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).

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.

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]

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]

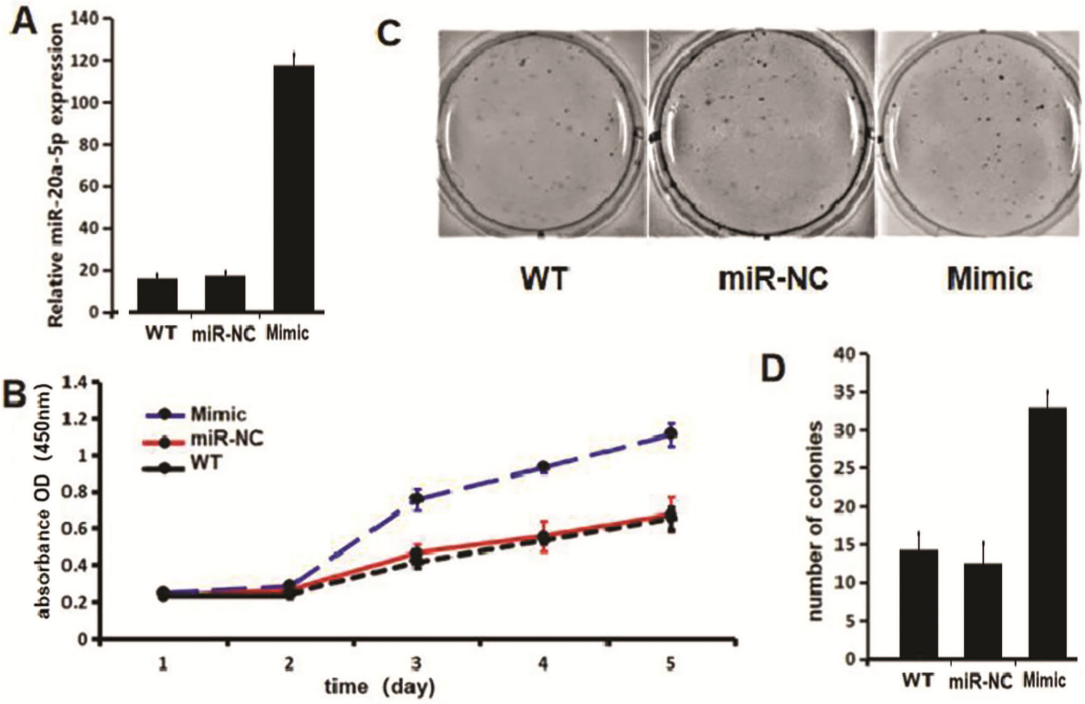

Fig. 3 — Effect of miR-20a-5p on cell growth and proliferation *in vitro* and on tumorigenicity *in vivo* of NCI-H446. (A) Transient transfection with specific mimics significantly upregulated the expression of miR-20a-5p (P <0.05); (B) Growth curves of miR-20a-5p high expression and miR-NC WT by Cell 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.

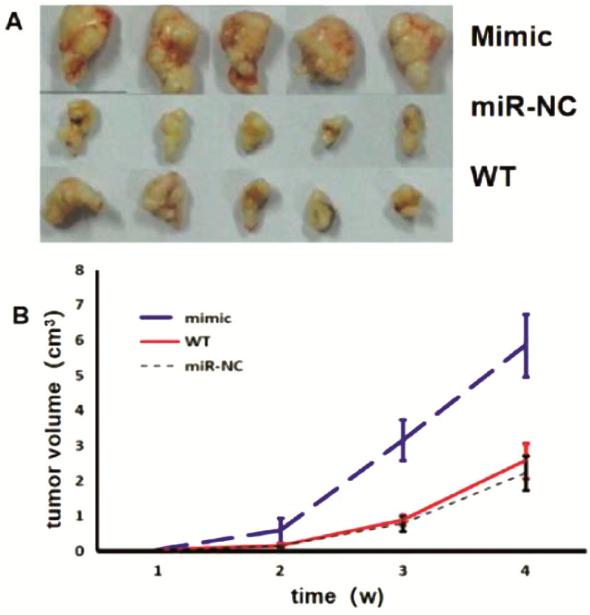

Fig. 4 — (A)The size of the tumors formed by miR-20a-5p transfectants; and (B) Results represent the mean size of tumors of five mice in each group. [The tumors generated by transfectants with higher miR-20a-5p expression were significantly larger than those of control transfectants and WT, mean volume,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

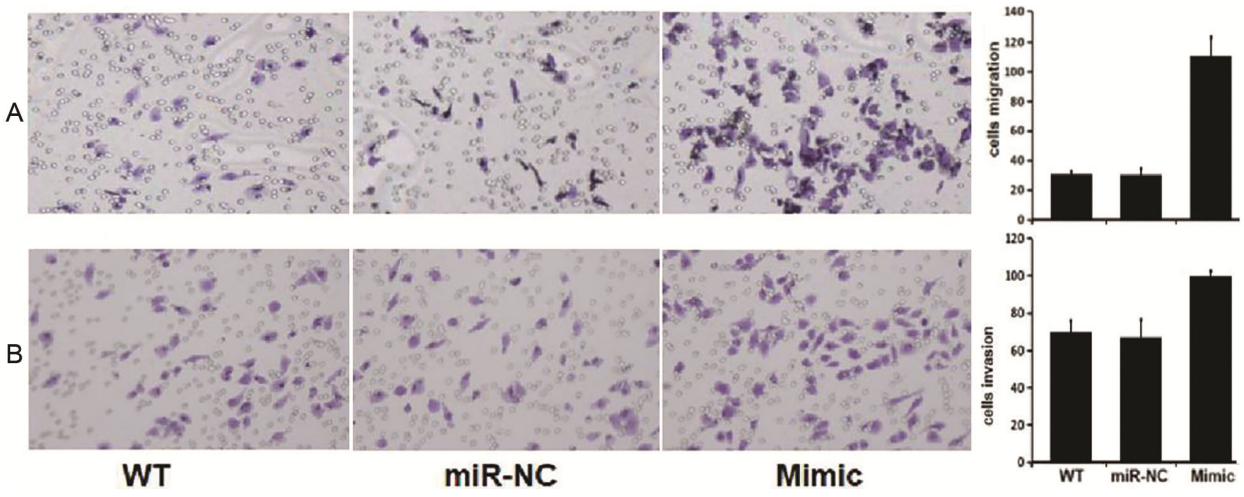

Fig. 5 — Modulation of migration and invasion by miR-20a-5p high expression in SCLC cells. (A) Increased cell migration of miR-20a-5p high expression. Columns, mean calculated from three independent experiments; p =0.026; and (B) Increased cell invasion of miR-20a-5p high expression. [Columns, mean calculated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

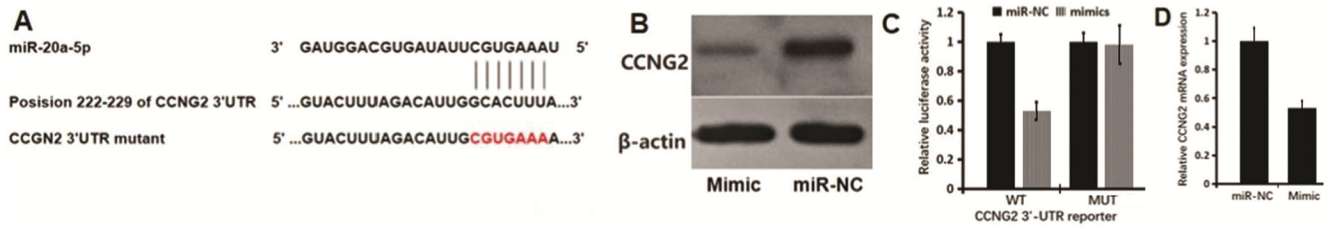

Fig.6 — Identification of CCNG2 as a target of miR-20a-5p. (A) Wild-type and mutated sequences of the CCNG2 3'-UTR (nucleotides 222-229); (B) Western blot analysis was used to detect CCNG2 protein level in cells transfected with miR-20a-5p mimic or unrelated mimic control; (C) Luciferase activity was 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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