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Prognostic significance of microRNA-20a-5p levels which promotes proliferation and invasion by targeting cyclin G2 in small cell lung cancer

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MicroRNA-20a-5p (miR-20a-5p) has been shown to function as a tumor promoter factor in several cancers. However, its role in small cell lung cancer (SCLC) remains unclear. In this study, we have made an attempt to measure the tumor tissue levels of miR-20a-5p in patients with SCLC using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The biological function of miR-20a-5p in SCLC cells was investigated *in vitro* and *in vivo* studies, including cell proliferation, migration assays and tumorigenicity in nude mice. Meanwhile, we conducted the luciferase reporter assay to verify the biological relationship between miR-20a-5p and CCNG2. The expression of miR-20a-5p was significantly upregulated in human SCLC compared to that in normal tissues. Kaplan-Meier analysis indicated that patients with high expression of miR-20a-5p are closely related with the shorter survival of SCLC. Further, multivariate analysis showed that miR-20a-5p was an independent prognostic factor. Increasing miR-20a-5p expression promotes the proliferation, migration and invasion of the NCI-H446 cells *in vitro* and *in vivo*. Dual-luciferase reporter gene assay demonstrated that miR-20a-5p directly targets CCNG2. These findings suggest that miR-20a-5p levels might be a novel diagnostic and prognostic marker of SCLC. Inhibiting miR-20a-5p could be a promising therapeutic strategy for SCLC.

Keywords: Cancer suppressor, Luciferase reporter gene assay, Tumor promoter factor, Tumorigenicity

Although small cell lung cancer (SCLC) accounts for approximately 15% in all primary lung cancers, it is the most aggressive malignancy due to poor prognosis[1]. SCLC is characterized by genomic instability, early metastatic dissemination, high vascularity and rapid tumor growth, which contribute to exceedingly poor survival[2]. Despite rapid progress in unraveling the biology of SCLC, the first-line treatment for conventional chemo- and radiation therapy for SCLC has remained unchanged for decades in clinics[3,4]. Thus, it is urgent to uncover the novel biomarkers for early disease detection and better outcome of SCLC patients.

MiR-20a-5p coded by the miR-17-92 cluster was also known as miR-20a[5]. In accordance with their targets, miRNAs are classified into onco-miRs and cancer suppressors. In some tumors, miR-20a-5p could be grouped as onco-miR[6,7]. However, in oral squamous tumor[8] and hepatic tumor[9], miR-20a-5p acts as a cancer suppressor. These findings evidenced that miR-20a-5p performed various functions in different tumors. To date, some miRNAs have been revealed in SCLC, such as miR-26b, miR-886-3P[10,11]. Although these miRNAs play an important role in the occurrence and development in SCLC, there are still sensitivity limitations in clinical diagnosis and treatment. Therefore, it is very necessary to find novel and effective miRNA markers for the patients with SCLC.

Cell cycle regulation performs the core function in cell proliferation and is closely related to cell carcinogenesis. Cyclin G2 (CCNG2) encodes an unconventional cyclin homolog, cyclin G2 (CycG2)[12]. Similarly, CCNG2 has the function of growth inhibition and tumour suppression[13]. In recent years, the dysregulation of CCNG2 has been revealed in a variety of human tumors such as breast, oral, pancreatic, and thyroid cancer[14-17]. Liu *et al.*[18] reported that CCNG2 can inhibited proliferation of gastric cell. In addition, other researchers have demonstrated that CCNG2 could increase apoptosis and reduce proliferation、colony formation and invasion[19]. However, the involved underlying mechanism of CCNG2 in SCLC is not yet clear. Hence, in the present study, we tried to assess the clinical significance of miR-20a-5p in SCLC and investigate the effects of miR-20a-5p on proliferation,

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migration and invasion of SCLC cells, and to further understand the mechanisms of action of miR-20a-5p by identifying its potential target gene.

Materials and Methods

Tissue samples

Thirty-six human SCLC tumor tissues and 36 matched normal tissue (located >5 cm away from the tumor) FFPE samples with histologically confirmed limited-stage SCLC、TNM classification: $T_1N_0M_0$ 21 patients, $T_2N_0M_0$ 15 patients and Eastern Cooperative Oncology Group (ECOG)[20] performance status of 0 to 1, were collected from patients (26 males and 10 females, age range: 38-82 years; mean age: 58.78 years) who underwent thoracic operation in XuanWu Hospital (Beijing, China) during February 2008 and July 2018. None of the patients with SCLC had received radiotherapy or chemotherapy prior to the surgery and followed by adjuvant chemotherapy according to the standard of therapy after surgery. This study was approved by the Medical Ethics Committee of XuanWu Hospital and informed consent was obtained from the patients upon samples collection.

Cell line culture, transfection

NCI-H446, NCI-H209, NCI-H345 and normal bronchial epithelial cell line 16HBE were purchased from the Chinese Academy of Sciences (Shanghai, China). At 37°C with 5% CO_2, the cells were cultured in DMEM (Dulbecco's modified Eagle medium, Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco) . The four cell lines were used to detect the expression of miR-20a-5p and NCI-H446 was conducted for further functional analysis.

The miR-20a-5p unrelated mimic control and mimic were obtained from Genechem. Lipofectamine 2000 (Invitrogen) transfection was conducted as previously described[21]. Twenty-four hours after transfection, the cells were harvested and subjected to further analysis.

RNA extraction and qRT-PCR

Both cultured cells and FFPE specimens were isolated from TRIzol using a polyethylene glycol (PEG) solution precipitation method[22] and were labeled according to the method described by Thomson and colleagues[23]. We use the Taqman microRNA assay kit (Applied Biosystems, CA, USA) to determine the expression of miR-20a-5p and set U6B be the endogenous control. QRT-PCR was performed using SYBR Premix Ex Taw (Takara, Dalian, China). ß-actin was set as the endogenous control. PCR amplification of CCNG2 gene was conducted using the following set of primers: forward: 5'-CTTTGGGCATTATTAGGA-3'; and reverse: 5'-GAG GAGGAAACAGTAGCAG-3'; ß-actin upstream primer: AGCACAATGAAGATCAAGATCAT; ß-actin downstream primer: ACTCGTCATACTCCTGCTTGC. The relative miR-20a-5p and mRNA expression was determined using the $2^{-\Delta\Delta}$CT method.

Cell proliferation assay

To investigate the growth rate, 3×10^3NCI-H446 cells were seeded into 96-well plates 24 h after transfection. Using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc. Japan) and incubating by 10 µL CCK-8 solution after 2 h, then measured by a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at an absorbance of 450 nM in triplicate independently.

Colony formation assay

With 0.25% trypsin plus 0.5 mM ethylenediaminetetraacetic acid solution at 37°C for 3 min 500 cells (miR-20a-5p mimic or unrelated mimic control, NCI-H446 cells) were seeded in 6-well plates and cultured for two weeks, separately. Cell colonies were then photographed and counted using an AID iSpot Reader (Autoimmun Diagnostika GmbH, Strassberg, Germany). Assays were independently conducted three times.

Migration and invasion assay

For the migration assay, 2×10^5 untreated or transfected cells were suspended in 200 µL serum free medium, then placed into the upper chamber with 8-µM pore of a Transwell filter (Costar, Cambridge, MA, USA). And 500 µL of 10% FBS-DMEM was used as a chemoattractant in the lower chamber. After incubation at 37°C, 5% CO_2 for 24 h, cells that had migrated to the bottom chamber were stained with crystal violet for 30 min at 37°C and counted under a light microscope in five random fields per chamber. For the invasion assay, BD coated invasion system was used to measure the invasion ability. All groups of cells were incubated for 48 h, with the remaining experimental procedures same to the migration experiments which were performed in triplicate.

Xenograft studies

Female, 4-5 weeks old, nude athymic BALB/c mice were purchased from Vital River Laboratory (Beijing, China). 1×10^7 cells (miR-20a-5p mimic or unrelated mimic control, NCI-H446 cells) were

washed twice with antibiotic-free and re suspended by serum-free 1640 in 0.2 mL, then injected subcutaneously into the left flanks of the mice. After 4 weeks, mice were sacrificed and tumor was weighed. Once a week, tumor diameters were measured using a digital caliper and tumor volume=(length×width2)/2. All the mice were sacrificed on the 28th day post-injection and the xenografts tissues were collected.

Target prediction

We used TargetScan[24], microRNA[25] and miRDB[26] to predict the candidate targets of miR-20a-5p and selected the gene which was simultaneously predicted by these three algorithms for further analysis.

Western blot analysis

Cell lysis, protein extraction and Western blot analysis were performed according to standard protocols. For primary antibodies, a dilution of 1:1000 was used for anti-CCNG2 (cat. no. ab251826; Abcam) and anti-β-actin antibody (cat. no. ab16039; Abcam), while a dilution of 1:2000 was used for goat anti-rabbit secondary antibodies (cat. no. ab150077; Abcam). β-actin was set as the internal control.

Dual luciferase reporter assay

A fragment of the CCNG2 3′-UTR containing either the predicted putative binding site or a mutant 3'-UTR was cloned into the psiCHECK-2 vector (Promega Corporation). Using a Lipofectamine 2000, the plasmids were used for transfecting NCI-H446 cells with or without synthetic miR-20a-5p mimic. After 48 h, cells were harvested and luciferase activity was assessed using the Dual-Luciferase® Reporter Assay system (Promega, Madison, USA). Firefly luciferase activity was normalized using renilla luciferase activity.

Statistical analysis

All data are expressed as the mean ± standard deviation. Association of miR-20a-5p expression and clinical character of the specimens were accessed by $\chi 2$ test. The Kaplan-Meier method was used to analyze the survival curves. The joint effect of covariables was examined by the Cox proportional hazard regression model. Pair-wise comparisons were conducted by t-test, while differences among multiple groups were performed using one-way analysis of variance (ANOVA). All statistical tests were done with SPSS 16.0 (SPSS Inc., Chicago, IL USA).

Results

Expression of miR-20a-5p is upregulated in SCLC tissues and cell lines

Compared to the normal tissues, the expression of miR-20a-5p was significantly up regulated in human SCLC by qRT-PCR (P <0.05, Fig. 1A). The three candidate SCLC cells lines exhibited higher expression level than the normal control16HBE cell line (P <0.05, Fig. 1B).

Clinicopathologic characteristics and prognosis of SCLC patients

The clinicopathologic characteristics of 36 patients are summarized in Table 1. Poor survival related with high expression of miR-20a-5p in SCLC patients by Kaplan–Meier analysis (P = 0.003, Fig. 2). Furthermore, univariate Cox analysis with miR-20a-5p expression level and clinicopathologic factors (smoking, age, gender, ECOG performance status) showed that the miR-20a-5p expression level had prognostic significance (P = 0.007; risk ratio: 4.043; 95% CI, 1.473–11.099; Table 2).

High miR-20a-5p expression was a significantly unfavourable prognostic factor independent of all

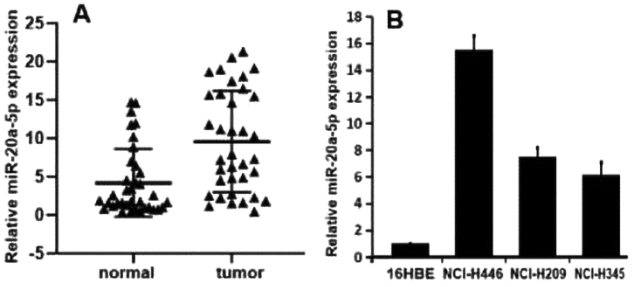

Fig. 1 — Kaplan–Meier analyses of OS according to the miR-20a-5p expression in FFEP specimens of 36 SCLC cases assessed by qRT-PCR.（P = 0.03）

Table 1 — Clinicopathologic features of patients

Clinicopathologic features	Number	Percentage
Age		
≥60 yr	19	53%
<60 yr	17	47%
Gender		
Male	26	72%
Female	10	28%
Smoking		
Smoking	26	72%
No	10	28%
ECOG performance status		
0	22	61%
1	14	39%
TNM classification：		
$T_1N_0M_0$	21	58%
$T_2N_0M_0$	15	42%

other clinicopathologic factors by multivariate Cox proportional hazard regression analysis (P = 0.008; risk ratio: 4.3362; 95% CI, 1.47-12.745; Table 2). The results revealed that poor survival of Chinese SCLC patients correlated strongly with high levels miR-20a-5p expression.

MiR-20a-5p induced proliferation and tumorigenicity of NCI-H446 cells both in vitro and in vivo

We chose the NCI-H446 cell to investigate the biologic functions. MiR-20a-5p expression level was significantly increased after transfected with miR-20a-5p mimic (termed Mimic) compared with those following transfection with unrelated mimic control cells (termed miR-NC) and parental cells (termed WT) 24 h later. (P <0.05, Fig. 3A). CCK-8 assay showed that the cells of Mimic group grew faster at 24, 48, 72, 96 and 120 h compared with the cells of miR-NC and WT groups (P = 0.02, Fig. 3B). We next compared the colonies form in soft agar as an additional evaluation of tumorigenicity *in vitro* and found that miR-20a-5p overexpression could increase both colony number and size compared with the miR-NC and WT (P = 0.036, Fig. 3 C &D). Furthermore, we investigated the affects of miR-20a-5p high expression on tumor growth in the nude mice model and found that the treatment with miR-20a-5p mimics significantly promote the growth of NCI-H446 xenografts (P <0.001, Fig. 4).

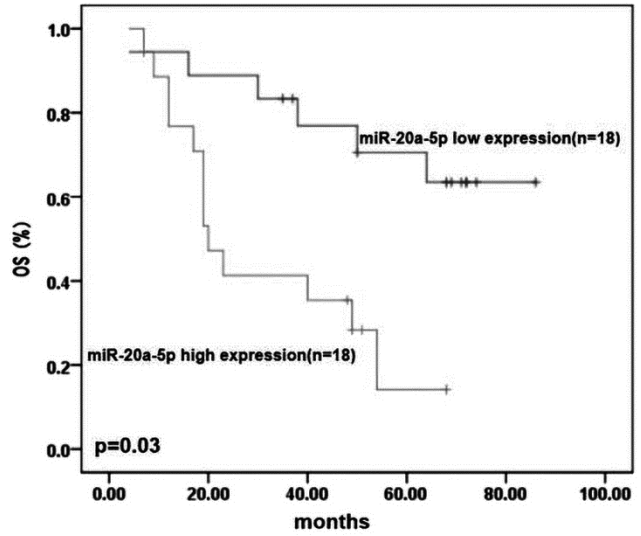

Fig. 2 — Expression of miR-20a-5p in SCLC tissues and cell lines (A) The miRNA expression of miR-20a-5p was assessed by qRT-PCR using 36 SCLC tissue samples and matched adjacent non-tumor normal tissues; and (B) Average relative expression of miR-20a-5p in three SCLC cell lines: NCI-H446, NCI-H209, NCI-H345 compared with normal control 16HBE cells. [P <0.05. miR-20a-5p, microRNA-20a-5p; SCLC, small cell lung cancer]

Overexpression miR-20a-5p promotes NCI-H446 cells invasion and migration

Cell capacity of invasion and migration was marked following transfection with mimics compared with that following transfection with miR-NC and WT, as shown in Fig. 5, P=0.026 and P=0.049, respectively.

MiR-20a-5p negatively regulated CCNG2

Three miRNA target prediction programs (TargetScan, microRNA and miRDB) were indicated that miR-20a-5p targets CCNG2 3′UTR (Fig. 6A). To identify the effect of miR-20a5p on the expression of CCNG2, a luciferase reporter vector containing the CCNG2 3'-UTR or the mutants was generated. As Fig. 6C showed, a significantly reduction in luciferase activity that was found in cells transfected with Mimic compared with miR-NC (P <0.05), whereas mutation of the miR-20a-5p binding sites reversed this reduction in the NCI-H446 cells. Furthermore, we observed miR-20a-5p decreased CCNG2 expression at protein and mRNA level (P=0.024) (Fig. 6 B &D). All of these results suggested that miR-20a-5p was a negative regulator of CCNG2 in SCLC.

Discussion

Small cell lung cancer (SCLC) is well known for its rapid progression and high 2-year recurrence rate,[27]. Earlier studies have shown that miRNA is one of the key factors leading to the invasive phenotype of

Table 2 — Postoperative survival of patients in relation to clinicopathological characteristics and miR-20a expression analyzed by Cox proportional hazard regression model in 36 cases

Clinicopathological characteristics		Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Smoking	+/-	0.593 (0.237-1.481)	0.263	0.349 (0.110-1.104)	0.101
Age	≥60	0.944 (0.380-2.345)	0.902	0.558 (0.215-1.444)	0.251
Sex	M/F	1.067 (0.382-2.980)	0.902	2.586 (0.740-9.031)	0.131
ECOG performance status	0/1	2.232 (0.878-5.674)	0.092	2.956 (0.977-8.943)	0.401
T stage	T1/T2	2.321 (0.866-6.222)	0.094	2.051（0.574-7.697）	0.287
miR-20a	High/Low	4.043 (1.473-11.099)	0.007	5.043 (1.646-15.454)	0.005

[CI, confidence interval]

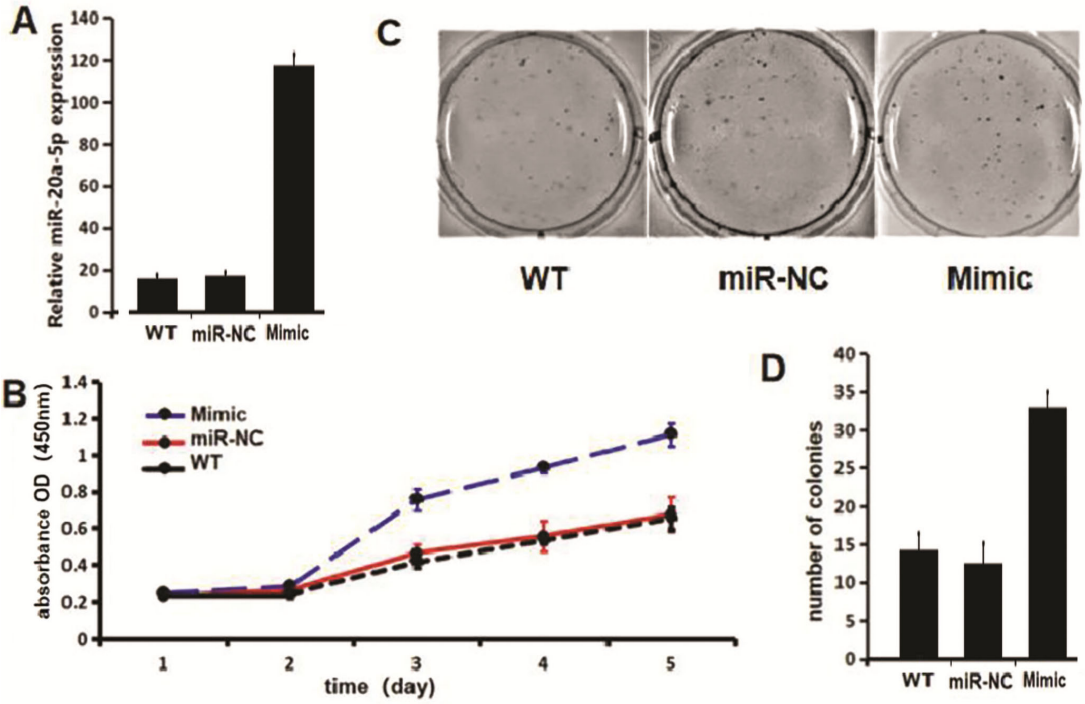

Fig. 3 — Effect of miR-20a-5p on cell growth and proliferation *in vitro* and on tumorigenicity *in vivo* of NCI-H446. (A) Transient transfection with specific mimics significantly upregulated the expression of miR-20a-5p (P <0.05); (B) Growth curves of miR-20a-5p high expression and miR-NC WT by Cell Counting Kit-8 assay. The data at each time point were derived from three independent experiments and the error bars represent standard deviations. The growth rates were significantly higher in overexpression of miR-20a-5p than the miR-NC and WT (P = 0.02); (C) The size of the colonies was formed by miR-20a-5p transfectants; and (D) Colony formation assay, showing overexpression of miR-20a-5p leading to an increase in colony formation, P = 0.036.

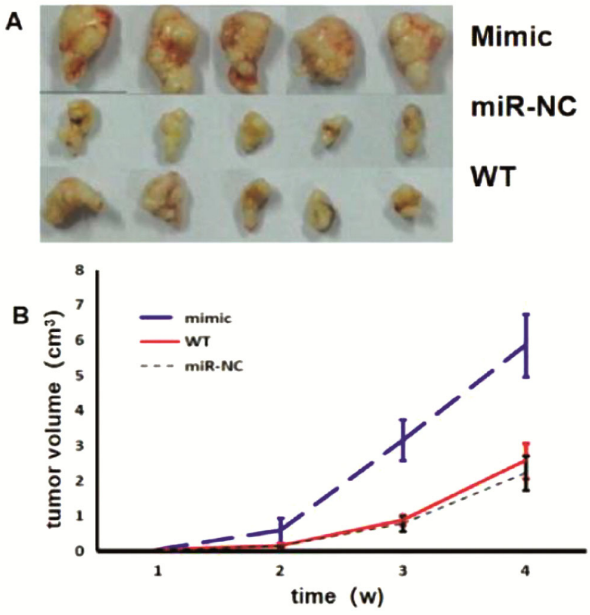

Fig. 4 — (A)The size of the tumors formed by miR-20a-5p transfectants; and (B) Results represent the mean size of tumors of five mice in each group. [The tumors generated by transfectants with higher miR-20a-5p expression were significantly larger than those of control transfectants and WT, mean volume, P <0.001 at all time points after 4 weeks]

SCLC[28,29]. MiR-20a-5p has been found that it can inhibit tumorigenesis or promote tumor development, depending on the environment of different cancers. In some human tumors, miR-20a-5p is expressed high and deregulates important cancer related genes[15,16], whereas, in a subset of human tumors, mir-20a-5p inhibits proliferation paradoxically[17,18]. Although more and more evidence shows that mir-20a-5p plays a function as oncomiR, the carcinogenic role of mir-20a-5p in SCLC has not been fully elucidated.

The occurrence of tumors is a process of long-term, complex and multistage accumulation. It is well known that cell migration and the proliferation states of SCLC cells perform core functions in the SCLC progression. Since miR-20a-5p is up-regulated in tissue samples of SCLC, it is deduced that miR-20a-5p may have carcinogenic effect in SCLC. The survival rate of patients with high mir-20a-5p expression level in tumors is low, denoting that high miR-20a level is a marker of poor prognosis in SCLC patients.

Although overexpression of miR-20a-5p has been demonstrated, a direct carcinogenic and metastasis

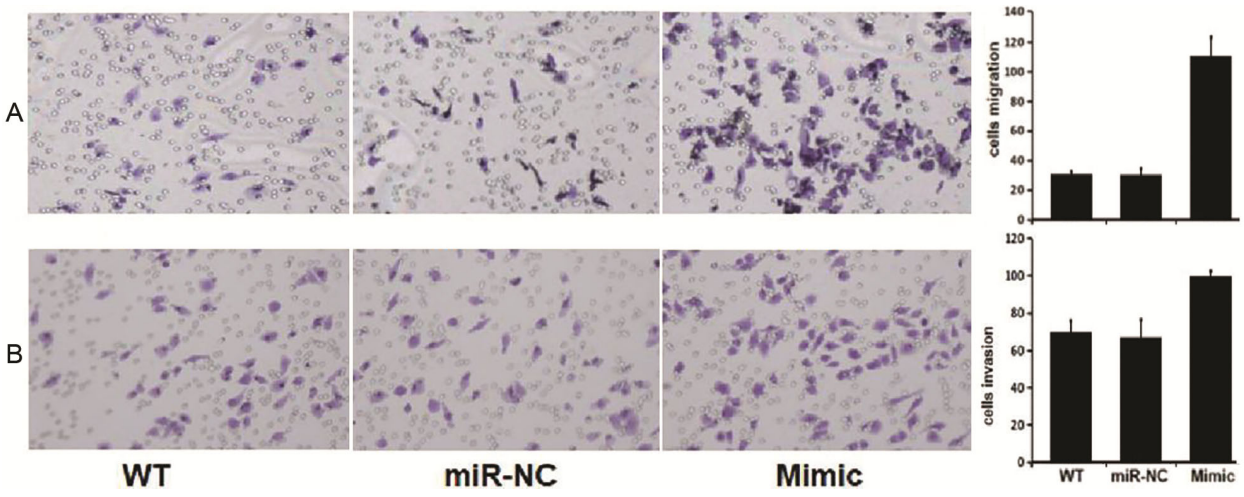

Fig. 5 — Modulation of migration and invasion by miR-20a-5p high expression in SCLC cells. (A) Increased cell migration of miR-20a-5p high expression. Columns, mean calculated from three independent experiments; p =0.026; and (B) Increased cell invasion of miR-20a-5p high expression. [Columns, mean calculated from three independent experiments; $P = 0.049$]

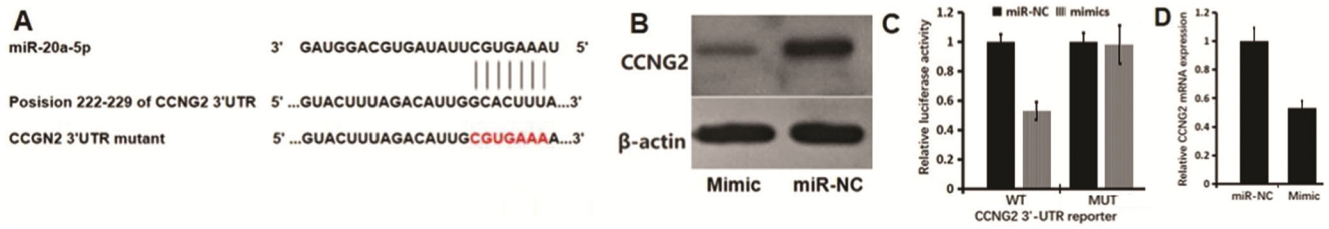

Fig.6 — Identification of CCNG2 as a target of miR-20a-5p. (A) Wild-type and mutated sequences of the CCNG2 3'-UTR (nucleotides 222-229); (B) Western blot analysis was used to detect CCNG2 protein level in cells transfected with miR-20a-5p mimic or unrelated mimic control; (C) Luciferase activity was detected once NCI-H446 cells had been co-transfected with unrelated mimic control or miR-20a-5p mimic with CCNG2 3'-UTR fragment containing either the miR-20a-5p target sequence (WT) or a mutant; and (D) qRT-PCR was used to detect expression of CCNG2 mRNA in cells transfected with miR-20a-5p mimic or the corresponding control. [B-actin was used as an internal control. $P = 0.024$ compared with control samples]

role for miR-20a-5p in SCLC was not clear. The oncomiR function of the miR-20a-5p was further confirmed by experiments that high expression of miR-20a-5p by Mimic transfection promot more colonies and produce larger tumors in nude mice. These results first showed that miR-20a-5p high expression influenced not only the growth of tumor cells but also oncogenic phenotypes of SCLC cells both *in vitro* and *in vivo*.

Postoperative distant metastasis is one of the poor prognostic factors of SCLC. Cells lose these epithelial characteristics and gain development, wound repair, angiogenesis and invasion, which permit these cells, to enter into the systemic circulation for metastasis and proliferation[30]. Our studies demonstrated miR-20a-5p high expression in NCI-H446 cells promoted significantly cell migration and invasion. Consistent with the findings, in colorectal cancer[31], non-small cell lung cancr[32] and cervical cancer[33], miR-20a-5p also play a crucial role in metastasis.

Some signal pathways or genes have been discovered to be targeted by miR-20a-5p in tumor biological character, such as ABL2 in prostate cancer[34], LIMK1 in anaplastic thyroid cancer[35], RB1CC1/FIP200 in breast cancer[36], KIF26B in osteosarcoma[37]. Therefore, it is imperative to study the target of miR-20a-5p in SCLC to determine its underlined molecular mechanism in the progression of SCLC. We searched three miRNA target prediction programs and produced luciferase reporter vectors and found miR-20a-5p repressed CCNG2 expression not only in luciferase reporter assay but also in Western blot and qRT-PCR in NCI-H446 cells. These results indicate that CCNG2 play a significant role on the SCLC progression mediated by miR-20a-5p.

Conclusion

In this present study, we demonstrated the expression of miR-20a-5p which was upregulated in human SCLC compared to that in normal tissues significantly. Dual-luciferase reporter gene assay showed that miR-20a-5p targets CCNG2 directly. Kaplan-Meier analysis indicated that patients with high expression of miR-20a-5p are closely related with poor survival of SCLC and multivariate analysis showed that miR-20a-5p was an independent prognostic factor. Increasing miR-20a-5p expression promotes the proliferation, invasion and migration of the NCI-H446 cells both *in vitro* and *in vivo*. Furthermore, we demonstrated that miR-20a-5p directly targets CCNG2 by dual-luciferase reporter gene assay. These results suggest that miR-20a-5p might play important roles in the development and progression of SCLC. Inhibiting miR-20a-5p could be a promising therapeutic strategy for SCLC therapy.

Conflict of interest

Authors declare no competing interests.

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