MicroRNA 141 represses nasopharyngeal carcinoma growth through inhibiting BMI1

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Abstract. Distant metastasis is the primary cause of mortality in patients with nasopharyngeal carcinoma (NPC). Unveiling the mechanism of metastasis will aid in shedding light on the clinical therapeutic strategies in NPC treatment. The present study revealed that the expression of microRNA 141 (miR-141) was downregulated in NPC tumor cells, particularly in metastatic ones. Ectopic expression of miR-141 blocked the proliferative and invasive ability of the tumor cells *in vitro*, and inhibited NPC tumor growth *in vivo*. Mechanistic studies identified that BMI1 served as the direct target of miR-141, and overexpression of BMI1 reversed the tumor repressor effect of miR-141. Prognostic analysis revealed that this miR-141/BMI1 signaling axis correlated with the clinical stage of patients with NPC. The study of miR-141 provided novel insight into the mechanism of NPC progression, which was correlated with the stage and metastatic state of patients. This miR-141/BMI1 axis may serve as a novel pharmacological target in NPC treatment.

Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck cancer with a relatively high rate of malignancy. The tumor cells in NPC are derived from nasopharynx epithelium, and invade the surrounding tissues with the tumor growth. Facilitated by the progress of radiotherapy, the curative effect on NPC has increased. However, there are certain patients with advanced stages of disease with a poor prognosis, owing to recurrence or distant metastasis (1,2). Therefore, understanding the molecular mechanisms governing NPC tumor cells proliferation and metastasis will aid in identifying novel and more effective therapeutic methods.

MicroRNA (miRNA), which is widely expressed in numerous organisms, is a small non-coding RNA that is 19-25 nt in length. Usually, miRNA takes part in the post-transcriptional regulation of gene expression through base-pairing 3'-untranslated region (UTR) of the target mRNAs (3-5). The important regulatory role of miRNAs during biological processes have been revealed in recent decades. Recent studies also demonstrated that certain miRNAs were aberrantly expressed in different types of cancer, and served critical roles in carcinogenesis (6-8), indicating that miRNAs can serve clinically as an important indicator of cancer developmental stage. Furthermore, manipulating miRNA expression was an effective therapeutic strategy for cancer treatment (9-12). miR-141 was first identified to function in prostate cancer (13). Subsequent studies also revealed that it served critical roles in gastric and ovarian cancer (14,15). However, no previous studies has focused on miR-141 in NPC.

The present study investigated the expression and function of miR-141 in NPC, and revealed its significance in early diagnosis and clinical treatment. It was demonstrated that miR-141 was a tumor repressor and was downregulated in the NPC biopsy samples. Functional studies revealed that ectopic expression of miR-141 inhibited NPC tumor cell proliferation and invasion. Further analysis revealed that BMI1 served as the direct target of miR-141. This miR-141/BMI1 signaling cascade provided a novel therapeutic strategy for NPC treatment.

Materials and methods

Ethics statement. The Research Ethics Committee of the Nanshan Affiliated Hospital of Guangdong Medical College provided ethical approval for the present study, and all patients provided written informed consent. All specimens were handled and stored anonymously according to ethical and legal standards.

Patients and specimens. Tumor samples were obtained from patients (average age of 51 years, ranging from 22 to 74 years

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Abbreviations: NPC, nasopharyngeal carcinoma; miRNA, microRNA

Key words: nasopharyngeal carcinoma, microRNA-141, BMI1, metastasis

old, consisting of 38 males and 13 females) with pathologically confirmed NPC (metastatic, n=10; non-metastatic, n=41) or nasopharyngeal mucosa chronic inflammation (n=4) at the Department of Otorhinolaryngology, Nanshan Affiliated Hospital of Guangdong Medical College between June 2015 and August 2016. No patient had undergone any antitumor therapy prior to sampling. All patients were staged according to the 7th edition of the American Joint Committee on Cancer Staging Manual (16).

Cell lines. The human immortalized nasopharyngeal epithelial NP69 cell line was maintained in Keratinocyte/serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with bovine pituitary extract (BD Biosciences, Franklin Lakes, NJ, USA). Human NPC 6-10B, C666, 5-8F and SUNE1 cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). For transfection, the cells were seeded into 6-well plates the day before transfection using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). 150 pM (pmol/l) miR-ctrl (5'-GGUCUGGGUAGAUCACAAUCU AC-3') or miR-141 oligo mimics (5'-UAACACUGUCUG GUAAAGAUGG-3') was transfected per well. BMI1 coding sequence was cloned into pcDNA3.1 plasmid. 2.5 μg plasmids were transfected in each well. Approximately 36 h later, the cells were harvested for the subsequent experiments.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (qPCR). Total RNA was isolated from NPC cell lines using an EasyPure RNA kit (Beijing Transgen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed at 42˚C for 1 h using M-MLV reverse transcriptase reagents (Promega Corporation, Madison, WI, USA). For the qPCR assay, complementary DNA (cDNA) was PCR-amplified using a GoTaq qPCR Master mix (Promega Corporation) in the Light Cycler 480 II PCR system (Roche Diagnostics GmbH, Basel, Switzerland). GAPDH was used as the internal control. The sense and anti-sense primers of miR-141 were 5'-TGGGTCCATCTTCCAGTA-3' and 5'-GGGAGCCATCTT TACCAG-3', respectively. The GAPDH sense and anti-sense primers were 5'-GAAGGTGAAGGTCGGAGT-3' and 5'-GAA GATGGTGATGGGATTTC-3', respectively. The thermocycling condition of qPCR: initial denaturation 95˚C 30 sec, followed by 45 cycles: 95˚C 15 sec, 65˚C 1 min. Relative gene expression was presented as the comparative threshold cycle ($2^{-\Delta\Delta Cq}$) values (17) and was representative of at least three independent experiments.

Western blotting. Protein was extracted using a protein extraction kit (KGP250-2100; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. After examining the concentration by BCA method, 10 μg protein from samples were treated with Dual Color Protein Loading Buffer (Thermo Fisher Scientific, Inc.) containing reducing agent at 100˚C for 5 min respectively, resolved on 10% Tris-HCl polyacrylamide gels, and transferred to a polyvinylidene fluoride membrane. Then the membrane was blocked by 5% skim milk for 1 h at room temperature. Overnight incubation (4˚C) with primary antibodies against the following: BMI1 (dilution, 1:1,000; catalog no. ab14389; Abcam, Cambridge, UK) and GAPDH (dilution, 1:2,000; catalog no. ab8245; Abcam) was followed by incubation (37˚C) with horseradish peroxidase (HRP)-conjugated anti-rabbit (dilution, 1:1,000; catalog no. NBP2-30348H; Novus Biologicals, LLC, Littleton, CO, USA) or anti-mouse (dilution, 1:1,000; catalog no. NBP2-30347H; Novus Biologicals) secondary antibodies. Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) and a Tanon 5200 Luminescent Imaging Workstation (Tanon Science & Technology Co., Ltd., Shanghai, China) were subsequently used.

Mice tumor model. Mice are raised in individually ventilated cages (IVF) in the mouse room with temperature of 18-23˚C and 40-60% humidity. A 12 light/12 dark cycle is used. Water and food are accessible at all times. 10 BALB/c nude male mice (Biocytogen, Worcester, MA, USA) aged 4 weeks (18-22 g body weight) were used for the tumor growth experiments. SUNE1 cells transfected with miR-141 or scrambled control were resuspended in PBS, and $1x10^6$ cells were subcutaneously injected into the dorsal flank of the nude mice. Mice were observed and the tumor sizes were measured every two days. A total of 3 weeks later, the mice were sacrificed and the tumors were dissected and compared. For H&E staining, the tumor tissues were fixed by 4% paraformaldehyde at 4˚C overnight, and embedded in paraffin. Next, the tissues were sectioned into 5-μm tissues for H&E staining. For metastasis analysis, $2x10^6$ SUNE1 cells diluted in 200 μl PBS were injected into 10 nude mice via the tail vein. Mice were observed every two days. A total of 8 weeks later, mice were sacrificed and their lungs were dissected. The general humane endpoints for mice that required euthanasia are as follows: 20% decrease in normal body weight, the longest diameter of the tumor exceeded 20 mm, the inability to reach food or water for more that 24 h. All mice were euthanized by carbon dioxide with 10-30% chamber volume per minute. Animal feeding and research procedures were approved by the Animal Care and Use Ethic Committee of Nanshan Affiliated Hospital of Guangdong Medical College (Shenzhen, China).

Luciferase reporter assay. The wild-type and mutated 3'-UTR of miR-141 were cloned into firefly luciferase-expressing vector psiCHECK™ (Promega Corporation). For the luciferase reporter assay, NPC SUNE1 and 5-8F cells were co-transfected with miR-141 and BMI1 wt or mutated 3'-UTR reporter vectors by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). 36 h later, the luciferase activity was examined using a Dual-Luciferase® Reporter assay system (catalog no. E1910; Promega Corporation), and the reporter activity were normalized by comparing with *Renilla* luciferase activity.

MTT and colony formation assay. For the MTT assay, NPC SUNE1 and 5-8F cells transfected with miR-141 or scrambled control were counted and 1,500 of them were seeded into 96-well plates. DMSO was used to dissolve the formazan in each well. From day 1 to day 5, the absorbance of the cells was determined at 490 nm using a spectrophotometric plate

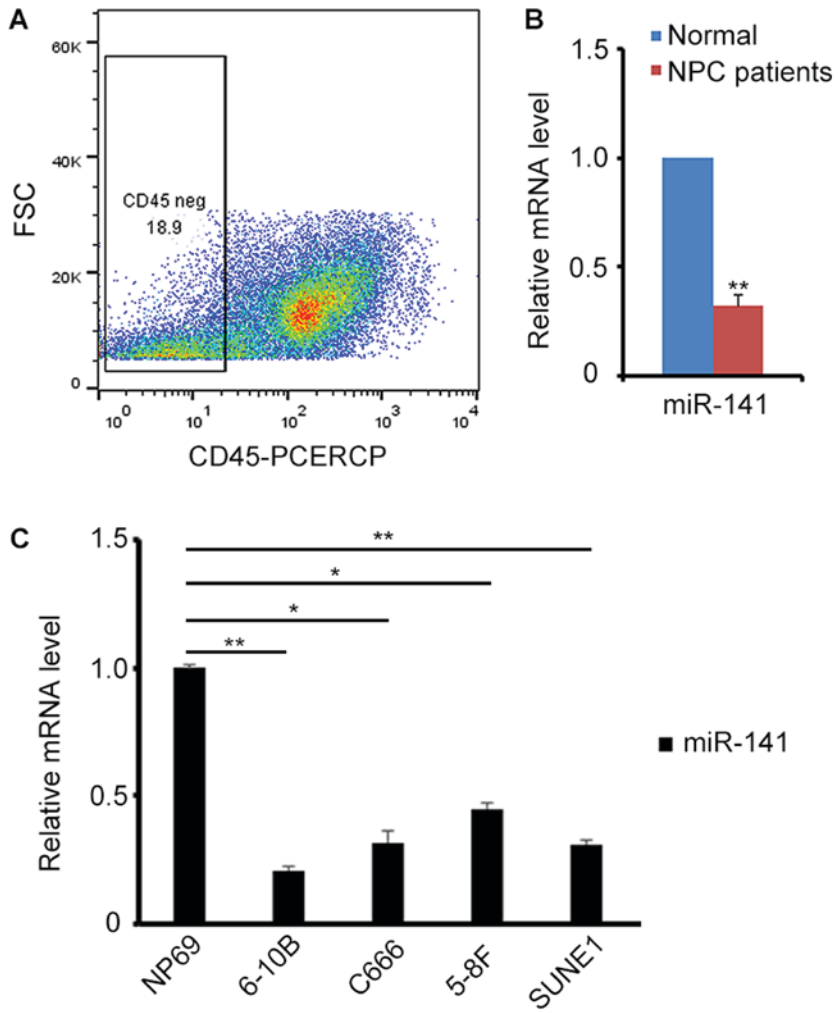

Figure 1. The expression of miR-141 is downregulated in NPC cells *in vivo* and *in vitro*. (A) FACS-sorted CD45- cells from NPC biopsy samples. (B) Expression of miR-141 in sorted NPC cells. (C) Quantitative polymerase chain reaction detection of miR-141 expression in NPC cell lines. Data are presented as the mean ± standard deviation. P-values were calculated relative to NP69 by analysis of variance with Bonferroni's correction. *P<0.05, **P<0.01. miR, microRNA; NPC, nasopharyngeal carcinoma; CD, cluster of differentiation.

reader. For the colony formation assay, 500 cells transfected with miR-141 or scrambled control were seeded into 6-well plates. The colonies were observed and counted 5-7 days later.

Cell invasion assay. A total of $5x10^4$ 5-8F or SUNE1 cells following transfection with miR-141 or scrambled control were resuspended in serum-free medium (DMEM, Thermo Fisher Scientific, Inc.). The Transwell chambers (Corning Incorporated, Corning, NY, USA) were placed on the upper surface of the 24-well plate, and the cells were seeded into the upper chamber. The medium supplemented with 10% FBS was placed in the lower chambers. After 10-16 h of culture, the chambers were collected and the cells on the lower surface of the chambers were fixed by absolute methanol for 20 min at room temperature and stained by 0.1% crystal violet for 30 min at room temperature. The cells on the chambers were captured at x100 magnification by an inverted microscope.

Statistical analysis. All data are presented as the mean ± standard deviation. χ^2 test, Fisher's exact test, analysis of variance with Bonferroni's correction and Student's t-test were used for comparisons between groups. Spearman's correlation analysis was used to examine the correlation between gene expression and disease staging. The prognostic factors were examined by univariate and multivariate analyses using the Cox proportional hazards model. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

miR-141 serves as a tumor repressor in NPC. To begin with, the endogenous expression of miR-141 in NPC biopsy samples was detected. To exclude the interference of lymphocytes in these samples, the $CD45^-$ cells were sorted for the subsequent experiments by flow cytometry (Fig. 1A). A total of 23 patients were examined. Compared with healthy people, the biopsy samples from patients with NPC exhibited relatively low expression of miR-141 (Fig. 1B). Next, endogenous miR-141 expression was detected in different NPC cell lines. Consistent with the results in biopsy samples, miR-141

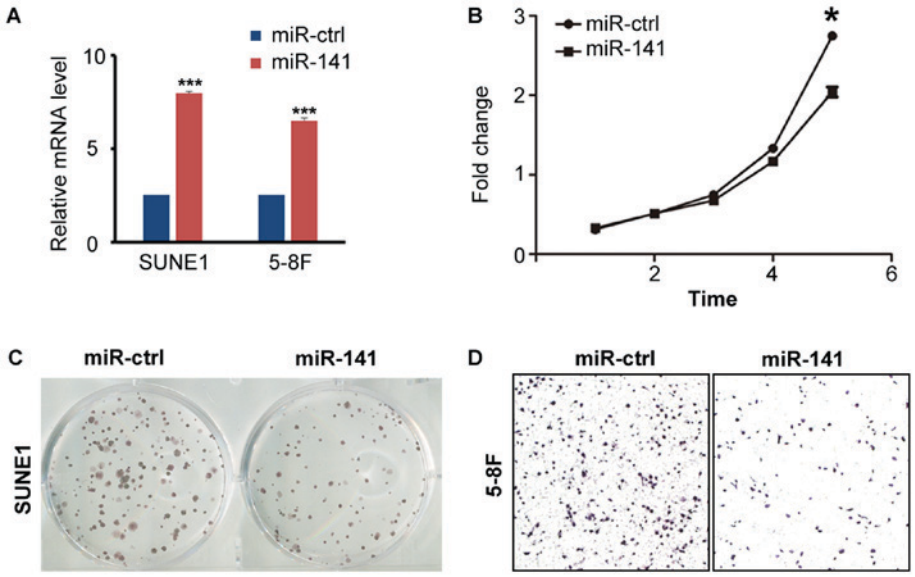

Figure 2. Overexpression of miR-141 inhibits NPC cell proliferation and metastasis. (A) Quantitative polymerase chain reaction detection of miR-141 expression following treatment with miR-141 mimics. (B) MTT assay of 5-8F cells with miR-141 overexpression. P-values were calculated by comparing with control groups at each time point using Student's t-test. (C) Plating assay of SUNE1 cells with miR-141-overexpression. (D) Transwell assay of 5-8F cells with miR-141-overexpression. Data are presented as the mean ± standard deviation. *P<0.05, ***P<0.001 (Student's t-test). miR, microRNA; NPC, nasopharyngeal carcinoma; ctrl, control.

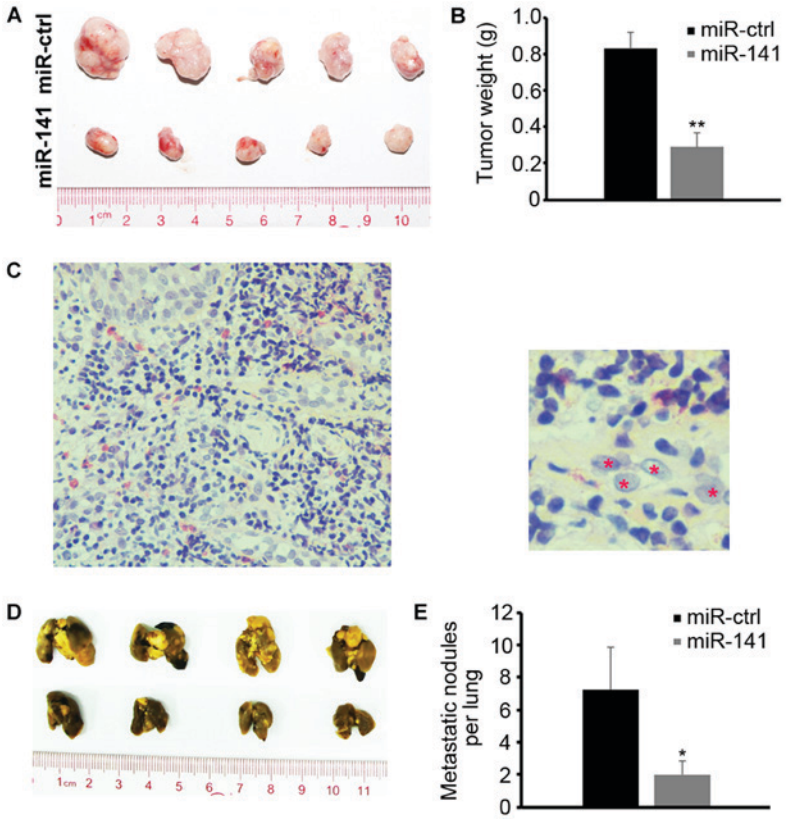

Figure 3. Ectopic expression of miR-141 suppresses NPC tumor growth *in vivo*. (A) SUNE1 cells with ectopic expression of miR-141 were injected into nude mice. A total of 3 weeks later, mice were sacrificed and tumor cells were dissected and observed. (B) The tumor cells were subsequently weighed. Data are presented as the mean ± standard deviation. **P<0.01 (Student's t-test). (C) H&E staining of tumor tissues dissected from mice (magnification, x400). The boxed region shows a higher magnified view of the image. Red asterisks indicate the tumor cells. The lungs from mice treated with (D) miR-ctrl or (E) miR-141, respectively. The metastatic nodules on the lung surface from each mouse were counted. Data are presented as the mean ± standard deviation. *P<0.05 (Student's t-test). miR, microRNA; NPC, nasopharyngeal carcinoma; ctrl, control.

expression was revealed to be decreased in NPC cell lines (Fig. 1C), implying that miR-141 may act as a tumor repressor in NPC.

To examine the role of miR-141 in NPC, the miR-141 oligo-mimics were designed to simulate the ectopic expression of miR-141 *in vitro*. Following transfection of miR-141

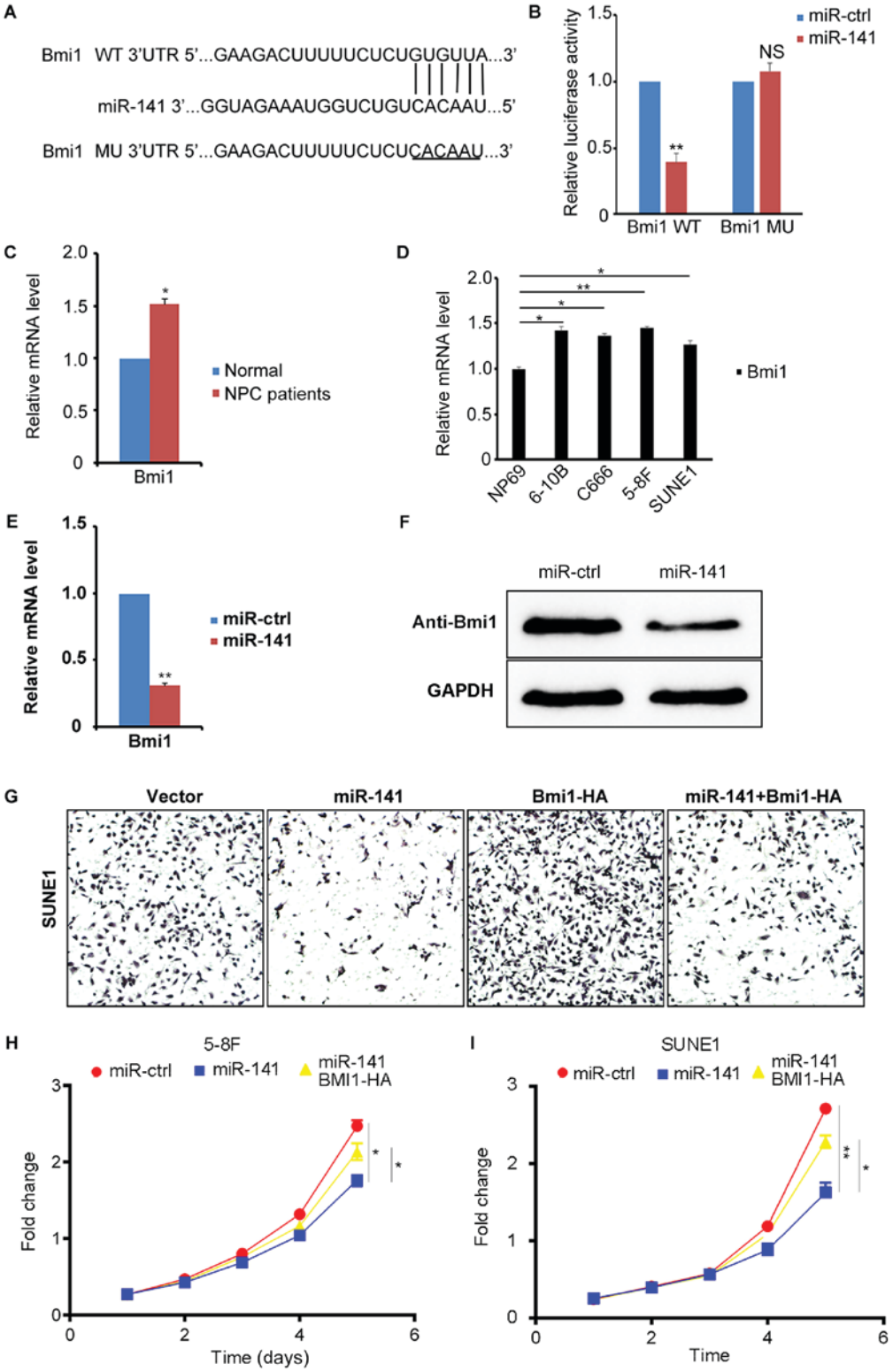

Figure 4. BMI1 served as the direct target of miR-141 in NPC cells. (A) Wild-type or mutant miR-141 target sequence of BMI1 3'-UTR. (B) Relative luciferase activity of SUNE1 cells following co-transfection with wild-type or mutant BMI1 3'-UTR reporter genes and miR-141 mimics (Student's t-test). (C) Expression of BMI1 in NPC biopsy samples (Student's t-test). (D) BMI1 expression in NPC cell lines. P-values were calculated by comparing with NP69 using ANOVA with Bonferroni's correction. (E) Quantitative polymerase chain reaction detection of BMI1 expression in SUNE1 cells with miR-141 overexpression (Student's t-test). (F) BMI1 expression in 5-8F cells with miR-141 overexpression by western blot analysis. (G) Transwell assay of SUNE1 cells with miR-141 or BMI1 overexpression. MTT assay of (H) 5-8F or (I) SUNE1 cells with miR-141-overexpression or combined with BMI1 ectopic expression. P-values were calculated by comparing with the miR-141/BMI1 group at each time point using ANOVA with Bonferroni's correction. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. miR, microRNA; NPC, nasopharyngeal carcinoma; ctrl, control; UTR, untranslated region; ANOVA, analysis of variance.

mimics into NPC SUNE1 and 5-8F cells, increased expression of miR-141 was detected (Fig. 2A). To identify whether miR-141 affects NPC cell viability, MTT and colony formation assays were performed. It was revealed that miR-141

Table I. Correlation between miR-141 expression and clinicopathological characteristics of nasopharyngeal carcinoma.

Characteristic	No. patients	Expression of miR-141		P-value
		Low	High	
Age, years				0.96
≤45	22	10	12	
>45	29	13	16	
Sex				0.67
Male	39	19	20	
Female	12	5	7	
VCA-IgA				0.74
<1:80	11	6	5	
≥1:80	40	24	16	
EA-IgA				0.54
<1:10	14	7	7	
≥1:10	37	15	22	
TNM stage				0.04
I-II	22	10	12	
III-IV	27	20	7	
Metastasis				0.03
Yes	10	9	1	
No	41	22	19	

miR, microRNA; VCA-IgA, viral capsid antigen immunoglobulin A; EA-IgA, early antigen immunoglobulin A; TNM, Tumor-Node-Metastasis. P-values were analyzed by Student's t-test or Fisher's exact test, as appropriate.

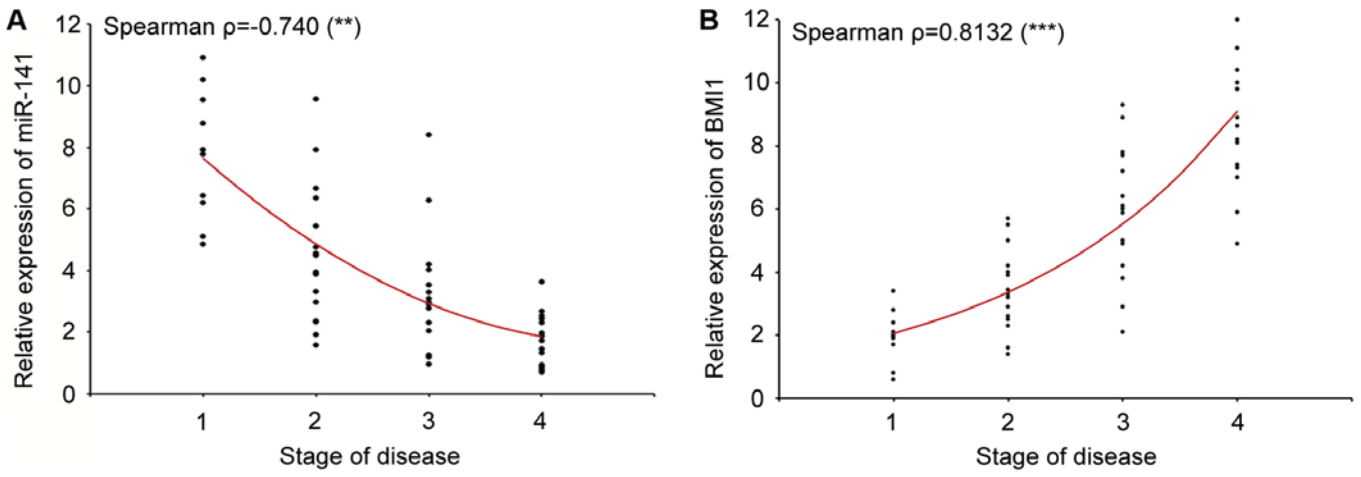

Figure 5. miR-141/BMI1 signaling axis was correlated with the disease stage of patients with NPC. (A) miR-141 expression in patients with different stages of NPC (n=51). (B) Quantitative polymerase chain reaction detection of BMI1 in patients with different stages of NPC (n=51). Spearman's correlation analysis was performed to analyze the correlation between mRNA expression and the disease stage of NPC. **P<0.01, ***P<0.001. miR, microRNA; NPC, nasopharyngeal carcinoma.

overexpression blocked tumor cells proliferation compared with their control counterparts (Fig. 2B). The colony formation assay also revealed that ectopic expression of miR-141 inhibited tumor cell growth (Fig. 2C). To determine whether miR-141 influenced NPC cell invasive ability, a Transwell assay was performed. The results revealed that overexpression of miR-141 decreased the number of invaded tumor cells (Fig. 2D).

Next, it was determined whether ectopic expression of miR-141 affects tumor growth *in vivo*. SUNE1 cells transfected with miR-141 mimics or scrambled control were subcutaneously injected into the dorsal flank of nude mice (n=10). A

Table II. Univariable and multivariable analyses of prognostic parameters in patients with nasopharyngeal carcinoma.

Variable	HR (95% CI)	P-value
Univariate analysis		
Age, ≤45 years/>45 years	1.212 (0.932-2.819)	0.082
Sex, male/female	1.878 (1.241-3.752)	0.241
VCA-IgA, <1:80/≥1:80	2.131 (1.381-3.543)	0.080
EA-IgA, <1:10/≥1:10	1.772 (1.373-2.949)	0.088
TNM stage, I-II/III-IV	2.308 (1.365-3.658)	0.028[a]
miR-141 expression, low/high	0.525 (0.035-1.384)	0.014[a]
Multivariate analysis		
TNM stage, I-II/III-IV	2.015 (1.103-3.223)	0.021[a]
miR-141 expression, low/high	0.423 (0.103-1.186)	0.018[a]

[a]P<0.05. Univariate analysis, Cox proportional hazards regression model; multivariate analysis, Cox proportional hazards regression model. Variables were adopted by univariate analysis. HR, hazard ratio; CI, confidence interval; miR, microRNA; VCA-IgA, viral capsid antigen immunoglobulin A; EA-IgA, early antigen immunoglobulin A; TNM, Tumor-Node-Metastasis.

total of 3 weeks later, the mice were sacrificed and the tumors were dissected. Compared with the control, miR-141-overexpression significantly inhibited tumor growth *in vivo* (Fig. 3A and B). H&E staining confirmed that the dissected tissues were derived from NPC tumor cells (Fig. 3C). To evaluate the inhibitory effect of miR-141 on metastasis *in vivo*, the SUNE1 cells were injected into nude mice via the tail vein. A total of 8 weeks later, the mice were sacrificed and their lungs were dissected. Compared with the control, ectopic expression of miR-141 effectively repressed the metastasis of NPC cells *in vivo* (Fig. 3D and E).

BMI1 functions as the direct target of miR-141 in NPC. To investigate the mechanism of miR-141 in repressing NPC growth, the present study aimed to identify the direct downstream target of miR-141. BMI1 is known as an important regulator in NPC (18). A previous study revealed that miR-141 represses BMI1 expression in diploid fibroblasts (19). Therefore, we hypothesized that BMI1 may serve as a potential miR-141 target. To verify this, BMI1 3'-UTR target sequences with and without mutations were constructed into the luciferase reporter vector (Fig. 4A). Next, the luciferase reporter assay was performed, and the results confirmed that BMI1 served as the direct target of miR-141 (Fig. 4B).

Since miR-141 is a NPC tumor repressor gene, it was investigated whether miR-141 functioned through inhibiting BMI1 expression. BMI1 expression was first detected in NPC biopsy samples and cell lines, and it was revealed that BMI1 was highly activated (Fig. 4C and D). Next, BMI1 expression was examined in SUNE1 cells with ectopic miR-141 expression, and the results demonstrated that Bmi1 was inhibited accordingly (Fig. 4E and F). Furthermore, overexpressing BMI1 partially rescued the inhibitory effect of NPC cells with ectopic miR-141 expression using Transwell and MTT assays (Fig. 4G-I), indicating that BMI1 functions downstream of miR-141.

The expression of miR-141 and BMI1 correlate with the staging status of patients with NPC. To evaluate the clinical significance of miR-141 and BMI1, their expression level was detected in 51 NPC biopsy samples. As demonstrated in Table I, it was demonstrated that miR-141 expression was correlated with the clinical stage of patients with NPC (P=0.03, Table I), and univariable and multivariable analyses confirmed this correlation (Table II). Patients with advanced stages of disease or metastasis exhibited relatively lower expression of miR-141 than patients with earlier stages of disease (Fig. 5A). Furthermore, patients with low expression of miR-141 had high expression of BMI1 (Fig. 5A and B), indicative of the regulatory role of miR-141 and BMI1 *in vivo*.

Discussion

The present study revealed that miR-141 was downregulated in NPC clinical samples and cell lines. Ectopic expression of miR-141 blocked tumor cell proliferation and invasion. Mechanistic analysis identified that BMI1 served as the direct target of miR-141, and overexpressing BMI1 partially rescued the tumor suppressive phenotype of miR-141. Furthermore, the miR-141/BMI1 signaling cascade was correlated with NPC clinical staging. Patients with metastases have relatively lower expression levels of miR-141 and higher expression levels of BMI1, which suggested that ectopic expression of BMI1 facilitated NPC tumor cell metastasis *in vivo*.

Tumor cell growth requires a series of gene transcription and modification networks. Understanding these regulatory networks may aid in identifying the mechanism of tumor cell initiation and growth, so as to develop precise medical strategies for clinical use. Previous studies have demonstrated that several miRNAs were aberrantly expressed and functioned in NPC tumor cells, as well as being involved in cell proliferation, migration and metabolism processes (6,7,9). Furthermore, recent studies have demonstrated that circulating free nucleic acid (cfNA) in patients with cancer were more highly concentrated (20-22). Detecting circulating miRNAs has been emerging as a novel early diagnosis strategy to identify the clinical stage of patients with cancer (22,23). Therefore, it

would be of great importance to study the whole miRNAs regulation network during NPC development.

Recent studies have revealed that miR-141 was active in prostate cancer and ovarian cancer (14,15), and served an inhibitory role in gastric cancer growth (13). Several miRNAs were reported to serve roles in manipulating NPC development (24-30). However, the expression and function of miR-141 in NPC has not been identified. The results of the present study established the tumor repressor effect of miR-141 in NPC, and confirmed that miR-141 functioned through regulating its target, BMI1. Previous results confirmed that BMI1 was an important oncogene in NPC. Inhibiting BMI1 was regarded as the effective method to block NPC proliferation and invasion (16,31,32). The results provided a direct BMI1 regulator in NPC, which may aid in shedding light on the clinical application of pharmacological inhibition of BMI1.

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Authors' contributions

FL and CG conceived the project. FL and WW carried out most of the experiments. SL, QY, JH, and NZ participated in data analysis and interpretation of results. All authors read and approved the manuscript.

Availability of data and materials

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

Ethics approval and consent to participate

This study was performed in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the ethics committee of Nanshan Affiliated Hospital of Guangdong Medical College. Patients provided written informed consent.

Patient consent for publication

All patients have provided written informed consent for the publication of this manuscript and any associated images.

Competing interests

The authors declare that they have no competing interests.

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