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ORIGINAL ARTICLE

MicroRNA-203 promotes the progression of non-small cell lung cancer via survivin

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Summary

Purpose: *MicroRNAs are involved in the occurrence and progression of tumors. Previous studies have confirmed that microRNA-203 serves as an oncogene. The specific role of microRNA-203 in non-small cell lung cancer (NSCLC) is rarely reported. This study aimed to explore the regulatory effect of microRNA-203 on NSCLC and its underlying mechanism.*

Methods: *MicroRNA-203 expression in 96 pairs of NSCLC tissues and paracancer tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR). Correlation between microRNA-203 expression and prognosis of NSCLC was further analyzed. Proliferative, migratory and invasive abilities of NSCLC cells after transfection with si-microRNA-203 or si-negative control (NC) were assessed by cell counting kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU), colony formation and Transwell assay, respectively. Rescue experiments were conducted to investigate the interaction between microRNA-203 and survivin in regulating NSCLC progression.*

Results: *MicroRNA-203 was highly expressed in NSCLC tissues than in paracancer tissues. Correlation analyses showed that microRNA-203 expression was positively correlated to tumor stage, lymph node metastasis and distant metastasis, whereas it was not correlated to age and sex of NSCLC patients. MicroRNA-203 knockdown inhibited proliferative, migratory and invasive abilities of NSCLC cells. Rescue experiments confirmed that microRNA-203 promotes the progression of NSCLC via targeting survivin.*

Conclusions: *MicroRNA-203 is highly expressed in NSCLC, and is closely related to tumor stage, lymph node metastasis, distant metastasis and poor prognosis of NSCLC patients. It is concluded that microRNA-203 promotes the progression of NSCLC via regulating survivin expression.*

Key words: *microRNA-203, surviving, NSCLC, proliferation, apoptosis*

Introduction

Lung cancer (LC) is a common malignancy of the respiratory system worldwide [1,2]. In China, there were 4.29 million new cases and 2.81 million death cases of LC in 2015 [3,4]. Non-small cell lung cancer (NSCLC) accounts for over 85% of all LC cases, with morbidity of up to 80-90% [5-7]. Although the treatment approaches for NSCLC have been greatly advanced, the 5-year survival is still less than 15% [8]. At present, targeted drugs greatly reduce the lesions to adjacent normal tissues and incidence of adverse events compared to traditional cytotoxic drugs [9,10]. With the development of molecular biology technology, targeted drug therapy has become one of the research hotspots in recent years [11]. It is believed that NSCLC results from long-term interaction between genetic and environmental factors. Disordered proliferation, apoptosis and differentiation, all lead to tumorigenesis [12-14]. Hence, it is of great significance to clarify the specific mechanism leading to NSCLC

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genesis, so as to improve the clinical outcomes of affected people.

MicroRNAs (miRNAs) are non-coding, small RNAs with about 22-25 nucleotides in length, which regulate expressions of target genes by complementary pairing. MicroRNAs participate in various biological processes, such as cell proliferation, differentiation and apoptosis [15,16]. MicroRNAs are widely present in organs and tissues, which are capable of regulating disease development at post-transcriptional level [17-19]. It is reported that microRNAs serve as oncogenes or tumor-suppressor genes, playing crucial roles in tumor progression [20-22]. So far, the role of microRNAs in cisplatin-resistance of LC cells and the molecular mechanisms remains unclear.

The purpose of our study was to provide a theoretical basis for developing novel approaches in diagnosing and treating NSCLC.

Methods

Patients and NSCLC samples

Surgically resected NSCLC tissues and paracancer tissues from 96 NSCLC patients were collected. All enrolled patients were pathologically diagnosed as NSCLC based on the 8th edition of UICC/AJCC. Patients did not receive preoperative anti-tumor treatments. This study was approved by the Ethics Committee of Tangdu Hospital and all patients signed informed consent.

Cell culture

Human LC cell lines (SKMES1, A549, H358, H1299 and SPCA1) and human bronchial epithelial cell line (16HBE) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Transfection

Corresponding plasmids of si-microRNA-203 and si-NC were purchased from GenePharma (Shanghai, China). Cell transfection was performed until 70% confluence following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell counting kit-8 (CCK-8) assay

Transfected cells were seeded in 96-well plates with 2000 cells per well. CCK-8 reagent was added (Dojindo Laboratories, Kumamoto, Japan), followed by determination of optical density (OD) at 490 nm wavelength using a microplate reader.

Colony formation assay

200 transfected cells were seeded in each well of 6-well plates for 2-week cell culture. DMEM containing 10% FBS was replaced once a week. Cells were then washed with phosphate buffered saline (PBS), fixed with methanol for 20 min and stained with 0.1% crystal violet for 20 min. Colonies were captured using a microscope.

5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay

Transfected cells were incubated with 50 μM EdU (RiboBio, Nanjing, China) for 2 h, followed by Apollo staining and DAPI (4',6-diamidino-2-phenylindole) staining. EdU-positive cells were accessed with a fluorescence microscope. The EdU incorporation rate was calculated as the ratio of EdU-positive cells to total DAPI-positive cells (blue cells).

Table 1. Association of miR-203 expression with clinicopathologic characteristics of hepatocellular carcinoma

Parameters	*Number of cases*	*miR-203 expression*		*p value*
		Low (%)	*High (%)*	
Age (years)				0.569
<60	42	25	17	
≥60	54	29	25	
Gender				0.143
Male	47	30	17	
Female	49	24	25	
T stage				0.032
T1-T2	53	35	18	
T3-T4	43	19	24	
Lymph node metastasis				0.035
No	55	36	19	
Yes	41	18	23	
Distant metastasis				0.023
No	70	45	25	
Yes	26	10	16	

Transwell assay

Transfected cells were centrifuged and resuspended in serum-free DMEM at a density of 2.0×10^5/mL. Transwell chambers pre-coated with Matrigel were placed in 24-well plates. 200 μL of cell suspension and 600 μL of medium containing 10% FBS were added in the upper and lower chamber, respectively. After cell culture for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Inner cells were carefully cleaned. Penetrating cells were captured in 5 randomly selected fields of each sample for cell counting.

Quantitative real-time polymerase chain reaction (QRT-PCR)

TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into complementary DNA (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes. Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method.

Western blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit (Abcam, Cambridge, MA, USA). Protein sample was separated by gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody (Cell Signaling Technology, Danvers, MA, USA), immunoreactive bands were exposed by enhanced chemiluminescence method.

Statistics

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for statistical analyses. The quantitative data were expressed as mean±standard deviation (x±s). The-t test was used for comparing differences between the two groups. Categorical data were compared using x^2 test. The prognosis of NSCLC patients was analyzed by Kaplan-Meier and differences between curves were compared by Log-rank test. P<0.05 was considered statistically significant.

Results

MicroRNA-203 was highly expressed in NSCLC tissues and cell lines

MicroRNA-203 was highly expressed in NSCLC tissues compared to paracancer tissues detected by qRT-PCR (Figure 1A and 1B). Similarly, microRNA-203 expression was higher in NSCLC cells compared with that of controls (Figure 1C). In particular, SPCA1 and H1299 cells showed a relative high expression of microRNA-203, which were selected for the following experiments.

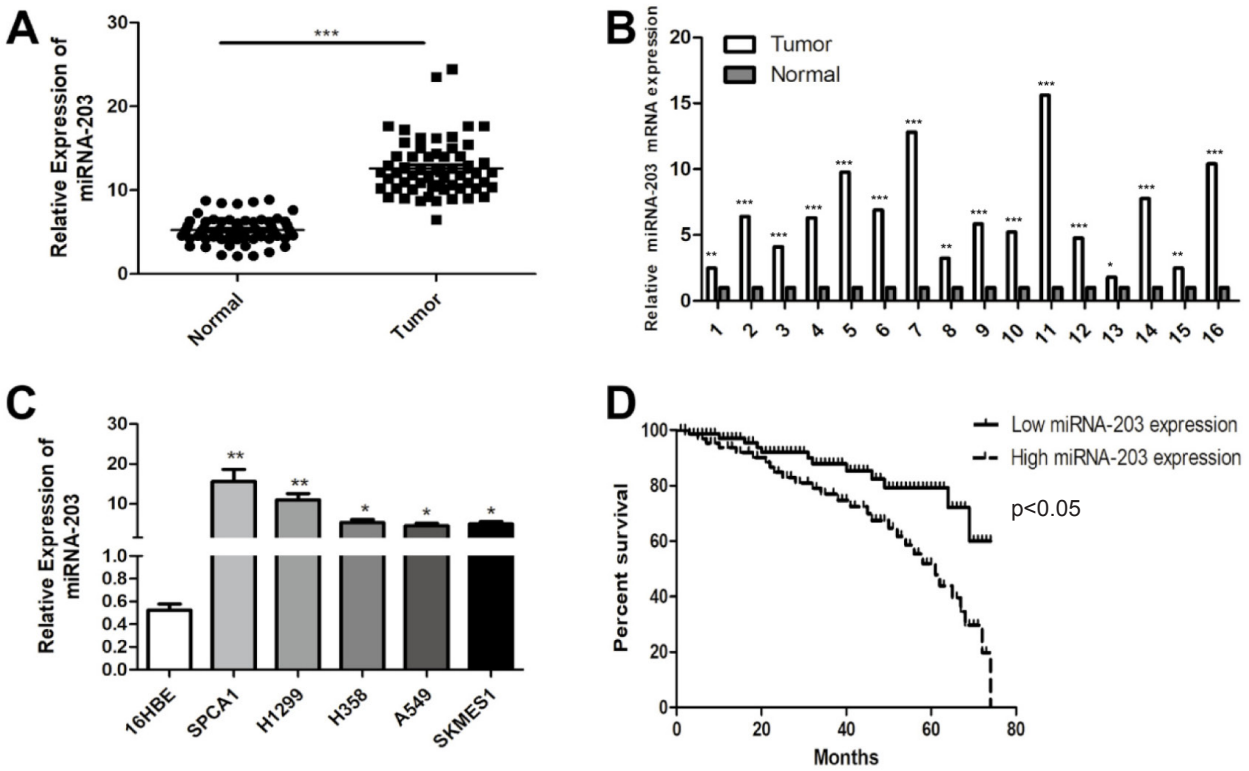

Figure 1. A,B: MicroRNA-203 expression in 96 pairs of NSCLC tissues and paracancer tissues. The results of qRT-PCR showed that microRNA-203 was highly expressed in NSCLC tissues compared to paracancer tissues. **C:** MicroRNA-203 expression in NSCLC cell lines (SKMES1, H358, SPCA1, H1299, and A549) and normal lung cell (16HBE). The results of qRT-PCR showed that microRNA-203 was highly expressed in NSCLC cell lines compared to normal lung cells. **D:** Kaplan-Meier survival curves of NSCLC patients based on microRNA-203 expression. (*p<0.05, **p<0.01, ***p<0.001).

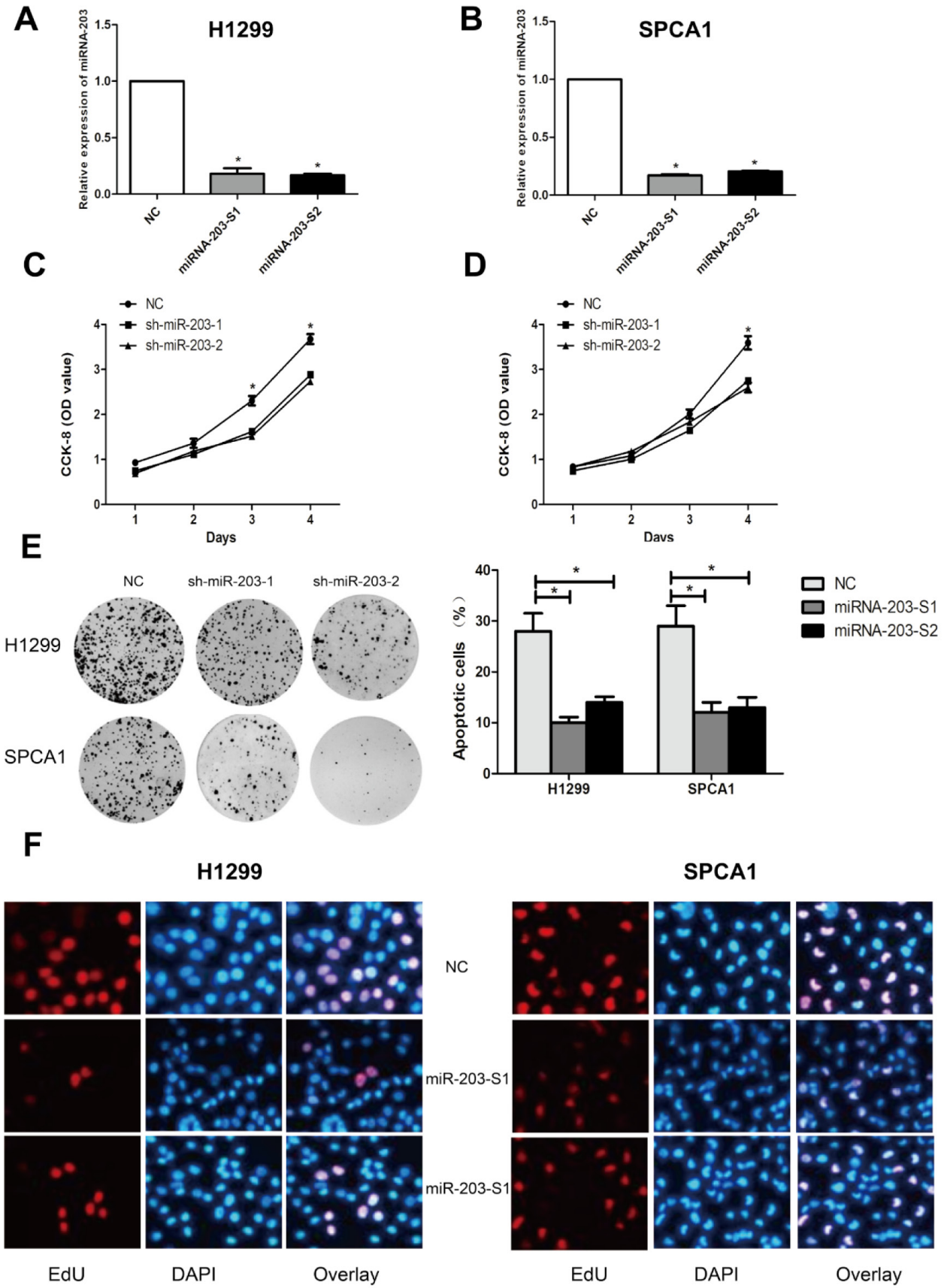

Figure 2. A,B: Transfection efficacy of si-microRNA in SPCA1 and H1299 cells. **C,D:** Proliferation of SPCA1 and H1299 cells after microRNA-203 knockdown. **E:** Colony formation in SPCA1 and H1299 cells after microRNA-203 knockdown showing that microRNA-203 knockdown inhibited the proliferation of NSCLC cells. **F:** Representative profiles of EdU-positive cells after microRNA-203 knockdown in SPCA1 cells and H1299 cells. The Figure shows that microRNA-203 knockdown inhibited the proliferation of NSCLC cells (*p<0.05).

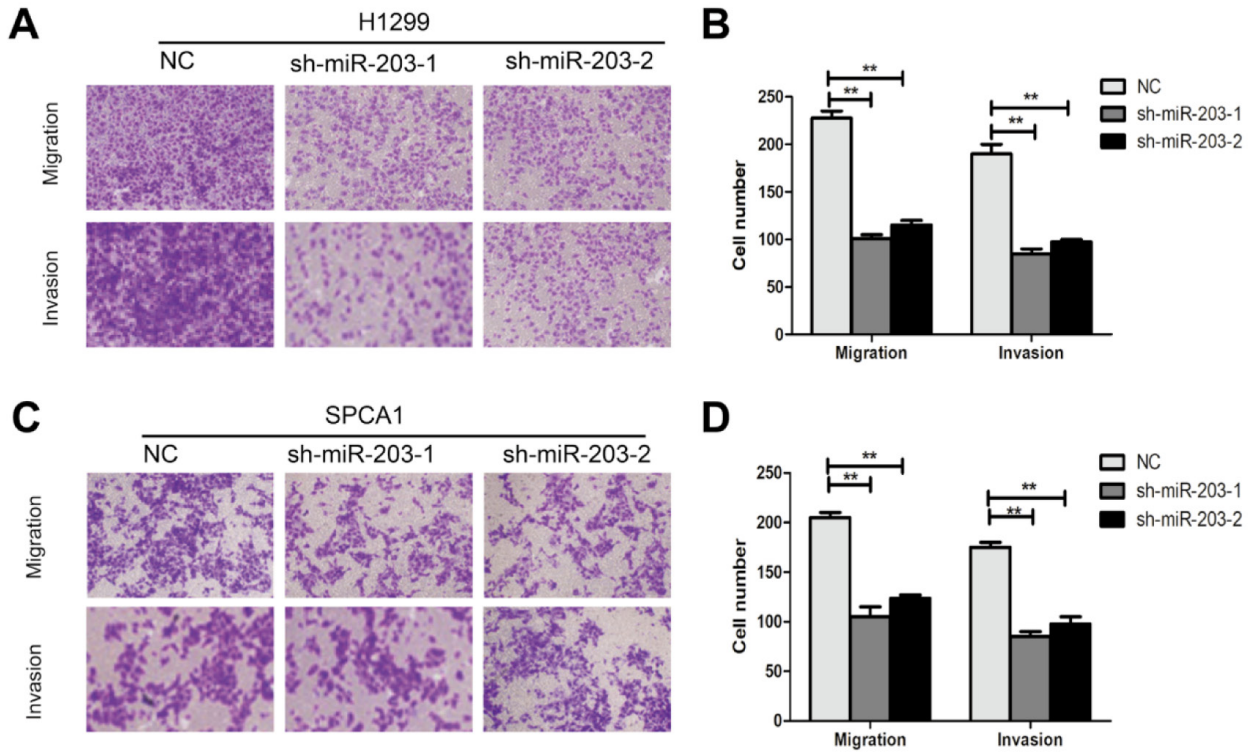

Figure 3. A,B: SPCA1 and H1299 cells transfected with si-microRNA-203 displayed significantly lower migration capacity. **C,D:** SPCA1 and H1299 cells transfected with si-microRNA-203 displayed significantly lower invasion capacity (**p<0.01).

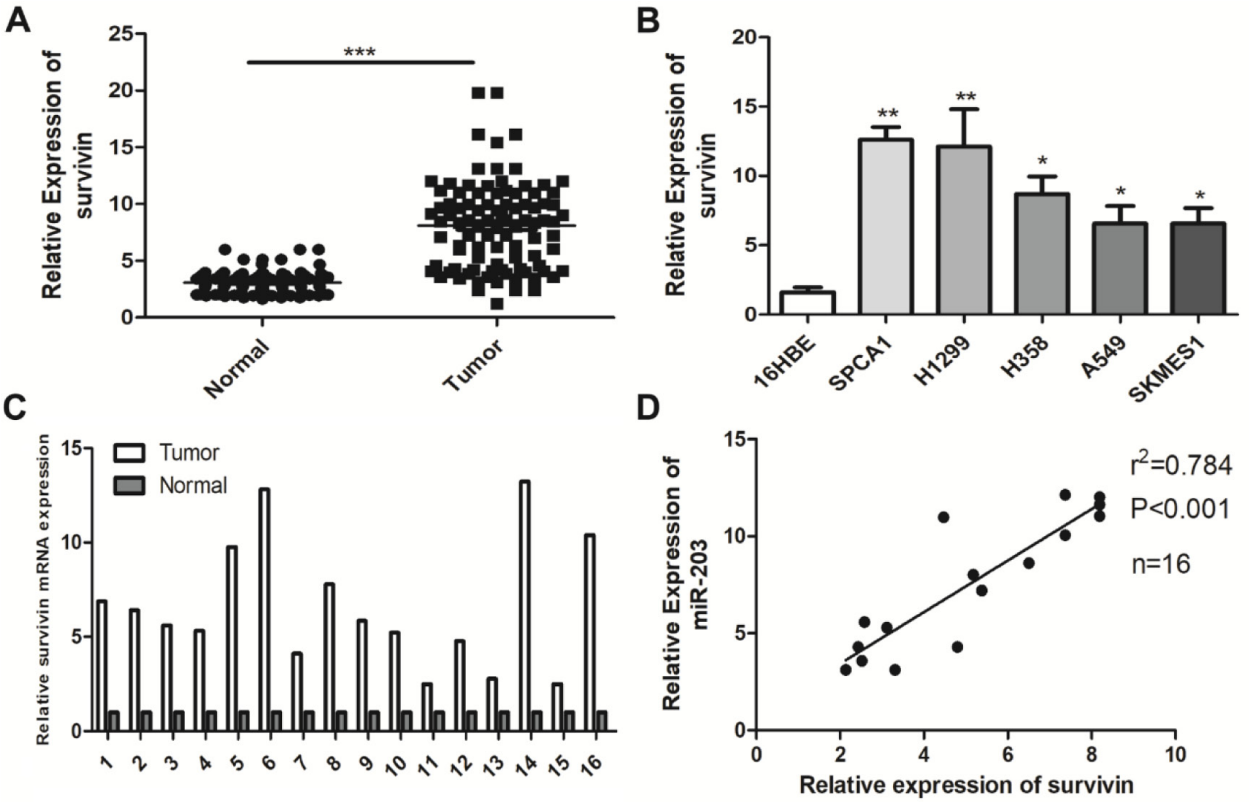

Figure 4. A-C: The mRNA expression level of survivin relative to GAPDH in human NSCLC tissues, paracancer tissues and NSCLC cell lines were detected by qRT-PCR. The Figure shows that survivin expression was significantly lower in NSCLC tissues compared to paracancer tissues and human bronchial epithelial cell line 16HBE, respectively. The Figure also shows that knockdown of survivin in NSCLC cells remarkably upregulated microRNA-203 expression. **D:** Negative correlation was found between microRNA-203 and survivin in tumor samples. (*p<0.05, **p<0.01, ***p<0.001).

MicroRNA-203 expression was correlated with clinical stage, lymph node metastasis, distance metastasis and overall survival of NSCLC patients

Correlation analyses showed that microRNA-203 expression was positively correlated to tumor stage, lymph node metastasis and distant metastasis, but not to age and sex of NSCLC patients (Table 1). Subsequently, NSCLC patients were divided into high-level group and low-level group according to their microRNA-203 expression. Survival analyses confirmed that NSCLC patients in the high-level group presented a worse prognosis compared with those in the low-level group (Figure 1D), suggesting that microRNA-203 may serve as a prognostic factor in NSCLC.

MicroRNA-203 knockdown inhibited cell proliferation

To explore the role of microRNA-203 in regulating biological functions of NSCLC cells, si-microRNA-203 and si-NC were first constructed (Figure 2A and 2B). CCK-8 results elucidated that microRNA-203 knockdown inhibited the proliferation of NSCLC cells (Figure 2C and 2D). Similar results were obtained in colony formation assay and EdU assay (Figure 2E and 2F).

MicroRNA-203 knockdown inhibited cell migration and invasion

Transwell assay demonstrated that the amount of penetrating cells after microRNA-203 knockdown was fewer than that of controls, indicating that microRNA-203 inhibited the migratory ability of LC cells (Figure 3A and 3B). We also confirmed that microRNA-203 knockdown suppressed the invasive ability of NSCLC cells (Figure 3C and 3D).

Survivin modulated microRNA-203 expression in human NSCLC cells

Through bioinformatics analyses, survivin was found to be related to microRNA-203. Both qRT-PCR and Western blot confirmed that survivin was downregulated in NSCLC tissues in comparison with that of paracancer tissues (Figure 4A). Survivin expression was also lowly expressed in NSCLC cells than that of 16HBE cells (Figure 4B).

Furthermore, knockdown of survivin in NSCLC cells remarkably upregulated microRNA-203 expression (Figure 4C). We selected 16 pairs of NSCLC tissues and paracancer tissues to detect the expression levels of microRNA-203 and survivin.

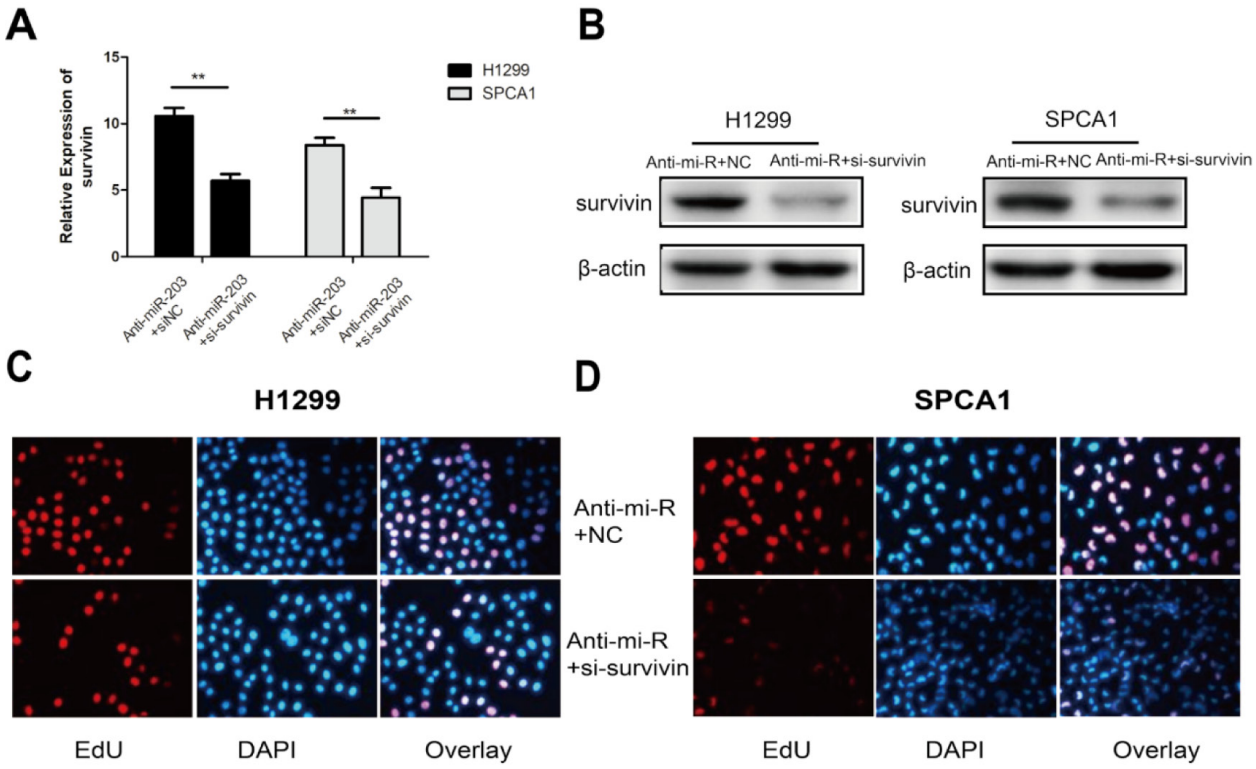

Figure 5. A: Survivin expression in NSCLC cells detected by qRT-PCR. The Figure shows that survivin mRNA was lowly expressed in NSCLC cells after transfection with si-survivin. **B:** Western blot was used to detect the protein expression of survivin. The Figure shows that survivin protein was lowly expressed in NSCLC cells after transfection with si-survivin. **C,D:** The roles of microRNA-203 and survivin in the representative profiles of EdU-positive cells. The Figure shows inhibited proliferation of SPCA1 and H1299 cells induced by microRNA-203 knockdown which was reversed by survivin knockdown. A representative data set is displayed as mean ± SD values (**p<0.01).

The data showed that microRNA-203 expression was negatively related to survivin expression in NSCLC tissues (Figure 4D). *In vitro* experiments also confirmed that survivin was lowly expressed in NSCLC cells (Figure 5A and 5B). After construction of si-survivin, the results demonstrated that the inhibited proliferation of SPCA1 and H1299 cells induced by microRNA-203 knockdown was reversed by survivin knockdown (Figure 5C and 5D).

Discussion

The incidence and mortality of LC have been astonishingly risen in the world. The incidence of LC in China is also increasing year by year [1-3]. Traditional radiotherapy and chemotherapy regimens exert low activity and serious adverse reactions. It has been reported that the 5-year survival rate of NSCLC patients undergoing cisplatin chemotherapy is only 15.7% [4,5]. NSCLC poses a great challenge in clinical treatment and it also poses a serious burden on affected patients and their families [6,7]. So far, radical surgery is still the preferred therapeutic method for NSCLC. However, part of NSCLC patients cannot be operated due to advanced disease stage. These patients receive conservative treatments, such as chemotherapy, radiotherapy, and targeted drug medication [8-11].

Studies have shown that about 65% of NSCLC patients are already in advanced stage of disease when diagnosed, and the onset age is over 65 years in 50% of affected patients. Due to the poor physical function, cisplatin chemotherapy is the preferred option for them [12]. However, the 5-year survival rate is still unsatisfactory [13]. It is reported that chemotherapy resistance is the leading cause for the treatment failure [7,14]. With the in-depth understanding of tumorigenesis, molecular targeted therapy has been well recognized in tumor treatment [10-13].

Recent studies have found that certain microRNAs are differentially expressed in NSCLC, which are crucial for diagnosis, treatment and prognosis of NSCLC [15-17]. Therefore, finding aberrantly expressed microRNAs in NSCLC and analyzing their correlation with clinical prognosis will contribute to improvement of the diagnosis and treatment of NSCLC [20]. This study investigated the regulatory effects of microRNA-203 on progression of NSCLC. We first detected microRNA-203 expression in NSCLC tissues and paracancer tissues. Our results confirmed that high expression of microRNA-203 in NSCLC was positively correlated to tumor stage and metastasis. As a result, we considered that microRNA-203 may function as an oncogene in NSCLC. Furthermore, microRNA-203 knockdown inhibited the proliferation, migration and invasion of NSCLC cells.

Survivin exerts anti-apoptosis function that is selectively expressed in malignancies [23,24]. Functionally, survivin could directly inhibit caspase-3 and caspase-7 or indirectly inhibit caspases' expression via p21 [25,26]. Previous studies pointed out that survivin is highly expressed in NSCLC, which may be utilized as a tumor biomarker for early diagnosis [27,28]. It is shown that the positive rate of survivin is closely related to tumor differentiation, TNM stage and lymph node metastasis, indicating the specific role in tumor development. Higher level of survivin indicates a worse prognosis of NSCLC [8]. This study indicated that NSCLC tissues with high level of survivin presented poor differentiation, frequent metastasis and high tumor stage, which were consistent with previous conclusions [28]. Survivin overexpression promotes metastasis of tumor cells through upregulating the levels of adhesion factors or initiating metastasis pathway [29-31]. Our rescue experiments demonstrated the interaction between microRNA-203 and survivin, suggesting that microRNA-203 regulates the progression of NSCLC via targeting survivin.

Conclusions

MicroRNA-203 is highly expressed in NSCLC, which is closely related to tumor stage, lymph node metastasis, distant metastasis and poor prognosis of NSCLC patients. It is concluded that microRNA-203 promotes the progression of NSCLC via regulating survivin expression.

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Conflict of interests

The authors declare no conflict of interests.

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