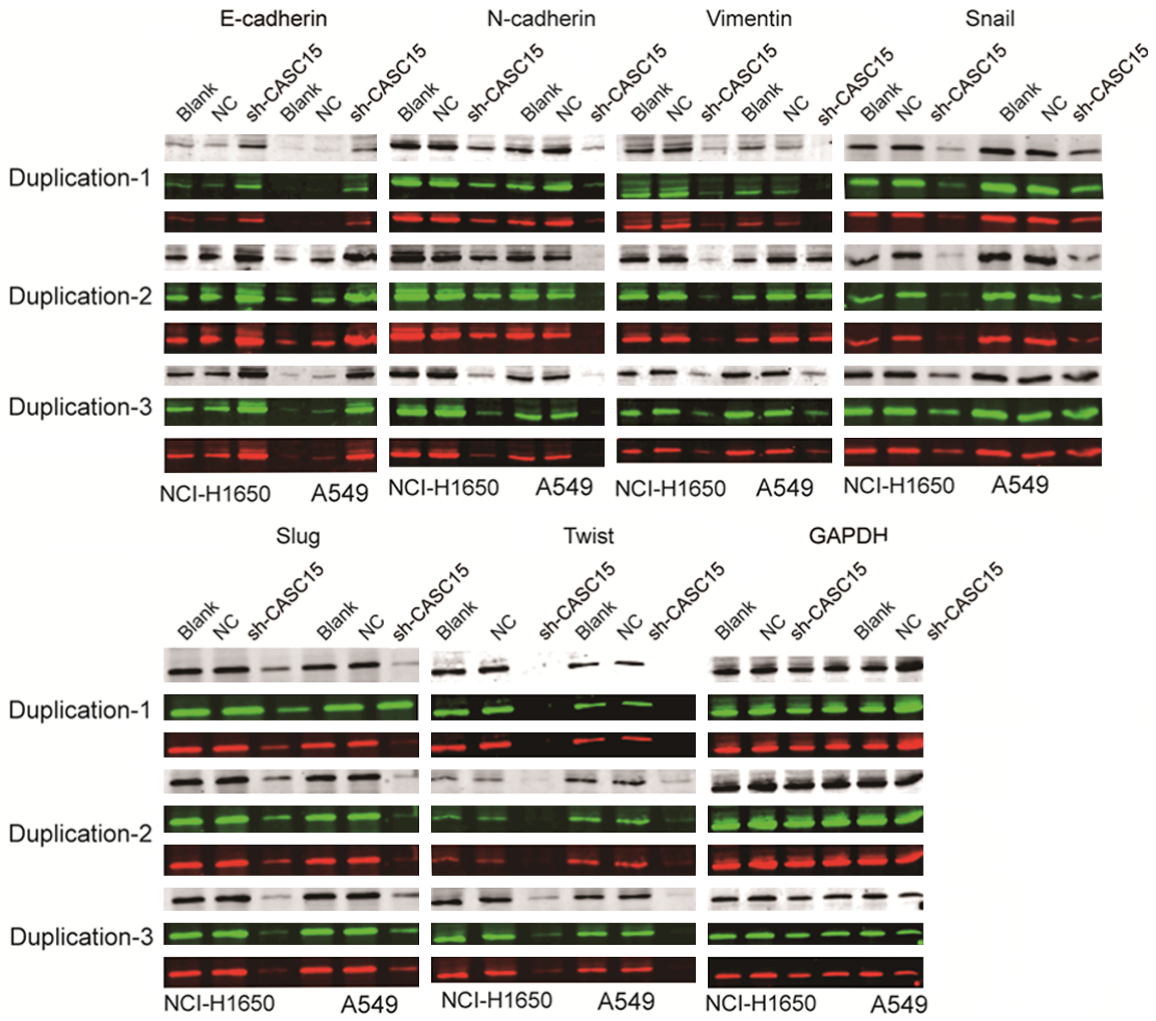

Figure S1. Original western images for all relevant western blots.