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RESEARCH ARTICLE

SP1-induced overexpression of LINC00520 facilitates non-small cell lung cancer progression through miR-577/CCNE2 pathway and predicts poor prognosis

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Abstract

Long noncoding RNAs (lncRNAs) have gained much attention in the past few years. Long intergenic non-protein coding RNA 520 (LINC00520) was one of the newly discovered lncRNA which has been demonstrated to be dysregulated in several cancers. So far, the function and mechanism of LINC00520 in non-small cell lung cancer (NSCLC) are unclear. In this paper, our group first showed that LINC00520 level was elevated in non-small cell lung cancer (NSCLC) tissue and cells. In addition, SP1 could bind directly to the promoter region of LINC00520 and thus promote its transcription. Increased LINC00520 was distinctly correlated with advanced tumor stage and shorter survival time in NSCLC patients. Further functional investigations provided evidences that forced down regulation of LINC00520 inhibited NSCLC cell proliferation, invasion, metastasis and EMT, while contributing to cells apoptosis. Mechanistically, we found that LINC00520 serving as a competing endogenous RNA to be involved in the modulation of miR-577 expressions, and thus affected the expression of CCNE2 which was a target gene of miR-577. Moreover, in NSCLC cells with si-LINC00520, up regulation of CCNE2 led to an increase of cell growth and invasion. Taken together, LINC00520 displayed its tumor-promotive roles through modulating the miR-577/CCNE2 axis, highlighting a potential therapeutic strategy for NSCLC patients.

Keywords LncRNA LINC00520 · miR-577 · CCNE2 · SP1 · NSCLC · Tumorigenesis

Introduction

Lung cancer is the most commonly occurring cancer and the leading cause of cancer deaths worldwide, with ~ 1.5 million novel cases diagnosed each year [1, 2]. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases [3]. In China, the incidence and mortality of NSCLC are the highest among malignant tumors [4]. Despite improvements in surgery, radiotherapy, chemotherapy and targeted therapies including EGFR targeting therapies, the long-term outcome remains poor [5, 6]. Major reasons of high mortality rate in NSCLC are the high rate of recurrence and metastasis [7]. Thus, further elucidating the underlying mechanisms of metastatic NSCLC is urgently required.

Long noncoding RNAs (lncRNAs) are a newly discovered class of noncoding RNA (ncRNA) > two hundred nucleotides in length which lack an open reading frame [8, 9]. LncRNAs have recently received a lot of attention for their effects in diverse biological progression including cell differentiation, apoptosis and metastasis [10]. More and more studies show that the dysregulation of lncRNAs is involved in various diseases, such as cancers, cardiac diseases and immunolgically mediated diseases [11–13]. MicroRNAs (miRNAs), a class of endogenous short noncoding RNAs, has been well studied and served as tumor promoter or oncogenes in cancer progression [14, 15]. Interestingly, lncRNAs is reported to interact with miRNAs as it competes with endogenous RNA [16]. Although at least 3000 lncRNAs have been identified, less than 1% of them have been characterized.

Long intergenic non-protein coding RNA 520 (LINC00520) was a newly discovered diseases-related lncRNA whose abnormal expression had been demonstrated in several cancers [17, 18]. However, its biological function and molecular mechanism in progression tumors remains

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unknown. In this paper, for the first time, we analyzed the expression pattern, clinical significance and biological function of LINC00520 in NSCLC. Our study provided important evidence that LINC00520/miR-577/CCNE2 axis regulatory network might provide a potential novel therapeutic strategy for NSCLC treatment.

Materials and methods

Patients and tissue samples

NSCLC specimens and noncancerous adjacent normal samples were collected from 150 NSCLC patients who had not received any radio- or chemotherapy. The NSCLC tissue samples were obtained from the Henan Provincial Chest Hospital from February 2013 to September 2015. Tissue samples were frozen using liquid nitrogen, followed by being preserved at − 80 °C. The patients signed the written informed consents and the Ethics Committee of the Henan Provincial Chest Hospital granted approval for the present study (CXJH-2018-167).

Cell transfection

Human NSCLC cell types (H1975, H1299, PC 9, HCC827, A549) and A human immortalized normal epithelial cells (BEAS-2B cells) were purchased from BeNa corporation (Suzhou, Jiangsu, China). The cells were maintained at 37 °C in RPMI-1640 medium (EallBio, Daxing, Beijing, China) with 10% FBS.

A riboFect transfection reagent (Ruibo, Nantong, Jiangsu, China) was utilized to conduct the cell transfection. SiRNAs were designed by Applygen Technologies Inc. (Changping, Beijing, China) to target human LINC00520 (siRNA#1 and siRNA#2). The NC or miR-577 mimics, NC or miR-577 inhibitors were all obtained from HanBio Co. Ltd. (Pudong, Shanghai, China). The LINC00520 or CCNE2 sequence was subcloned into pcDNA3.1 empty vector to overexpress LINC00520 (pcDNA3.1-LINC00520) or CCNE2 (pcDNA3.1-CCNE2) by Beijing Syngentech Co., Ltd. (Changping, Beijing, China).

qPCR analyses

Total RNAs of the NSCLC specimens or treated cells were isolated using TRIzol Reagent (JiningShiye Co., Ltd., Jinshan, Shanghai, China). The RNA was firstly reverse transcribed to complementary DNA (cDNA) which was followed by qRT-PCR analyses using SuperScript III Platinum One-Step qRT-PCR kit (Unique Biotech, Chaoyang, Beijing, China). The YRBIO miRNA qPCR Detection kits (Jinan, Shandong, China) were applied to examine miR-577 levels. The primers for LINC00520, CCNE2 and miR-577 were presented in Table 1. GAPDH or U6 was employed as endogenous control. The $2^{-\Delta\Delta Ct}$ methods were utilized for calculating the relative expression.

Cell proliferation detetction and clonogenic formation assays

Briefly, after LINC00520 siRNAs or pcDNA3.1-CCNE2 plasmids were transfected into cells, 2000 treated cells were placed into each well of the 96-well plates. Thereafter, we placed 10 µl CCK-8 reagents (Vazyme Biotech, Nanjing, Jiangsu, China) into each well and sequentially used a LumiStation 1800Plus microreader (Flash Spectrum, Minhang, Shanghai, China) to examine the absorbance (450 nm).

For cell colony formation assays, appropriate 500 LINC00520 siRNAs or pcDNA3.1-CCNE2 plasmids transfected A549 or H1299 cells were then put into six-well plates. Two weeks later, 0.1% crystal violet reagents (JissKang Biotech, Qingdao, Shandong, China) were utilized to treat the colonies. After washing for three times, a Caikon XSP-11CC microscope (Jiading, Shanghai, China) was sued for the observation of the images.

In vivo tumorigenesis assays

Four-week-old nude mice were purchased from the Pulate Technology (Changsha, Hunan, China). 3×10^6 A549 cells after transfection of sh-LINC00520 or sh-control were subcutaneously injected into the flanks of 4-week-old nude mice. Subcutaneous tumor volumes were detected every 7 days with caliper for 28 days and calculated as length × width^2)/2.

Western blotting

We used protein isolation kits which were bought from BestBio company (Pudong, Shanghai, China) to extract the

Table 1 The sequences of primers for RT-PCR in this study

Names	Sequences (5′–3′)
LINC00520: forward	AACAAATGAGGGAATGAATGAG
LINC00520: reverse	TAGAAGCCAAAACAGAAGGAAC
miR-577: forward	TGCGGTAGATAAAATATTGG
miR-577: reverse	GTGCAGGGTCCGAGGT
CCNE2: forward	TCAAGACGAAGTAGCCGTTTAC
CCNE2: reverse	TGACATCCTGGGTAGTTTTCCTC
GAPDH: forward	GGAGCGAGATCCCTCCAAAAT
GAPDH: reverse	GGCTGTTGTCATACTTCTCATGG
U6: forward	CTCGCTTCGGCAGCACA
U6: reverse	AACGCTTCACGAATTTGCGT

total proteins from the treated NSCLC cells. Subsequently, the protein lysates from corresponding treated groups were firstly separated using 7–12% SDS-PAGE, followed by being transferred to PVDF membranes. After 1 h blockage with 5% bovine serum albumin (BSA), the membranes from different treatment groups were immunoblotted with corresponding primary antibodies against anti-Caspase-3 (ab32351, Abcam, Rabbit), anti-Caspase-9 (ab185719, Abcam, rabbit), vimentin (ab92547, Abcam, Rabbit), *N*-cadherin (ab18203, Abcam, Rabbit), GAPDH (ab9485, Abcam, Rabbit) and CCNE2 (ab32103, Abcam, Rabbit) overnight at 4 °C. After being probed with corresponding secondary antibodies, the membranes were utilized to show the protein bands using ECL kits (Haotian, Qingdao, Shandong, China).

Cell apoptosis analyses

In brief, the A549 or H1299 cells treated with LINC00520 siRNAs were digested by trypsinization and sequentially resuspended in corresponding binding buffer in apoptosis detection kits which were bought from Meilun Biological corporation (Dalian, Liaoning, China). Following staining with PI as well as FITC-annexin V for 25 min, the apoptotic H1299 or A549 cells were detected using a FACScan flow cytometer (BD Biosciences, Jingan, Shanghai, China).

Wound-healing assays

To determine the influence of LINC00520 on the mobility of H1299 or A549 cells, we seeded 2×10^4 treated A549 or H1299cells (70 µl) into each well of an ibidi culture-insert (Thundersci, Minhang, Shanghai, China) and cultured for 24 h. Afterwards, the culture medium in each well was discarded before the insert was removed. After washing with PBS for three times, 500 µl medium was added into the dish. Finally, a Caikon XSP-11CC microscope (Jiading, Shanghai, China) was used for the observation of the images at different time intervals (0 h, 24 h).

Transwell invasion assays

Briefly, after various treatments, the A549 or H1299 cells (2×10^4 cells) were suspended in 100 µl culture media without FBS. Subsequently, the cells were placed into the up sides (pre-coated with Matrigel) of the transwell inserts (BD, Xuhui, Shanghai, China), while 450 µl of the RPMI-1640 complete medium (15% FBS) was placed into the lower sides of the chambers. After 24 h, cells that invaded the membranes were treated using 0.1% crystal violet (JissKang Biotech, Qingdao, Shandong, China) and observed by a Caikon XSP-11CC microscope (Jiading, Shanghai, China).

RNA immunoprecipitation (RIP) assays

In short, A549 cells were treated using LINC00520 overexpressing vectors, pcDNA3.1-LINC00520, or miR-577 mimics. Then, we use EZ-Magna RIP kits (Haoran, Nantong, Jiangsu, China) to performed the RIP assays in accordance with the manufacture's protocols. The anti-Argonaute-2 (Ago2) antibody was bought from CST corporation (Trask Lane, Danvers, MA, USA).

Luciferase activity determination

For the clarifying that miR-577 was interacted with LINC00520, the wild-type LINC00520 or mutant LINC00520 sequence that incorporated miR-577 binding site were constructed into pGL3 vector to form a reporter vector named LINC00520 wt or LINC00520 mut, respectively. Then, the A549 or H1299 cells were co-treated with the LINC00520 wt or LINC00520 mut luciferase reporter vector with or without miR-577 mimics. After 48 h, Bosunlife Dual luciferase kits (Jinan, Shandong, China) were utilized to examine the luciferase activities. Similarly, the wild-type CCNE2 or mutant CCNE2 sequence that incorporated miR-577 binding site were also constructed into pGL3 vector to form a reporter vector named CCNE2 WT or CCNE2 MUT. The demonstration that CCNE2 was a target of miR-577 was also conducted with dual-luciferase reporter assays in agreement with the above methods. All these plasmids vectors were constructed by Shandong Vigene Biotechnology Co., Ltd. (Jinan, Shandong, China).

ChIP assays

ChIP analyses were carried out using EZ ChIP assay kits (Millipore, Bedford, MA, USA). Briefly, A549 cells were collected and fixed using formaldehyde reagents (1%) at 27 °C. for 15 min. After quenching the reactions by adding glycine buffer (10×), DNA fragments (200-500 bp) were obtained by sonication. Then, anti-SP1 antibodies (Abcam, Cambridge, MA, USA) were applied to precipitate the chromatin, and anti-IgG (Sangong, Songjiang, Shanghai, China) was used as negative control. Lastly, the precipitated DNAs were determined by qPCR assays.

Statistical analyses

SPSS (version 19.0, Chicago, IL, USA) was applied for statistical analyses. Differences (two groups) were evaluated for significance using Student's *t* test or differences (more than two groups) were determined using one-way ANOVA methods. The correlation between clinicopathological parameters and LINC00520 expression level was evaluated using χ^2 tests. Survival curve was generated using the Kaplan–Meier

methods, and evaluated by log-rank tests. Univariate and multivariate analysis was conducted to explore the independent risk factors for NSCLC. $p < 0.05$ was considered to be significant.

Results

LINC00520 was highly expressed and correlated with poor prognosis of NSCLC patients

Firstly, we analyzed TCGA dataset from NSCLC tissues ($n = 502$) and normal lung tissues ($n = 49$). Hierarchical clustering and volcano plots showed the dysregulated lncRNAs in NSCLC tissues (Fig. 1a, b). Besides, we also discovered that LINC00520 was markedly overexpressed in NSCLC specimens compared with lung tissues according to TCGA datasets (Fig. 1c). Then, we further carried out RT-PCR to assess the levels of LINC00520 in NSCLC samples and matched normal lung specimens form our hospital and the data presented that LINC00520 expression levels were increased in NSCLC specimens compared with matched normal tissues ($p < 0.01$, Fig. 1d). Similarly, up regulation of LINC00520 was also observed in NSCLC cells compared to BEAS-2B cells (Fig. 1e). Our results were consistent with the results of online microarray data.

Then, we further explored the clinical significance of LINC00520 in NSCLC patient by dividing all patients into two groups (high group; Low group) according to the median value of LINC00520. As shown in Table 2, the data of chi-square test demonstrated that high LINC00520 levels were distinctly correlated with advanced tumor stage ($p = 0.016$) and positively lymph nodes metastasis ($p = 0.011$). However, no obvious correlations of LINC00520 expression with other clinical features were observed. In addition, we next performed survival analysis to evaluate whether LINC00520 could influence the prognosis of NSCLC patients: the data showed that NSCLC patients with high LINC00520 levels showed shorter survivals compared with that had low LINC00520 expression ($p < 0.0001$). More importantly, univariate and multivariate analyses revealed that high LINC00520 expression was an independent predictor of

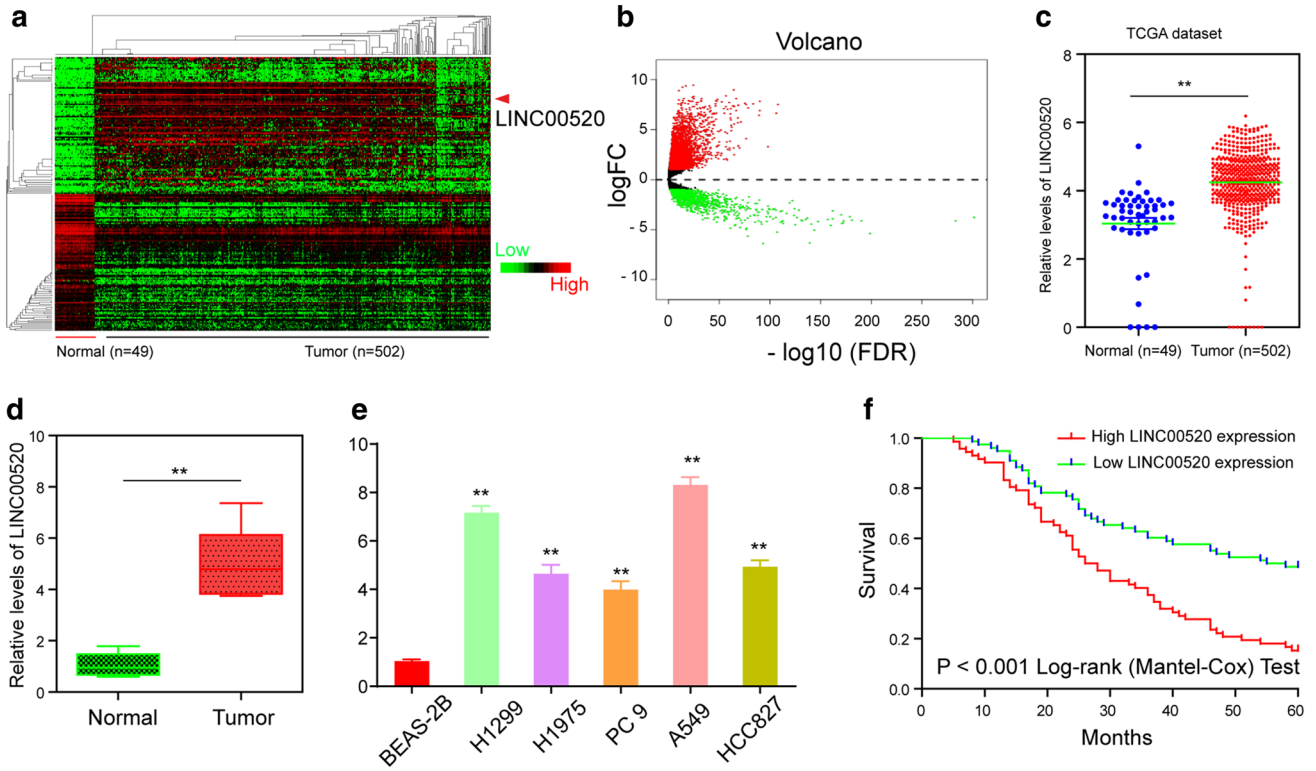

Fig. 1 LINC00520 was highly expressed in lung cancer and associated with poor prognosis. **a** Heatmap of dysregulated lncRNAs in NSCLC tissues compared to normal tissues according to the TGCA datasets. **b** Volcano plot of the aberrantly expressed lncRNAs between NSCLC and normal lung tissues according to the TGCA datasets. **c** The expression of LINC00520 in NSCLC tissues and normal brain tissues using the TGCA datasets. **d** The expression of LINC00520 in NSCLC tissues and normal brain tissues was determined by qRT-PCR. **e** qRT-PCR analysis indicated LINC00520 was highly expressed in NSCLC cell lines compared to BEAS-2B cells. **f** Kaplan–Meier overall survival curves for 150 patients with NSCLC classified according to relative LINC00520 expression level. $^{*}p < 0.05$, $^{**}p < 0.01$. The experiment was repeated three times

Table 2 LINC00520 expression and clinicopathologic features in NSCLC patients

Variable	Number	LINC00520 expression		*p* value
		Low	High	
Age (years)				0.325
< 60	74	33	41	
≥ 60	76	40	36	
Gender				0.151
Male	94	50	44	
Female	56	23	33	
Tumor size (cm)				0.241
< 3	81	43	38	
≥ 3	69	30	39	
Histologic type				0.947
SqCC	90	44	46	
Ad	60	29	31	
Tumor stage				0.016
I–II	90	51	39	
III	60	22	38	
Lymph nodes metastasis				0.011
No	100	56	44	
Yes	50	17	33	

We defined LINC00520 expression > 4.53 as high expression and LINC00520 expression ≤ 4.53 as low expression

SqCC squamous cell carcinoma, *Ad* adenocarcinoma

poor survival for NSCLC patients (RR = 3.138, 95% CI 1.211–4.895, $p = 0.006$, Table 3). Token together, our results firstly reported that LINC00520 was frequently up-regulated in NSCLC patients and may serve as a new indicator for NSCLC.

SP1 accelerated LINC00520 expression via binding with its promoter

Mounting evidences had demonstrated that transcription factors (TFs) might contribute to lncRNAs aberrant expression [19]. Therefore, in the subsequent experiments, we sought to find which TF might induce LINC00520 aberrantly high expression. To achieve that, we employed Jaspar program to predict the possible TFs which could bind with the promoter of LINC00520. The results revealed that SP1 might be a potential TF, and three possible binding sites with high scores were exhibited in Fig. 2a. Additionally, qPCR analyses revealed that SP1 was up-regulated in NSCLC specimens from 150 NSCLC patients (Fig. 2b). We thereby synthesized siRNAs targeting SP1 (si-SP1) and cloned overexpressing vectors of SP1 (ov-SP1). Real-time PCR analyses confirmed that si-SP1 was able to repress SP1 expression and ov-SP1 could remarkably elevate SP1 levels in A549 cells (Fig. 2c). Hence, si-SP1 or ov-SP1 was separately transfected into A549 cells and then LINC00520 levels were determined by qPCR. The data validated that repressing SP1 levels could inhibit LINC00520 expression, while elevating SP1 expression was capable to promote LINC00520 levels (Fig. 2d). Next, ChIP assays were conducted and the results indicated that SP1 might bind with the BS2 site of LINC00520 promoter (Fig. 2e). Subsequently, luciferase activity detection assays were performed. The results suggested that increasing SP1 expression by transfecting ov-SP1 vectors could markedly accelerate luciferase activity in A549 cells transfected with BS2 wild-type (BS2 wt) reporters, while there were no influences on luciferase activities in cells when they were co-treated with ov-SP1 vectors and BS2 mutated-type (BS2 mut) reporters (Fig. 2f). Collectively, these data validated that SP1 stimulated LINC00520 expression via binding with its promoter.

Silencing of LINC00520 impeded NSCLC cell proliferation and promoted apoptosis

In order to explore the effects of LINC00520 on NSCLC cell proliferation and apoptosis, we carried out loss-of-function studies through transfecting LINC00520 siRNAs into NSCLC cells. The silencing efficiencies of LINC00520 siRNAs were evaluated by qRT-PCR and the results suggested that LINC00520 siRNAs transfection remarkably reduced the expression of LINC00520 (Fig. 3a). According to CCK-8 assays, knockdown of LINC00520 markedly

Table 3 Univariate and multivariate analysis of overall survival in NSCLC patients

Variable	Univariate analysis			Multivariate analysis		
	RR	95% CI	*p*	RR	95% CI	*p*
Age	1.856	0.742–2.312	0.213	–	–	–
Gender	1.643	0.538–2.446	0.148	–	–	–
Tumor size	1.775	0.832–2.541	0.239	–	–	–
Histologic type	2.125	0.978–2.554	0.175	–	–	–
Tumor stage	3.665	1.438–5.027	0.003	3.017	1.233–4.478	0.009
Lymph nodes metastasis	3.863	1.564–5.667	0.001	3.231	1.277–4.432	0.008
LINC00520 expression	3.547	1.327–5.133	0.004	3.138	1.211–4.895	0.006

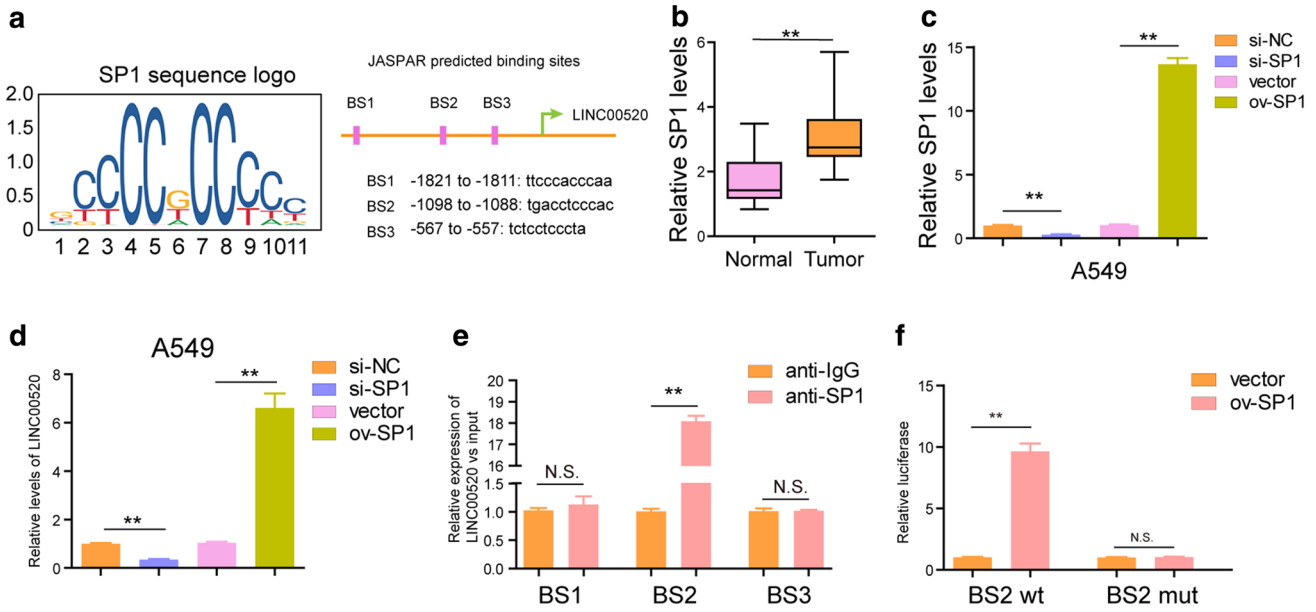

Fig. 2 SP1 induced overexpression of LINC00520 via acting as a transcription activator. **a** DNA motif of SP1 and three binding sites of SP1 in LINC00520 promoter. **b** The SP1 levels in NSCLC tissues and matched normal lung tissues were determined using RT-PCR. **c**, **d** qRT-PCR analysis of SP1 and LINC00520 expression in A549 cells after transfection with si-SP1, ov-SP1 or their controls or their controls. **e** ChIP-qPCR assays of SP1 occupancy in LINC00520 promoter regions in A549 cells. **f** Dual luciferase reporter assays for the examination of the SP1 targeting LINC00520. $*p<0.05$, $**p<0.01$. The experiment was repeated three times

depressed the viability of NSCLC cells compared with the controls (Fig. 3b, c). Similarly, colony formation assays also revealed that suppression of LINC00520 using siRNAs significantly repressed the clonogenic capacity of NSCLC cells (Fig. 3d). Moreover, the cell apoptosis was further determined using flow cytometry and we observed that silence of LINC00520 in NSCLC cells markedly accelerated the apoptotic rates (Fig. 3e). Besides, western blot confirmed that the expressing levels of cleaved-caspase-3 and cleaved-caspase-9 were notably elevated in above two tumor cells after the transfection of si-LINC00520, while the expressions of caspase-3 and caspase-9 were decreased (Fig. 3f). Overall, our data suggested that LINC00520 affected the tumor behaviors of NSCLC cells. Then, we further performed the nude mouse tumorigenicity assays to examine the potential effects of LINC00520 on tumor growth. As shown in Fig. 4a, LINC00520 expression was distinctly suppressed in A549 cells after the transfection of sh-LINC00520. Importantly, we observed that the tumor volume and weight in LINC00520-knockdown mice were distinctly smaller and lighter than those in the control mice (Fig. 4b–d). Taken together, our data suggested that LINC00520 knockdown suppressed the NSCLC cell growth.

LINC00520 modulated the mobility of NSCLC cells

To investigate the functions of LINC00520 on mobility in NSCLC cells, we conducted the wound-healing and transwell assays. As indicated in the results of wound-healing assays, depression of LINC00520 could remarkably reduce the migratory ability of NSCLC cells (Fig. 5a). Similar results could also be observed in the transwell invasion assays. The data revealed that knocking down LINC00520 in NSCLC cells resulted in a distinct decline of the invaded cell number (Fig. 5b). To further unravel the molecular mechanism that LINC00520 regulated the migration and invasive capacity of NSCLC cells, we carried out western blot to detect the protein expression of two cell metastasis relevant proteins: *N*-cadherin, vimentin. The results validated that transfection of LINC00520 siRNAs markedly impaired the expression of these two molecules in NSCLC cells (Fig. 5c, d). Therefore, our data shed light on that LINC00520 could modulate the progression of NSCLC and the underling mechanism might be that silence of LINC00520 activated epithelial mesenchymal transition signaling.

MiR-577 is a target of LINC00520

To study the molecular mechanism underlying the roles of LINC00520 on NSCLC development and progression, we firstly searched for LINC00520 associated miRNAs using "starbase", and we discovered that miR-577 might be a potential target of LINC00520 (Fig. 6a). To figure out whether LINC00520 could exactly bind with miR-577, we cloned luciferase reporters with wild-type (wt) or mutant-type (mut) miR-577 binding sequence of LINC00520.

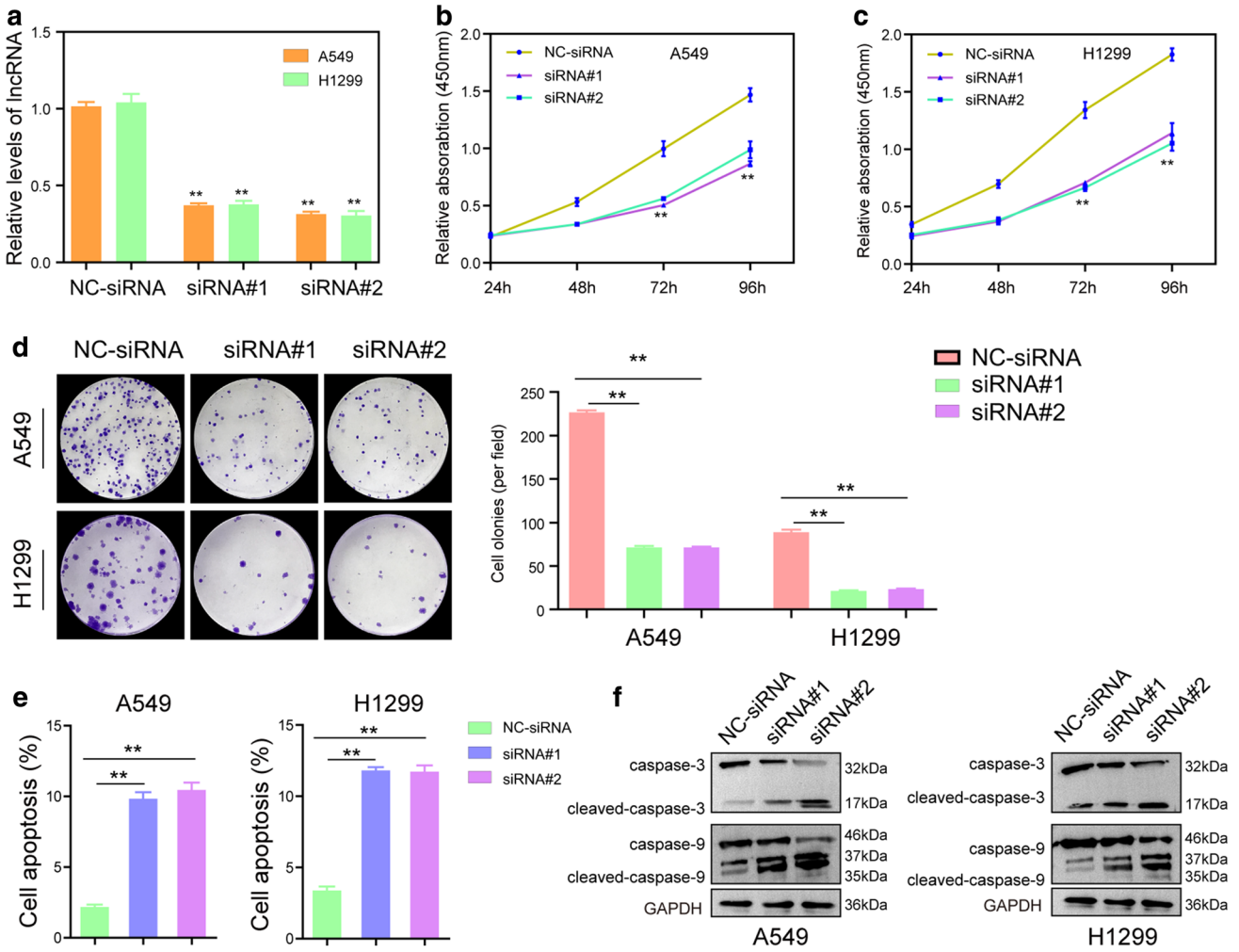

Fig. 3 LINC00520 knockdown suppresses NSCLC cell proliferation. **a** H1299 and A549 cells were transfected with LINC00520 siRNAs or negative control siRNAs (NC-siRNAs), and the relative expression of LINC00520 was detected by RT-PCR. **b, c** The cell viability of NSCLC cells was measured by CCK-8 assays. **d** In vitro proliferative ability of NSCLC cells was significantly decreased in LINC00520-suppressed cells compared to sh-control cells using colony formation assays. **e** The cell apoptosis was detected by flow cytometry. **f** Western blot examined the expressing levels of cleaved-caspase-3 and cleaved-caspase-9. $*p<0.05$, $**p<0.01$. The experiment was repeated three times

Subsequently, luciferase activity detection assays were carried out to determine the changes of luciferase activities. The data revealed that, when co-transfection with miR-577 mimics and wild-type LINC00520 reporters, the relative luciferase activities in NSCLC cells were significantly decreased (Fig. 6b). Moreover, RIP assays validated that there was notable enrichment of LINC00520 or miR-577 in Ago2-containing beads compared with the input groups and the IgG groups (Fig. 6c). Next, miR-577 levels were measured by the use of qRT-PCR assays. The results demonstrated that LINC00520 ectopic expression caused a remarkable decrease in miR-577 expression in H1299and A549 cells, while silence of LINC00520 dramatically promoted the miR-577 expressing levels (Fig. 6d). In summary, these data certified that miR-577 was a downstream target of LINC00520.

CCNE2 was a target of miR-577 and LINC00520 modulated CCNE2 expression via miR-577

MiRNA exerted its regulatory function through targeting the 3′UTR of corresponding genes. Therefore, to further ascertain the downstream target gene of miR-577, we performed bioinformatics prediction using “miRDB” and CCNE2 was found as a possiable target gene of miR-577 (Fig. 7a). To verify that, wild-type CCNE2 3′UTR (CCNE2 WT) and mutant-type CCNE2 3′UTR (CCNE2 MUT) luciferase reporter plasmids were firstly cloned, and respectively co-transfected

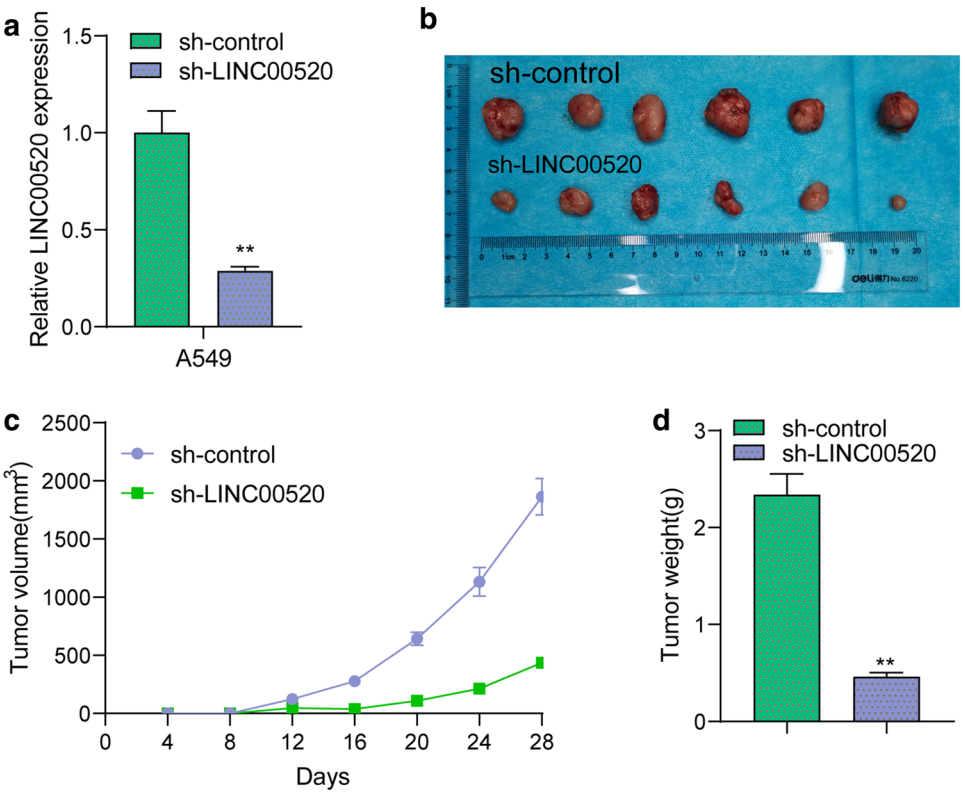

Fig. 4 LINC00520 accelerates in vivo tumor growth. **a** RT-PCR confirmed the expressing suppression of LINC00520 in A549 cells. **b** Tumors derived from mice in two different groups were presented. **c**, **d** Volume and weight of tumors obtained from two groups were measured and shown. $*p < 0.05$, $**p < 0.01$. The experiment was repeated three times

with miR-577 mimics into A549 or H1299 cells. Thereafter, luciferase activity detection assays were performed, and the data revealed that the luciferase activity in the CCNE2 WT plasmids and miR-577 mimics co-transfecting cells was remarkably decreased, whereas the luciferase activities were not affected in cells co-transfected with CCNE2 MUT plasmids and miR-577 mimics (Fig. 7b). Therefore, these data suggested that CCNE2 was a direct target of miR-577. For further confirming the regulatory relationship of LINC00520, CCNE2 and miR-577, we conducted qRT-PCR assays to evaluate the expression levels LINC00520 and CCNE2 in A549 cells afer the transfection of miR-577 mimics or miR-577 inhibitors. Our data confirmed that forced expression of miR-577 notably inhibited the levels of LINC00520 and CCNE2, while silencing miR-577 expression significantly enhanced the expression of LINC00520 and CCNE2 (Fig. 7c). Besides, western blot analysis demonstrated that forcing expression of LINC00520 remarkably abrogated the negative roles of miR-577 on CCNE2 expression (Fig. 7d). Overall, these data indicated that LINC00520 modulated the expression of CCNE2 via miR-577.

Knockdown of LINC00520 impaired NSCLC cell proliferation and metastasis through inhibiting CCNE2 expression

In the subsequent experiments, we attempted to explore whether CCNE2 was critical for the functional regulation of NSCLC cells upon LINC00520. We first cloned the CCNE2 overexpressing plasmid, pcDNA3.1-CCNE2, and the plasmid was then transfected into A549 and H1299 cells. The qPCR analyses demonstrated that treatment of pcDNA3.1-CCNE2 dramatically increased the levels of CCNE2 (Fig. 8a). Afterwards, we utilized CCK-8 assays to examine the cell proliferation. The data suggested that forced expression of CCNE2 in NSCLC cells remarkably abrogated the decrease of cellular viability caused by LINC00520 silencing (Fig. 8b). In addition, treatment of pcDNA3.1-CCNE2 dramatically restored the cell colony number of H1299 and A549 cells transfected with si-LINC00520 (Fig. 8c). Furthermore, we also investigated whether depression of LINC00520 impeded the metastatic potentials by inhibiting CCNE2. According to the results of transwell invasion assays, enhancing expression of

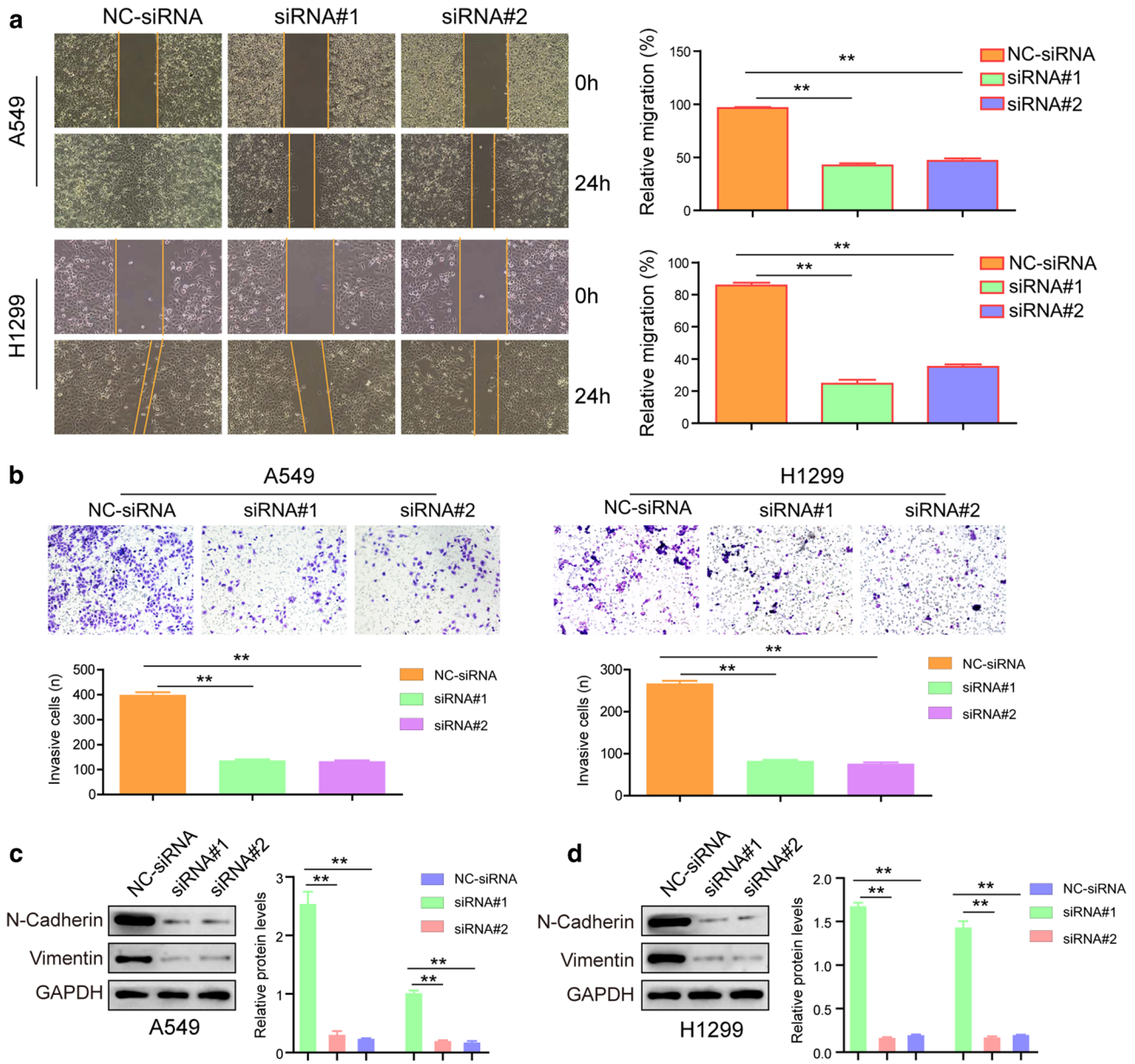

Fig. 5 LINC00520 promoted NSCLC cell metastasis in vitro. **a** Knockdown of LINC00520 reduced the migration of NSCLC cells. **b** Silence of LINC00520 impaired the invasive abilities of NSCLC cells. **c** The protein levels of *N*-cadherin and vimentin were detected by western blot. $*p<0.05$, $**p<0.01$. The experiment was repeated three times

CCNE2 could effectively reverse the suppressive function of LINC00520 silencing on the invasive abilities of NSCLC cells (Fig. 8d). Overall, our observation provided strong evidences that LINC00520 affecting the development and progression was mainly mediated via modulating miR-577/CCNE2 axis.

Discussion

NSCLC remains a serious healthy problem in China [20]. Up to date, limited efficiency of present therapeutic

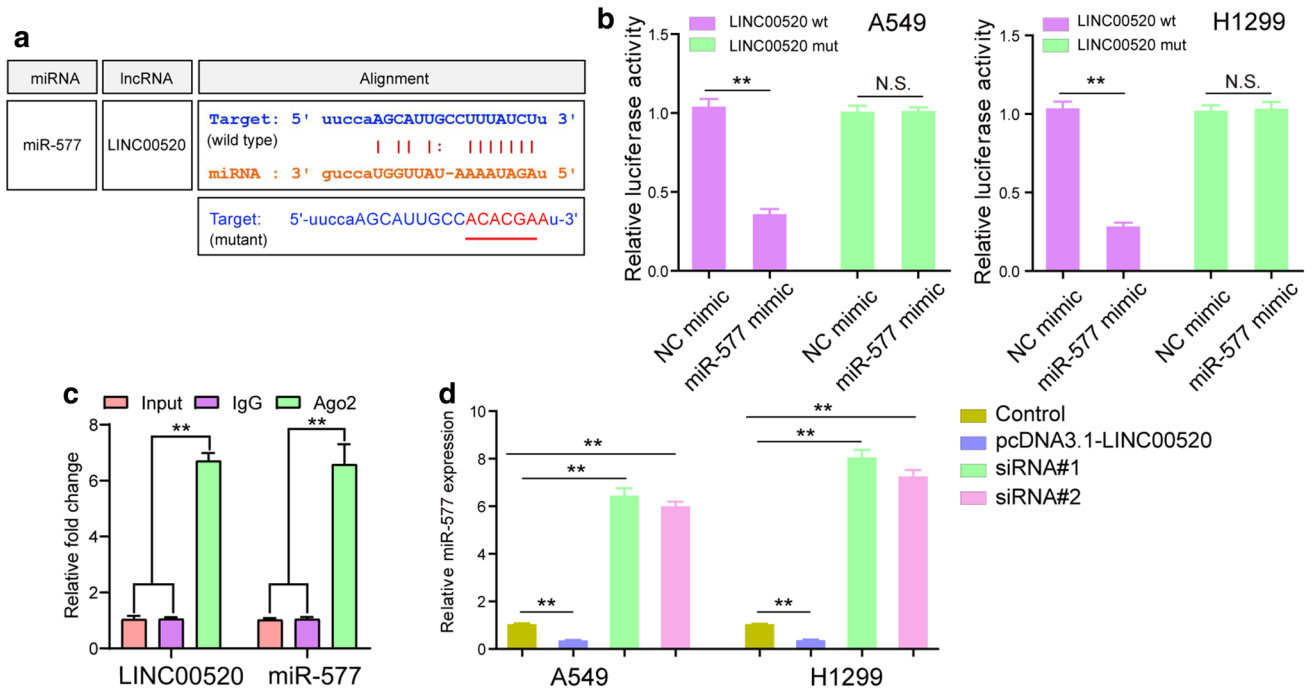

Fig. 6 miR-577 was directly targeted by LINC00520. **a** Binding region between miR-577 and LINC00520. **b** Luciferase reporter assay confirmed the molecular binding. **c** RIP assays determined the enrichments of LINC00520 and miR-577. **d** The relative expression of miR-577 in NSCLC cells after the transfection with pcDNA3.1-LINC00520 plasmids or si-LINC00520. $*p<0.05$, $**p<0.01$. The experiment was repeated three times

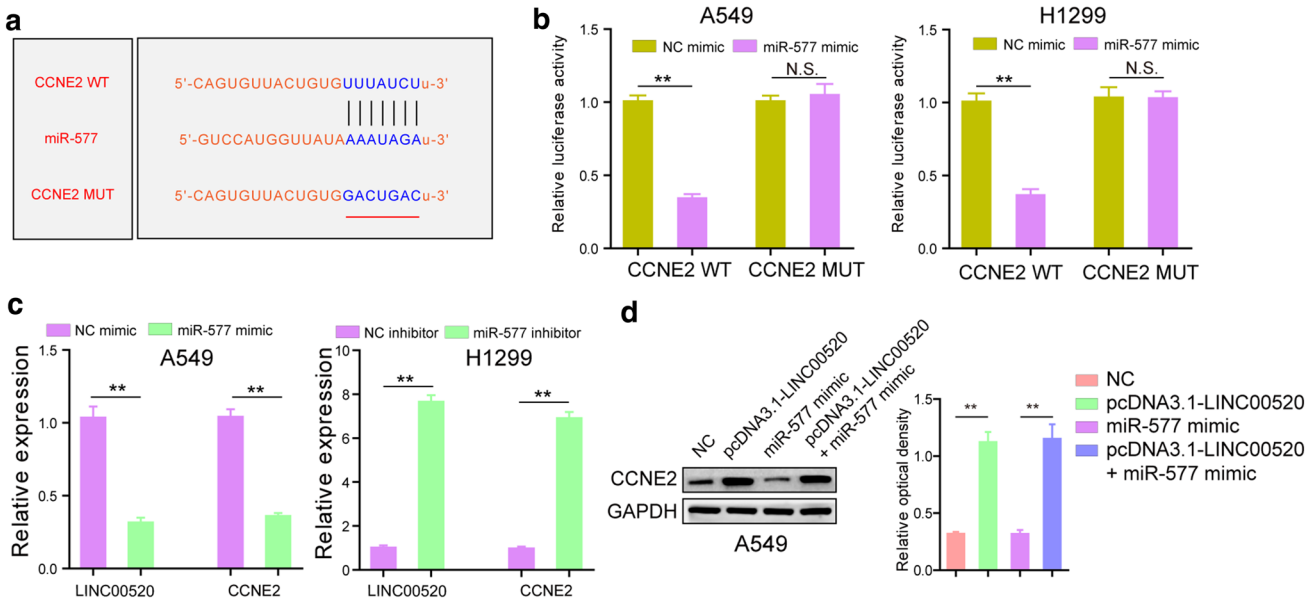

Fig. 7 Bioinformatical predication showed that CCNE2 was a putative target gene of miR-577. **a** The binding site of miR-577 was predicted by "starbase". **b** Luciferase activity assaysin A549 cells showed that miR-577 inhibited the expression of CCNE2. **c** The expression levels of LINC00520 and CCNE2 in A549 cells after transfecting with miR-577 mimics or inhibitors. **d** The relative protein levels of CCNE2 in A549 cells after various treatments. $*p<0.05$, $**p<0.01$. The experiment was repeated three times

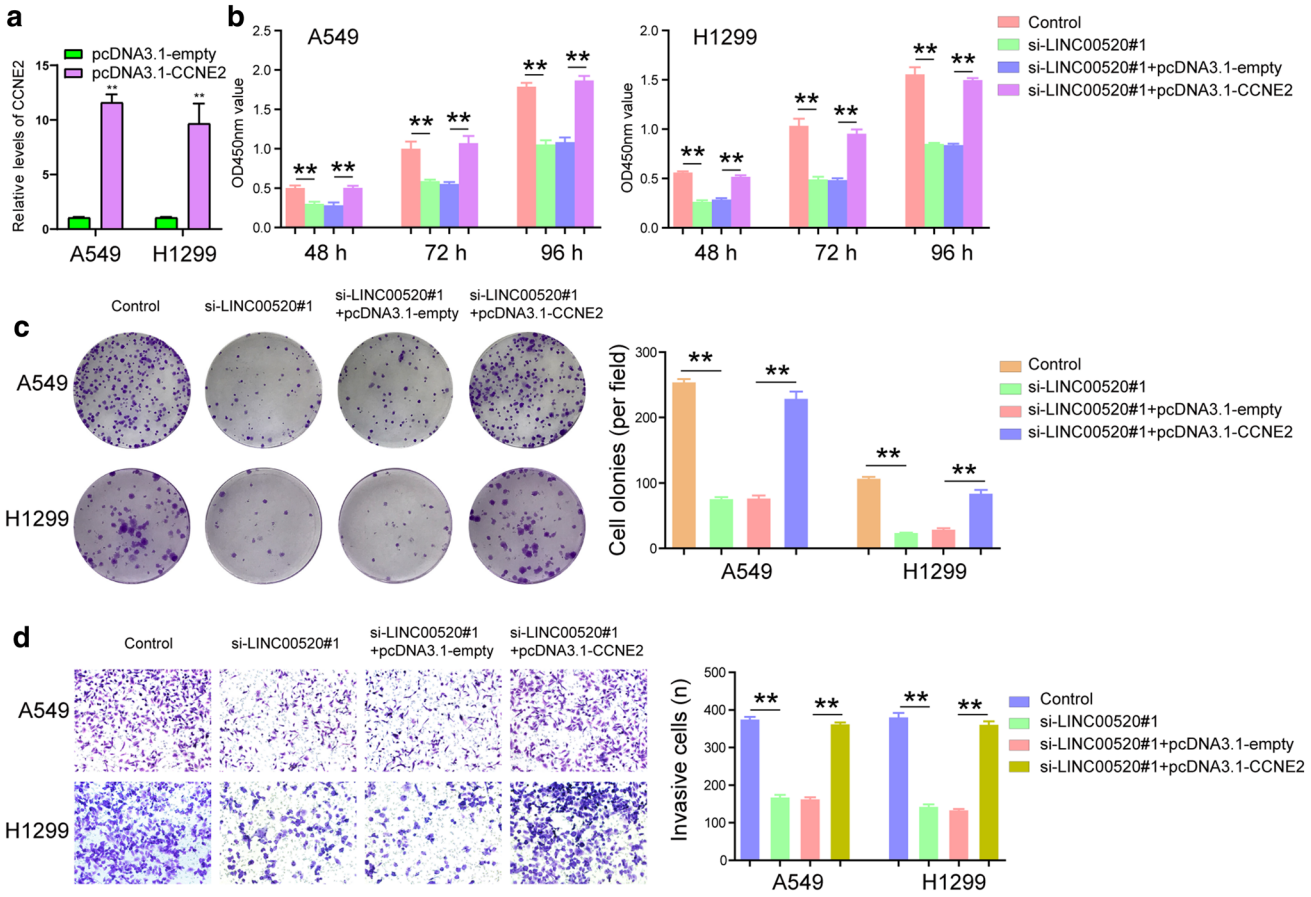

Fig. 8 CCNE2 reversed the inhibitory effects of LINC00520 knockdown on lung cancer cell proliferation and invasion. **a** The relative expression of CCNE2 in A549 and H1299 cells after transfecting with CCNE2 overexpressing plasmid, pcDNA3.1-CCNE2. **b** CCK-8 assays assessed the growth of NSCLC cells transfected with siRNAs or plasmids. **c** The cell colonies of NSCLC cells treated with various experimental conditions were detected by cell colony formation assays. **d** Tranwell invasion assays examined the invasive capacities of NSCLC cells after various treatments. $*p<0.05$, $**p<0.01$. The experiment was repeated three times

measure for advanced NSCLC and lack of sensitive markers utilized for targeted therapy [21, 22]. Exploration of novel sensitive markers for early diagnosis and outcome predication is very imperative for clinical management of NSCLC patients [23]. In this study, we firstly analyzed the TCGA datasets and found many dysregulated lncRNAs. LINC00520 was one of most up-regulated lncRNAs in NSCLC. Then, using RT-PCR, we demonstrated that LINC00520 was distinctly highly expressed in NSCLC. Subsequent clinical investigations showed that high LINC00520 expression in NSCLC patients was strongly correlated with advanced tumor stage, lymph nodes metastasis and shorter five years survival, suggesting that LINC00520 may play a positive regulator in clinical prognosis of NSCLC patients. Moreover, multivariate study demonstrated that increasing LINC00520 levels was an independent prognostic indicator for NSCLC. Taken together, these findings firstly demonstrated that LINC00520 was overexpressed in NSCLC and had potential to be a novel clinical marker for NSCLC patients. However, because of the small number of NSCLC patients evaluated, further studies on more NSCLC patients are necessary to demonstrate our conclusion.

Recently, several studies have suggested that the dysregulation of lncRNAs are involved in the modulation of transcription factors just like many protein coding genes [19, 24]. For instance, upregulation of lncRNA MIR100HG was induced by ELK1 and predicted a poor prognosis of osteosarcoma patients [25]. SP1-induced lncRNA ZFAS1 resulted in accelerated metastasis of colorectal cancer cells by modulating miR-150-5p/VEGFA [26]. In this paper, we used JASPAR to predict a possible transcription factor targeting LINC00520, SP1 whose levels was found to be up-regulated in NSCLC tissues. Previous studies have confirmed the activation of SP1 as an important modulator in tumor progression, including NSCLC. Hence, using the ChIP assays and luciferase assays, we proved that SP1 directly bound with the promoter region of LINC00520 to further promote the levels

of LINC00520. Our findings revealed that the upregulation of LINC00520 was mediated by SP1 in NSCLC.

As a newly identified lncRNA, the expression and function of LINC00520 were just reported in a few studies. Recently, LINC00520 was reported to be highly expressed in breast cancer and acted as a tumor promoter according to the cells experiments [18]. In addition, up regulation of LINC00520 was shown in laryngeal squamous cell carcinoma [27], renal cell carcinoma [17] and tongue squamous cell carcinoma [28]. However, the function of LINC00520 in NSCLC had not been investigated. In this study, to study the function of LINC00520 in NSCLC progression, we down-regulated the levels of LINC00520 in NSCLC cell lines, and performed a series of cell experiments. Our results indicated that down regulation of LINC00520 significantly suppressed NSCLC cells proliferation in vitro and in vivo, and promoted apoptosis by promoting the expression of cleaved-caspase-3 and cleaved-caspase-9. On the other hand, we also observed that suppression of LINC00520 inhibited the ability of migration and invasion of NSCLC cells by modulating EMT pathway. Thus, our findings revealed that LINC00520 may functioned as a positive regulator in progression of NSCLC.

Inspired by the 'competitive endogenous RNAs' regulatory network and growing studies that revealed that lncRNAs may be involved in this novel molecular mechanism, we focused on the effects of LINC00520 acting as a ceRNA [29, 30]. In this study, we showed that LINC00520 is a target of miRNA-577 by bioinformatics analysis and luciferase reporter assays. Previously, miR-577 was reported to be lowly expressed in various tumors, including NSCLC [31–33]. In addition, its tumor-suppressive roles were also confirmed by in vitro and in vivo. Interestingly, we found that overexpressing LINC00520 reduced miR-577 expression in both A1299 and A549 cells. However, knockdown of LINC00520 displayed opposite effects. Then, using bioinformatics analysis, we selected CCNE2 as a target gene from several putative miR-577 target genes, since CCNE2 had been confirmed to play an important role in development and progression of NSCLC [34]. In addition, using Western blot assay and luciferase reporter assay, we confirmed that CCNE2 was the target gene of miR-577. Finally, we performed rescue experiments to explore the association between LINC00520 and CCNE2; our results showed that overexpression of CCNE2 could effectively reverse the suppressive effects of LINC00520 silencing on the proliferation and invasion in NSCLC cells, suggesting that knockdown of LINC00520 exhibited a tumor-suppressive role in NSCLC by inhibiting CCNE2 expression. Taken together, our findings firstly suggested that LINC00520/miR-577/ CCNE2 will provide a novel insight into the potential mechanism of NSCLC proliferation, migration and invasion.

In conclusion, our results confirmed that LINC00520 may be involved in NSCLC with tumorigenic features and contributes the progression of NSCLC through the miR-577/CCNE2 axis. Our findings suggested a clinical strategy for targeting LINC00520 as a potential marker and a therapeutic target in NSCLC patients.

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Author contributions J-fW and Y-hQ conceived and designed the experiments, J-fW, Z-nX and ZB analyzed, and interpreted the results of the experiments, J-fW, Z-nX and H-jS performed the experiments.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest No potential conflict of interest was reported by the authors. The authors declare that they have no competing interests, and all authors should confirm its accuracy.

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