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Original article

Effects of long non-coding RNA (lncRNA) cancer susceptibility candidate 2c (CASC2c) on proliferation, metastasis and drug resistance of non-small cell lung cancer (NSCLC) cells through ERK1/2 and β-catenin signaling pathways

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ABSTRACT

Objectives: This study was aimed to investigate the effects of long non-coding RNA (lncRNA) cancer susceptibility candidate 2c (CASC2c) on the proliferation, metastasis and drug resistance of non-small cell lung cancer (NSCLC) cells.

Methods: The expression of CASC2c in NSCLC tissues and cell lines was detected by real-time fluorescence quantitative PCR (RT-qPCR). MTT and Transwell assay were used to determine the proliferation and migration of NSCLC cells in the experimental group and the control group respectively. The drug sensitivity test was used to confirm whether increasing the CASC2c expression level could reverse the resistance of NSCLC cells to the chemotherapy drug cisplatin. The effects of CASC2c on the expression levels of p-ERK1/2 and β-catenin were detected by western blot.

Results: The results of RT-qPCR showed that CASC2c was under-expressed in NSCLC tissues and cells compared with normal adjacent lung tissues cells ($p < 0.05$). In addition, the CASC2c expression was remarkably correlated with TNM staging, tumor cell differentiation, lymph node metastasis, smoking and other pathological indicators of patients with NSCLC ($p < 0.05$). MTT and Transwell assay showed that the high-expression of CASC2c significantly reduced the proliferation and migration of NSCLC cells compared to that of the control group ($p < 0.05$). Western blot assay showed that the high-expressed CASC2c can decrease the expression of phosphorylated-ERK1/2 (p-ERK1/2) and β-catenin.

Conclusions: CASC2c was low expressed in NSCLC tissues and cells. What's more, it inhibited the proliferation and migration of NSCLC cells by inhibiting the expression of p-ERK1/2 and β-catenin and reversed NSCLC cells' resistance to the chemotherapy drug cisplatin. Therefore, CASC2c may serve as a new biomarker and therapeutic target in the diagnosis and treatment of NSCLC.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for 80%–85% of lung cancer cases, which is the leading cause of death worldwide [1]. Currently, surgery therapy, radiation therapy and chemotherapy are the most common and also most effective treatments for NSCLC. Among them, surgical treatment is still the most preferred way, which can obviously improve the patients' life and extend their postoperative survival. However, the surgical resection rate for patients with advanced NSCLC is less than 30%. What's worse, the 5-year survival rate is very low [2] and the recurrence rate is high after surgery [3]. Therefore, the further exploration of the pathogenesis of NSCLC and the search for new therapeutic targets are of great significance for the diagnosis and treatment of NSCLC.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules longer than 200 nucleotides, which are involved in gene expression regulation through chromosome remodeling, transcription and post-transcriptional processing [4]. Besides, their abnormal expression is closely related to a variety of diseases, especially tumors [5]. For instance, LncRNA LINC00336 inhibits ferroptosis in lung cancer by functioning as a competing endogenous RNA [6]. LncRNA LINC319 aggravates lung adenocarcinoma carcinogenesis by modulating miR-450b-5p/EZH2 [7]. Long non-coding RNA cancer susceptibility candidate 2 (lncRNA CASC2) is a recently discovered lncRNA located on

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chromosome 10q26. Baldinu et al firstly identified lncRNA CASC2 in endometrial cancer and also found that CASC2 generated three transcriptional variants (CASC2a, CASC2b and CASC2c) through variable clippings [8]. Their further study [9] showed that CASC2a was expressed at a low level in endometrial cancer and acted as a tumor suppressor [9]. What's more, recent studies have reported that CASC2a/b is involved in the genesis and development of various malignant tumors such as NSCLC [10] and colorectal cancer [11]. However, little is known about the effects of CASC2c on cancers. Therefore, this study was aimed to investigate the expression, clinical significance, function and mechanism of CASC2c on NSCLC.

The extracellular signal-regulated kinase (ERK) is an important subfamily of mitogen-activated protein kinases (MAPKs), which contains two important members (ERK1 and ERK2) [12]. ERK can be activated by mitogen (such as various growth factors) and then translocated into the nucleus, thus promoting the transcription and expression of genes in charge of cell proliferation, differentiation, migration, invasion and apoptosis [13]. The over-expression of ERK plays an important role in tumorigenesis and tumor progression [14]. Furthermore, β-catenin signal pathway composed of multiple action sites and links is related to the growth and development of cells. Under normal physiological conditions, the Wnt/β-catenin signaling pathway is orderly activated and inactive according to the cell program, and the signaling pathway also participates in the process of cell proliferation, differentiation and apoptosis during embryonic and tissue development [15,16]. Recent studies have shown that the expression of β-catenin in NSCLC is up-regulated [17]. However, whether lncRNA CASC2c could regulate the expression of ERK and β-catenin on NSCLC remains largely unclear.

In this study, we tried to explore the expression, clinical significances, functions and potential mechanisms of CASC2c in NSCLC. The experiment on over-expression of CASC2c confirmed the biological functions of CASC2c in the development of NSCLC *in vitro*. Further study proved that CASC2c inhibited the proliferation and metastasis of NSCLC cells by down-regulating the ERK1/2 and β-catenin signaling pathways and reversed the resistance of NSCLC to cisplatin. All in all, this work may provide a new biomarker in the diagnosis of NSCLC as well as a potential target for targeted therapy of NSCLC.

2. Materials and methods

2.1. Cell lines and reagents

A total number of 86 matched cancer tissues and corresponding non-cancer tissues in NSCLS patients were obtained from our hospital. The baseline clinical features of the patient were shown in Table 1. None of the patients received any neoadjuvant therapy before surgery. All the patients agreed to participate in this study. The sampling process was conducted under the supervision of the ethics committee of our hospital.

NSCLC cells (H292, H226, H1975 and H460) and normal human lung bronchial epithelial cells BEAS-2B were purchased from the American type culture collection (ATCC, USA). The RPMI-1640 medium and DMEM medium were purchased from Hyclone company (USA). Double-resistant penicillin and streptomycin was purchased from Invitrogen (USA). Transfection reagent Lipofectamine3000 was purchased from Invitrogen (USA). The full length of lncRNA CASC2c was designed and synthesized by Genechem (Shanghai) Co., Ltd. RNA extraction kits RNAiso Plus reagent and SYBR Premix Ex Taq™ were purchased from Dalian TaKaRa company (Japan). HotStarTaq DNA polymerase was purchased from QIAGEN (USA). Plasmid pcDNA3.1 was purchased from Invitrogen (USA). BI (Biological Industries) fetal bovine serum was purchased from Cromwell (USA). The protein strip was purchased from Eberhardzell (Germany).

Table 1
The correlation between CASC2c expression and pathological indexes of patients with NSCLC.

Characteristic	N	CASC2c Expression		Chi-Square	*p*-Value
		High expression	Low expression		
Age					
≤55	50	26	24	1.2069	0.2720
>55	36	23	13		
Gender					
Male	54	28	26	1.5549	0.2124
Female	32	21	11		
Tumor Type					
Squamous Carcinoma	38	25	13	3.6075	0.1647
Adenocarcinoma	30	13	17		
Large cell carcinoma	18	11	7		
Lymph Invasion					
Negative	41	29	12	6.0477	0.0139
Positive	45	20	25		
T Stage					
T1-T2	21	17	4	6.5154	0.0107
T3-T4	65	32	33		
Smoking History					
No	23	10	13	2.3338	0.1266
Yes	63	39	24		

2.2. Cell culture

Lung cancer cell lines (H292, H226, H1975 and H460) were cultured in RPMI-1640 medium supplemented 10% FBS and 0.2% double-resistant penicillin and streptomycin with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Normal lung bronchial epithelial cell line BEAS-2B was cultured using DMEM medium while other conditions were consistent with lung cancer cell line culture conditions.

2.3. Vector construction and transfection

The human CASC2c sequence was obtained from the National Center for Biotechnology Information (NCBI). The CASC2c sequence was amplified by PCR using HotStarTaq DNA polymerase. DNA ligase was inserted between the KpnI and XbaII sites of the vector plasmid pcDNA3.1 to construct the overexpressed vector pcDNA-CASC2C. The primer sequence is as follows: 5′-CGGGGTACCCCGGGAGAACAGGATGGCCATGT-3′ (forward primer), 5′-TGCTCTAGAGCAGCCTTCTCCATGTTGGTCTC-3′ (reverse primer). The transfection reagent Lipofectamine 3000 was used to transfect the over-expression vector pcDNA-CASC2c when the cell fusion reached 80%. The transfection efficiency was detected by real-time fluorescence quantitative PCR (RT-qPCR).

2.4. RT-qPCR

Total RNA was extracted from the cells and tissues by using RNAiso Plus. The concentration and purity of extracted RNA were determined by spectrophotometer (SmartSpec Plus, Bio-Rad company, Shanghai, China). The extracted RNA was transcribed into complementary DNA (cDNA) using PrimeScript reverse transcriptase. RT-qPCR was performed on LightCycler480 in accordance with the reaction system and reaction time required by SYBR GREEN EX Taq™ instruction using cDNA as template. All experiments were repeated for 3 times. The results were calculated and statistically analyzed with $2^{-\triangle\triangle Ct}$. *GAPDH* was used as the internal reference gene. CASC2c forward primer: 5′-TTCCTCTCCCCTTTGGACTT-3′, reverse primer: 5′-TCTGCTTCTGCTGCTGTTGT-3′ ; GAPDH forward primer: 5′-TCATGGGTGTGAACCATGAGAA-3′; reverse primer: 5′-GGCATGGACTGTGGTCATGAG-3′.

2.5. Western blotting

The RIPA lysis buffer containing protease inhibitor and phosphatase inhibitor was used to lyse H292 and H226 cells. Pyrolysis products were boiled at 100 ℃ for 10 min. During this period, the product was violently shaken every 3 min to interrupt the DNA. BCA method was used for protein quantification. After polyacrylamide gel electrophoresis, the membrane was transferred by 0.3A constant current wet method for 1.5 h. It was then sealed with 50 g/L skim milk at room temperature for 1 h. After the primary antibody was incubated at 4℃ overnight, it was washed with TBST three times (10 min each time). Then the secondary antibody was incubated at room temperature for 1 h. All the antibodies (Anti-p-ERK1/2 and Anti-t-ERK1/2 (Abca, ab17942, 1:1000), Anti-β-catenin (Abcam, ab6302, 1:1000) and Anti-GAPDH (Abcam, ab9484, 1:1000)) were purchased from Abcam (Shanghai, China). Protein bands were used for scanning analysis in the imaging system.

2.6. MTT assay

The cells in different groups were plated in the density of 5×10^3 cells/well in 96-well plates at 37 ℃ in an atmosphere of 5% (*v/v*) of CO_2. Fresh serum-free medium 110 μL (containing 10 μL MTT assay) was added in each hole. Bio-rad450 enzyme mark (B–D company, Shanghai, China) was used to test wavelength absorbance (A) values at 570 nm after incubation for 30 min at 37 ℃. Changes of cell proliferation number at 0, 24, 48, 72 and 96 h were then detected. In the drug resistance experiments, different concentrations of cisplatin (0.0001–100 μmol/L) were added to the growth medium after 24 h of culturing. Each concentration was set 3 repeated wells. SDS solution was then added to terminate the experiment 4 h later. Cell survival rate (VR) at different concentrations was calculated according to the following formula:

$$\mathrm{VR}(\%) = \frac{\text{A value at experimental well}}{\text{A value at control well}} \times 100\%$$

Microsoft Excel was used to calculate the concentration of 50% inhibitory (IC_{50}). Each experiment was performed in triplicate.

2.7. Drug resistance assay

MTT assay was performed to determine the sensitivity of NSCLC cells to cisplatin. The transfected cells were firstly inoculated into 96-well plates (3000 cells/wells) and incubated overnight. These cells were treated with different concentrations of cisplatin for 24 h. Cisplatin was added into each well with the appropriate gradient(from 1.30–56 μmol/L). After that, the culture medium was removed and dimethyl sulfoxide (DMSO, Sigma) of 150 μL was then added. ELISA (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the optical density (OD) at 570 nm. The half-maximal inhibitory concentration (IC_{50}) of cisplatin was the concentration of the drug that induced 50% growth inhibition.

2.8. Transwell assay

The cells of different groups were inoculated in a 6-well plate with a small chamber. 2 multiple wells were set in each group. The cell lines of H292 and H226 were suspended in the upper chamber without serum medium (approximately density: 100,000 cells/well, pore diameter: 8 μm; BD Biosciences, San Jose, CA, USA). The lower chamber was 500 μL DMEM medium containing 10% (*v/v*) FBS. The cells on the surface of the upper chamber (*i.e.*, the cells that did not invade the lower chamber) were gently wiped with a cotton swab after 24 h. The cells that invaded the lower chamber were fixed with methanol and then stained with crystal violet. Five visual fields (including the center and periphery of the membrane) were randomly selected under an inverted microscope to count the number of cells invading the lower chamber. As for the invasion assay, matrigel gel was coated on the base of the Transwell chamber. The other steps are the same as that of migration.

2.9. Statistical analysis

All the obtained data were processed by statistical software SPSS12.0. The experimental results were expressed as $\bar{x}$EQ ± s and analyzed by *t*-test. A *p* value of less than 0.05 was considered to be statistically significant. Each experiment was repeated at least three times and a representative result was shown.

3. Results

3.1. Comparison of CASC2c expression in NSCLC and normal bronchial epithelial cells

To confirm the expression level of CASC2c in NSCLC, we firstly determined CASC2c expression in 86 randomly selected NSCLC, tissues and adjacent non-tumor tissues by qRT-PCR. The results showed that CASC2c expression was significantly down-regulated in NSCLC tissues compared to matched adjacent non-tumor tissues (2.765 ± 1.605 v.s. 1.137 ± 0.754, $p < 0.05$, Fig. 1a). Furthermore, the expression of CASC2c in NSCLC cell lines (including H292, H226, H1975 and H460) were significantly lower than that in BEAS-2B cells ($p < 0.05$).

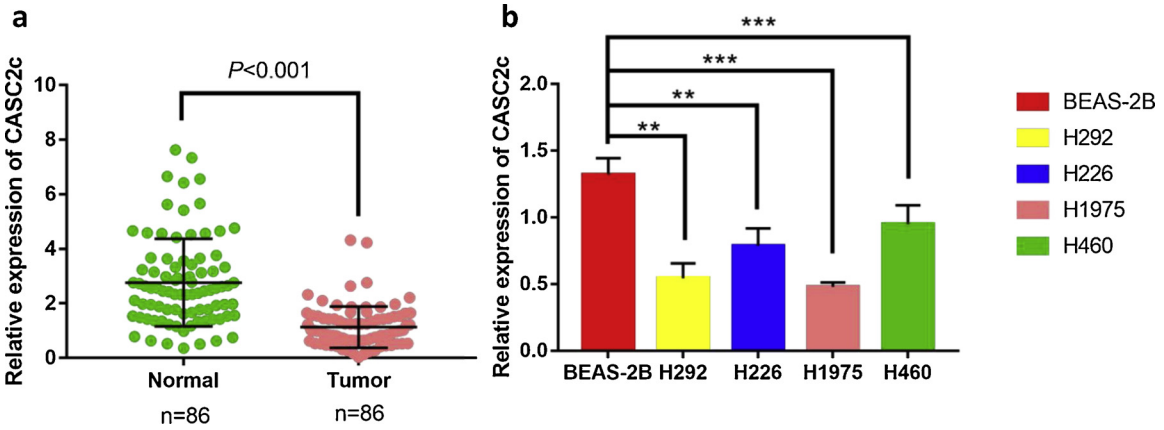

Fig. 1. CASC2c expression was decreased in non-small cell lung cancer (NSCLC) tissues. (a) The expression level of CASC2c in NSCLC tissues and adjacent (normal) tissues; (b) RT-qPCR was used to analyze the expression of CASC2c in different NSCLC cell lines and normal bronchial epithelial cells. *,** and *** respectively represented $p < 0.05$, $p < 0.01$, $p < 0.001$.

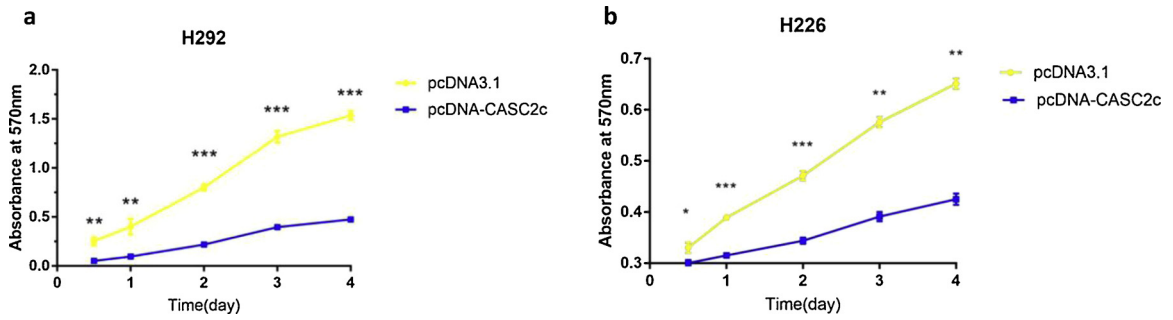

Fig. 2. The effects of CASC2c over-expression on the proliferation of H292 (a) and H226 (b) NSCLC cells were determined by MTT assay. *,** and *** respectively represented $p < 0.05$, $p < 0.01$, $p < 0.001$.

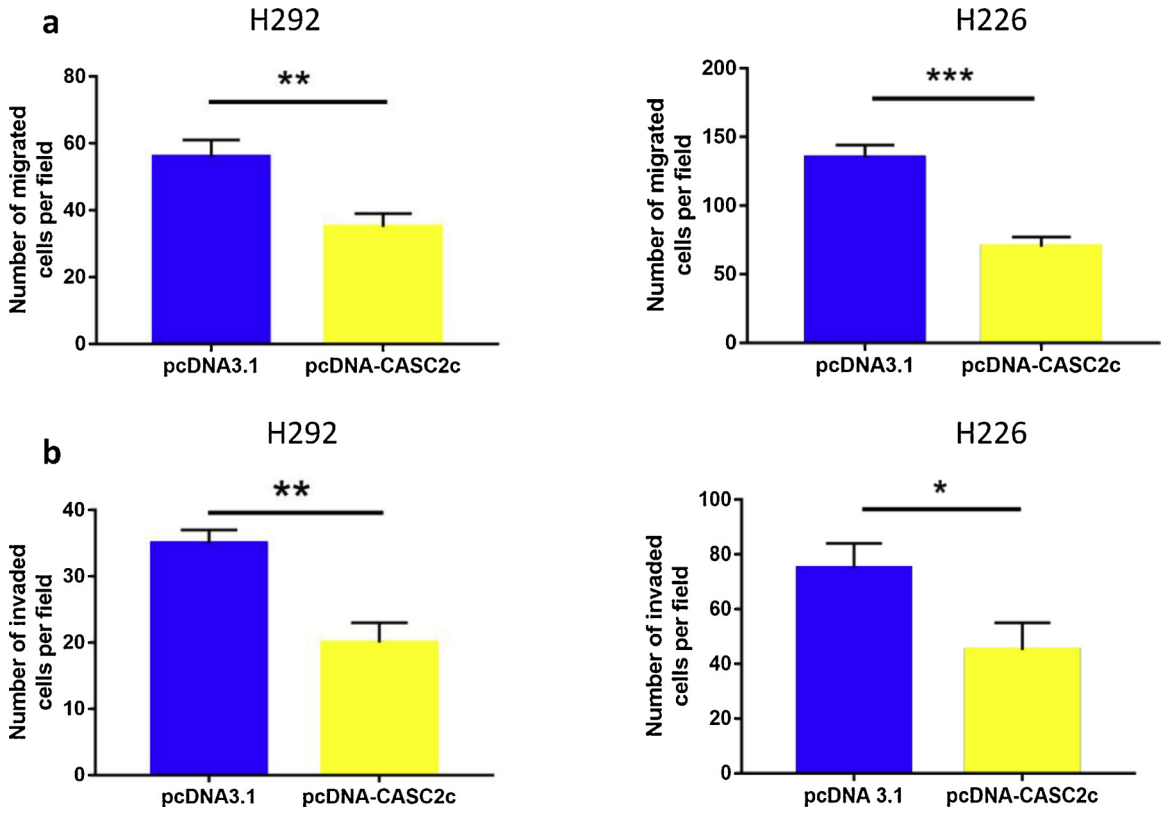

Fig. 3. Overexpression of CASC2c inhibited the migration and invasion of NSCLC cells. (a) Cell migration was assessed by Transwell assay in H292 (left) and H226 (right) cells 48 h after pcDNA-CASC2c transfection. (b) Cell invasion was assessed by Transwell assay using stromal gels from H292 (left) and H226 (right) cells 48 h after pcDNA-CASC2c transfection. *, ** and *** respectively represented $p < 0.05$, $p < 0.01$, $p < 0.001$.

Table 2
IC_{50} Values of H292 and H226 Cells against Cisplatin in Different Treatment Groups.

Groups	H292		H226	
	pcDNA3.1	pcDNA-CASC2c	pcDNA3.1	pcDNA-CASC2c
Cisplatin (IC_{50})	21.9 μmol/L	5.74 μmol/L	21.6 μmol/L	12.52 μmol/L

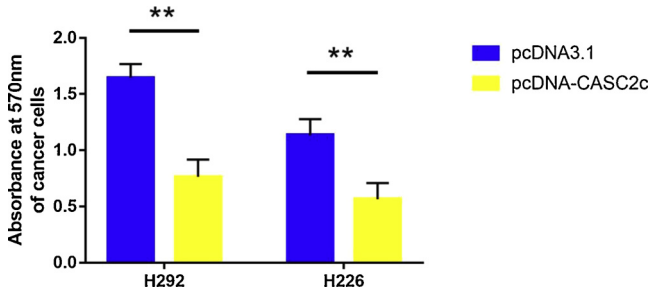

Fig. 4. MTT assay was conducted to determine the effects of CASC2c over-expression when 15 μmol/L cisplatin was used to treat H292 (left) and H226 (right) cells. *,** and *** respectively represented $p < 0.05$, $p < 0.01$, $p < 0.001$.

3.2. The CASC2c expression was related to multiple pathological indicators of patients with NSCLC

Next, we explored the correlation between CASC2c expression and various pathological indicators of 86 patients with NSCLC. The results indicated that the CASC2c expression was significantly correlated with the tumor T Stage ($p = 0.0107$) and lymph Invasion ($p = 0.0139$), but

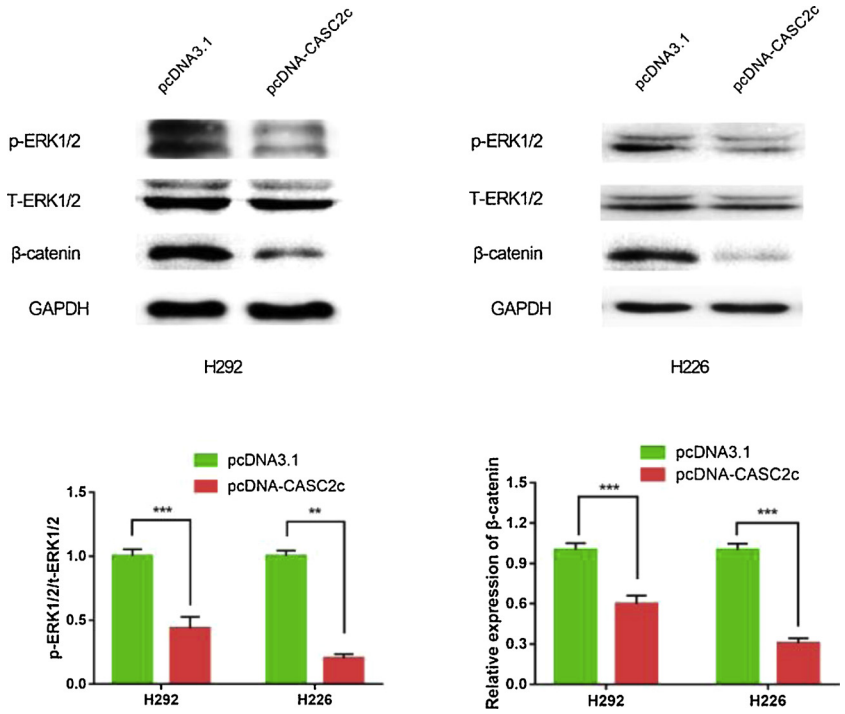

Fig. 5. Western blot was used to measure the expressions of ERK1/2 and β-catenin in H292 and H226 cells transfected with pcDNA3.1 or pcDNA-CASC2c. *,** and *** respectively represented $p < 0.05$, $p < 0.01$, $p < 0.001$.

without correlation ($p > 0.05$) with age, gender, tumor type or smoking history. The results suggested that CASC2c expression may be involved in the proliferation and metastasis of NSCLC (Table 1).

3.3. Enhancing the expression of CASC2c inhibitted the proliferation of NSCLC cells

To determine the effects of CASC2c on the proliferation of NSCLC cells, MTT assay was conducted. The results showed that compared with the pcDNA3.1 group, up-regulation of CASC2c dramatically decreased the proliferation of H292 and H226 NSCLC cells all at 12, 24, 48 and 96 h ($p < 0.05$) (Fig. 2).

3.4. Effect of CASC2c over-expression on NSCLC cell migration

Transwell Assay was performed to explore the migration and invasion of NSCLC cells. The results (Fig. 3) revealed that gain of CASC2c expression inhibited the migration and invasion both of H292 and H226 NSCLC cells ($p < 0.05$).

3.5. Increasing the CASC2c expression level could reverse the resistance of NSCLC cells to cisplatin

Cisplatin is widely used in the treatment of various cancers and tumors, including head and neck cancer, ovarian cancer, testicular cancer, bladder cancer, colorectal cancer and NSCLC [18,19]. However, many cisplatin-sensitive tumors at an early stage gradually develop to be cisplatin-resistant during treatment [20,21]. Such a high incidence of drug resistance is the most important factor limiting the clinical treatment of cisplatin. To verify the effect of CASC2c on the cisplatin resistance of NSCLC cells, we conducted MTT assay. The results showed that H292 and H226 cells with over-expressed CASC2c had lower IC_{50} value, and compared with control group, CASC2c overexpression significantly reduced the viability of NSCLC cells with 15 μmol/L cisplatin treatment, which indicated that the over-expression of CASC2c could reverse the drug resistance of NSCLC cells to cisplatin (Table 2 and Fig. 4).

3.6. Effect of CASC2c on expression of p-ERK1/2 and β-catenin

To explore the effects of over-expressed CASC2c on ERK1/2 and β-catenin signaling pathway, western blot experiments were conducted. The results showed that compared with the control group, the expression of p-ERK1/2 and β-catenin in the experimental group was significantly decreased ($p < 0.05$) (Fig. 5). In addition, there was no significant difference in total ERK1/2 expression level between the two groups ($p > 0.05$).

4. Discussion

Many lncRNAs play a key role in cell growth, differentiation, proliferation, apoptosis and invasion by regulating related gene expression [22–24]. Some lncRNAs can be used for the investigation of tumor risk, diagnosis and prognostic biomarkers. Nearly 18% of human lncRNAs were recently found to be associated with tumors [25]. In addition, relevant functional studies have shown that some lncRNAs could act as oncogenes or tumor suppressor genes, which were involved in the occurrence and development of human cancer. For example, MO et al. [26] showed that lncRNA XIST was highly expressed in gastric cancer tissues and cell lines to promote the growth of cancer cells, while Yuan et al. [27] showed that lncRNA DANCR inhibited the growth of gastric cancer by inhibiting the expression of CTNNB1. In addition, Hen et al. [28] found that those NSCLC patients with high expression of MALAT1 had a poor prognosis. In NSCLC, many non-coding RNAs (both lncRNAs and microRNAs) can affect the biological behavior of cancer cells [29–35]. However, more knowledge about the functions, mechanisms and clinical significances of lncRNAs in NSCLC are not completely clear due to their complexity sequences and spatial structures.

Previous studies have shown that CASC2a/b is involved in the occurrence and development of various malignant tumors such as gastric carcinoma and colorectal cancer. For example, CASC2a/b can be used as endogenous RNA to competitively bind and inhibit the expression of miR-18a, thereby inhibiting the proliferation of colorectal cancer cells [10]. Nevertheless, little is reported about the effects of CASC2c on cancers. Current study found that CASC2c was low-expressed in NSCLC

tissues and cells, and associated with pathological indexes. What's more, our *in vitro* study showed that up-regulation of CASC2c expression could inhibit the proliferation and migration of NSCLC cell lines, and CASC2c over-expression also decreased the drug resistance of H292 and H226 to cisplatin. Those results suggested that CASC2c played the role of tumor suppressor in the pathological process of NSCLC. However, in another study, CASC2c as an unfavorable prognosis factor interacts with miR-101 to mediate astrocytoma tumorigenesis [36]. This suggests that CASC2c may play different roles in different types of tumors.

It is reported that overexpression of CASC2 can increase the sensitivity of prostate cancer cells to docetaxel by inhibiting ERK activation [37]. Since CASC2c is produced by CASC2 through variability splicing, there is a large similarity between their sequences. Therefore, we hypothesized that CASC2c can inhibit the malignant behavior of NSCLC cells, which might be achieved through the involvement of ERK and its downstream Wnt/β-catenin signaling pathways. The ERK kinase family belongs to the tyrosine protein kinase and can be activated after phosphorylation [38]. Previous studies have shown that ERK activation was not detected in normal tissues, but occurred in a variety of malignant tumors, suggesting that ERKl/2 is also involved in cell proliferation and differentiation of tumors [39]. For instance, Luo et al. [40] found that bFGF could stimulate gastric epithelial cell proliferation by activating ERK1/2 signaling pathways, thus increasing the COX-2 expression to cause cell proliferation. Another study found that the ERK1/2 signaling pathway could regulate cell proliferation mainly by influencing the cell cycle [41]. Additionally, ERKl/2 is also involved in metastasis of NSCLC cells, and the application of nimbolide to inhibit the ERK1/2 pathway eventually inhibited the invasion and migration [41,42] Abnormal expression of the β-catenin pathway could also promote tumorigenesis and tumor development. Many studies have reported that β-catenin was over-expressed in a variety of tumor tissues, and inhibiting its expression could reverse the malignant phenotypes [43]. For example, β-catenin was not only involved in the differentiation and proliferation of breast cancer stem cells, but also related to the growth and apoptosis of glioma cells [44,45]. Mounting studies have shown that there was abnormal expression of β-catenin in NSCLC tissues, and it was closely related to the type, differentiation and staging of NSCLC [46–48]. Herein, we demonstrated that over-expression of CASC2c could reduce the expression of p-ERK1/2 and β-catenin in NSCLC, which partly explained the reason of activation of ERK and β-catenin pathways during NSCLC progression.

In conclusion, this work confirmed that CASC2c was lowly expressed in NSCLC tissues and cells. This was related to the malignant clinical characteristics of NSCLC patients. Furthermore, the over-expression experiments confirmed that CASC2c could inhibit NSCLC cells' proliferation, migration, invasion and reverse the drug resistance of the cells to cisplatin through suppressing ERK and β-catenin signal pathways. Our founding showed that CASC2c had an inhibitory effect on the pathogenesis of NSCLC and could be identified as a promising therapeutic target for NSCLC. However, it should be noted that this study was only limited to basic experiments *in vitro*. To further verify the specific functions of CASC2c, *in vivo* animal experiments need to be conducted in the future.

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