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LncRNA HCG18 contributes to nasopharyngeal carcinoma development by modulating miR-140/CCND1 and Hedgehog signaling pathway

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Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) exhibit important roles in a variety of biological properties of tumors. LncRNA HCG18 (HCG18) is a newly identified lncRNA whose roles in tumor progression remains largely unclear. The objective of our current research was to explore the roles and the underlying mechanisms of HCG18 in nasopharyngeal carcinoma (NPC).

PATIENTS AND METHODS: Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to determine the levels of HCG18 in NPC tissue and cell lines. Clinical significances and prognostic values of HCG18 were analyzed using the statistical methods. Cell Counting Kit-8 (CCK-8) assays, clonogenic survival assays, flow cytometry, wound-healing assays, and transwell assays were used to examine the tumorigenesis functions of HCG18 *in vitro*. Insights of the mechanism of ceRNAs were gained from bioinformatic methods and Luciferase analysis. Western blot was performed to determine the expression of tumor-related pathways.

RESULTS: We found that HCG18 expression was upregulated in both NPC specimens and cell lines. Higher levels of HCG18 were associated with positive lymph node metastasis and poor prognosis of NPC patients. Importantly, the multivariate analysis confirmed that HCG18 was an independent risk factor for outcome. Functionally, the downregulation of HCG18 exhibited tumor-suppressive effects *via* the inhibition of cell proliferation and metastasis. Mechanistically, HCG18 may directly bind to miR-140 and effectively act as a ceRNA for miR-140 to increase the expression of cyclin D1 (CCND1). In addition, HCG18 may contribute to NPC progression *via* modulating Wnt/β-catenin signaling and Hedgehog pathway.

CONCLUSIONS: Our findings suggested that HCG18 served as an oncogenic lncRNA in NPC progression, which may provide a novel biomarker of an unfavorable outcome and a potential therapeutic target for NPC.

Key Words:
LncRNA HCG18, Nasopharyngeal carcinoma, Prognosis, MiR-140, CCND1, Metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant squamous cell carcinoma which is an Epstein-Barr virus (EBV)-related epithelial malignancy and arises from epithelial cells of nasopharynx[1,2]. In China, the incidence of NPC is rising and as high as 25-60 per 100,000 people-years[3]. Although various advancements in early screening and therapies have improved the survival of NPC patients, it is extremely poor for the treatments of NPC at an advanced stage[4,5]. The major reasons for treatment failures and NPC-associated death include local recurrence and positive metastasis[6]. Therefore, the further identification of novel functional signal pathways involved in the development and occurrence of NPC is very important for early diagnosis and the development of novel target therapies to overcome this tumor.

Novel researches have indicated that the majority of the human genomes is transcribed, but only approximately 2% possess protein-coding exons[7]. Long non-coding RNAs (lncRNAs) are frequently defined as non-protein-coding transcripts with length surpassing 200 nucleotides with limited protein-coding ability[8]. Biological studies[9] suggest that the potential molecular functions of lncRNAs are very diverse and their roles involved in the epigenetic modulation are one of the best learned parts. Authors[10,11] demonstrate lncRNAs as important modulators in a large range of biological processes, such as

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the regulation of metastasis and apoptosis, and parental imprinting[10,11]. They have also been shown to be involved in the regulation of cancer pathogenesis by acting as tumor promoters and oncogenes based on the tumor microenvironment and the functional targets which they modulate[12,13]. In addition, with the great development of massive parallel sequencing, the dysregulation of lncRNAs is substantially identified, which highlights their clinical potential used as novel biomarkers due to their roles in tumor progression[14,15]. However, the roles of lncRNAs in NPC development have only recently been examined and remains largely unknown.

Long non-coding HCG18 (HCG18) was a recently identified lncRNA whose roles in biological progress remained largely unclear. Recently, the dysregulation of HCG18 had been indicated in several tumors, such as bladder cancer and glioma[16,17]. In intervertebral disc degeneration, HCG18 was found to the disease development *via* the regulation of miRNA-146a-5p/TRAF6/NF-κB axis[18]. However, little is known about the expression and roles of HCG18 in NPC. In this study, we firstly provided evidence that HCG18 levels were upregulated in NPC, and this upregulation was associated with poor prognosis of NPC patients. Functionally, we showed that HCG18 served as a tumor promoter *via* the modulation of miR-140/CCND1. Our findings may have clinical value for developing lncRNA-related markers for predicting prognosis and therapeutics for NPC patients.

Patients and Methods

Specimens Collection

This present research included 109 NPC patients who had undergone surgeries at Tongde Hospital of Zhejiang Province between March 2008 and September 2012. Patients did not undergo radiotherapy or chemotherapy before surgery. The collected specimens were immediately frozen using liquid nitrogen and stored at -80°C. The study was approved by the Ethics Committee of the Tongde Hospital of the Zhejiang Province. Each patient who participated in this study provided a written informed consent.

Cell Transfection

NPC cells (C666-1, CNE-1, HONE-1, SUNE-1, HNE-1, and CNE-2) and nasopharyngeal epithelial cells (NP69) were obtained from the Xinfang Biotechnology company (Ningbo, Zhejiang, China). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) media with serum (10%). The cells were cultured in a 5% CO_2 incubator at 37°C. Cell transfection was conducted using Lipofectamine 3000 reagents kits (Longjun, Chengdu, Sichuan, China) in accordance with the kits' protocols. The control and HCG18 siRNAs (si-HCG18-1, si-HCG18-2), negative control (NC) and miR-140 mimics, NC and miR-140 inhibitors were all bought from Neo biological company (Shenzhen, Guangdong, China). HCG18 overexpressing plasmids were cloned by Weien Biotechnology company (Jinan, Shandong, China).

Real Time-PCR

The total RNAs were extracted using TRIzol reagents (Baibo, Qingdao, Shandong, China). Then, the cDNAs were obtained by reversely transcribing total RNAs (1 μg) using TaKaRa PrimeScript RT kits (Fukun, Hefei, Anhui, China). To determine the levels of HCG18 and CCND1, we used TransGen SYBR Green qPCR kits (Hanhu, Wuhan, Hubei, China) following the manufacturer's protocols. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used for normalization. For miR-140 detection, miRNAs were isolated using Qiagen miRNeasy kits (Hongwei, Xiamen, Fujian, China) and qPCR analyses for miR-140 were conducted using miRNA qPCR detection kits (Biosun, Nanjing, Jiangsu, China). The primer sequences were listed in Table I. The data were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

NPC cells after treatment were lysed using Radioimmunoprecipitation assay buffer (RIPA; Guhong, Hefei, Anhui, China) and the protein concentrations were quantified using bicinchoninic acid assay (BCA) kits (EndaBio,

Table I. The primers for PCR in this study.

Name	Primer sequence (5′-3′)
HCG18: forward	TCAAGCCATGGTGTCAAT
HCG18: reverse	GCTTTGAGACCGGATGTA
miR-140: forward	TGCGGCAGTGGTTTTACCCTATG
miR-140: reverse	CCAGTGCAGGGTCCGAGGT
CCND1: forward	GCTGCGAAGTGGAAACCATC
CCND1: reverse	CCTCCTTCTGCACACATTTGAA
GAPDH: forward	GGAGCGAGATCCCTCCAAAAT
GAPDH: reverse	GCTGTTGTCATACTTCTCATGG

Changsha, Hunan, China). The Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels (7-12%) were applied for separating these proteins. The incubation with 5% bovine serum albumin and the polyvinylidene difluoride (PVDF) membranes for 1 h were then conducted after the separated proteins were transferred onto the PVDF membranes. These membranes were then probed with primary antibodies respectively targeting SHH (1:750; PTG, Wuhan, Hubei, China), β-Catenin (1:800; CST, Danvers, MA, USA), GLI1 (1:500; Abcam, Cambridge, MA, USA), cyclin D1 (1:500; PTG, Wuhan, Hubei, China), and c-myc (1:600; PTG, Wuhan, Hubei, China). The primary antibodies were incubated with the membranes at 4°C for 12 h. On the second day, the membranes were washed using Tris-Buffered Saline and Tween-20 (TBST) buffer three times and the corresponding secondary antibodies were applied for 1-hour incubation with the membranes. Enhanced chemiluminescence (ECL) kits (Tianfeng, Xiamen, Fujian, China) were used to visualize the proteins after the membranes were washed using TBST buffer.

Proliferation Detection

SUNE-1 and CNE-2 cells (2000 cells/well), transfected with HCG18 or control siRNAs, were placed in 96-well plates and incubated at 37°C with 5% CO_2. Subsequently, at 24, 48, 72, and 96 h (1-4 days), respectively, the Cell Counting Kit-8 (CCK-8) reagents (100 μl; Kaijie, Nanjing, Jiangsu, China) were placed into each well. The cells were kept in an incubator with 37°C and 5% CO_2 for 2.5 h. Then, a micro-plate reader machine was employed to determine the absorbance at 450 nm.

EdU Assays

Cell proliferation was also assessed by using EdU (5-ethynyl-2'-deoxyuridine) assay kits (Ruibo, Guangzhou, Guangdong, China). NPC cells after HCG18 or control siRNAs transfection were collected and seeded onto 96-well plates. Thirty-six hours later, the cells were treated with 25 μl EdU reagents for 2.5 h at 37°C. Afterwards, they were treated using paraformaldehyde (4%) for 25 min and 0.5% Triton X-100 for 15 min. Then, 15 μl 1×Apollo reaction reagents were added into each well and incubated for 25 min. The cells were then stained using DAPI solution for 10 min. Finally, the cells were visualized with a fluorescent microscope after being washed using phosphate-buffered saline (PBS).

Clonogenic Assays

NPC cells after HCG18 or control siRNAs transfection were trypsinized and collected. Appropriate numbers of cells (500 cells/well) were put into 60 mm dishes and cultured for 10 to 14 days. The media were changed every two days. After washing using PBS, the colonies were treated using paraformaldehyde (4%) and crystal violet solution (0.2%), followed by washing for three times using PBS. The colonies were then counted and photographed using a microscope.

Cell Apoptosis Detection

NPC cells after treatment were cultured in the media and then harvested for flow cytometry analysis. After the collected-cells were washed with cold PBS twice, they were then incubated in 150 μl binding buffer containing PI (5 μl) and Annexin V-FITC (2.5 μl) for 20 min in lightproof condition. The cells were then washed using cold PBS twice and flow cytometry was used to analyze the cell apoptosis. The cell apoptosis detection kits were bought from Kaijie Biological company (Nanjing, Jiangsu, China).

Caspase 3/9 Activity Detection

Beyotime caspase 3 or 9 activity detection kits (YekunBio, Changsha, Hunan, China) were employed for detecting caspase 3/9 activities in NPC cells after HCG18 knockdown. In brief, NPC cells after HCG18 or control siRNAs transfection were trypsinized and collected in a 1.5 ml centrifuge tube. Then, the cell lysis buffer (200 μl) was added into the cells and the mixtures were incubated for 15 min. Subsequently, 2mM of Ac-DEVD-pNA reagents were placed into the cell lysates. Finally, a micro-plate reader machine was employed to determine the absorbance at 405 nm.

Wound-Healing Assays

NPC cells (about 60-70% cell confluent) were transfected with HCG18 or control siRNAs. The transfected cells were trypsinized and collected 12 hours later. The collected-cells were then placed into 24-well plate at high density. After 12-20 h, the cells (about 100% cell confluent) were subjected to scratch wounding. The wounds were generated by scraping the cell monolayer with 100 μl pipette tips. After washing the cells, the photographs of the wounding areas were acquired at 0 h and 48 h after scratching using a microscope.

Transwell Assays

Twenty-four-well Corning transwell chambers (pre-coated with Matrigel; Dongcheng, Ningbo, Zhejiang, China) were applied for the transwell assays. The treated-NPC cells (1.5×10^5 cells/well) were placed in 250 µl serum-free media, and the cell suspensions were then put into the top of each well. Then, 650 µl of complete media (with 15% FBS) was added to the lower chamber. Twenty-four hours later, the cells invaded through the membranes were treated using paraformaldehyde (4%) and crystal violet solution (0.2%) for 15 min. After washing using PBS, the cells were photographed by using a microscope.

Subcellular Fractionation Location Assays

The Life Technologies' PARIS kits (HehuanBio, Fuzhou, Fujian, China) were applied for separating the nuclear and cytosolic fractions. In brief, the SUNE-1 cells (1×10^7 cells) were washed using PBS and placed into 400 µl cell fractionation buffer, followed by incubation on ice for 10 min. The mixtures were then centrifuged at 4°C (500 × g/min; 5 min). The cytoplasmic fractions were aspirated in a new centrifuge tube, and the nuclear pellets were washed and added with disruption buffer. Subsequently, the RNAs in cytoplasmic or nuclear fractions were isolated following the protocols provided in the kits. Finally, RNAs from each of the fractions were subjected to qPCR analyses to determine the levels of U6 (nuclear control), GAPDH (cytoplasmic control), and HCG18.

Luciferase Reporter Assays

The complementary DNA fragment containing the wild-type (HCG18-wt) or mutant HCG18 (HCG18-mut) fragment was constructed into pGL3 Luciferase reporter plasmids. Similarly, the 3' untranslated region (UTR) of CCND1 (CCND1-WT) or corresponding mutant (CCND1-MUT) was respectively subcloned into pGL3 Luciferase reporter vectors. The control or miR-140 mimics were co-transfected with HCG18-wt, HCG18-mut, CCND1-WT, or CCND1-MUT Luciferase reporters into NPC cells using Lipofectamine 3000 reagents as described above. Forty-eight hours post-transfection, the Luciferase activities were consecutively determined by Promega Dual-Luciferase reporter kits (Dukai, Changsha, Hunan, China).

Statistical Analysis

Data analyses were conducted using SPSS 20.0 statistics software (IBM Corp., Armonk, NY, USA). Comparisons of data between the two groups were carried out using the Student's *t*-test. Multiple groups of measurement data were compared using One-way ANOVA methods. Tukey's post-hoc test was used to validate the ANOVA for comparing the measurement data between the groups. The Kaplan-Meier methods were applied for estimating the overall survival (OS) and disease-free survival (DFS), which were compared using the log-rank test. The multivariate assays of the prognostic factors were carried out with the Cox regression model. A p-value <0.05 was considered statistically significant.

Results

HCG18 Was Upregulated in NPC Tissues and Correlated with Poor Prognosis

To determine whether HCG18 was dysregulated in NPC, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed. As presented in Figure 1A, we found that HCG18 expressions were upregulated in NPC specimens compared with the matched normal specimens ($p < 0.01$). In addition, it was also observed that HCG18 expression in patients with stage III-IV NPC was distinctly higher than that in patients with stage I-II NPC ($p < 0.01$, Figure 1B). Next, we also detected the levels of HCG18 in several NPC cell lines, finding that PCAT-1 expression level in C666-1, CNE-1, HONE-1, SUNE-1, HNE-1, and CNE-2 were higher when compared with NP69 (Figure 1C). These results revealed that HCG18 might play a functional role in NPC progression.

To explore the clinical significance of HCG18 in NPC patients, 109 NPC patients were divided into a high HCG18 expression group and a low HCG18 expression group based on the median value of HCG18 in all NPC specimens. We found that high expression of HCG18 was distinctly associated with clinical stage ($p = 0.019$) and lymph node metastasis ($p = 0.009$) in NPC patients (Table II). Then, we further investigated whether the overexpression of HCG18 in NPC had prognostic value for NPC patients. The results of Kaplan-Meier methods indicated that patients with high expression of HCG18 had a shorter OS ($p = 0.005$) and DFS ($p < 0.0039$) as compared with the HCG18-low group (Figure 1D). The univar-

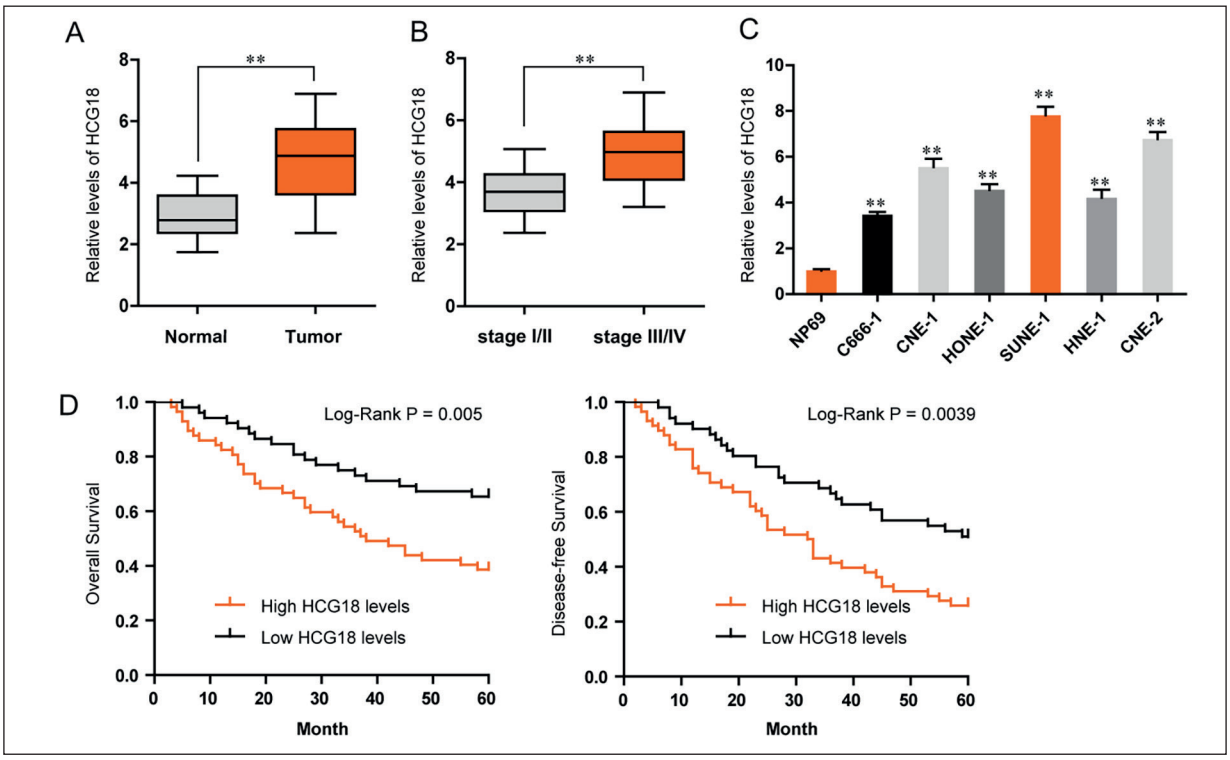

Figure 1. Expression levels of HCG18 in NPC and its clinical significance. **A**, The levels of HCG18 were analyzed by RT-PCR in NPC tissues and matched normal specimens. **B**, Higher expression of HCG18 was observed in the tissues of advanced stages. **C**, HCG18 expressions were measured by Real Time-PCR in six NPC cells and normal nasopharynx cell (NP69). **D**, HCG18 predicts the clinical outcome of NPC patients. Kaplan-Meier overall survival and disease-free survival curves according to HCG18 expression level. *$p < 0.05$, **$p < 0.01$.

iate analyses suggested that HCG18 expression, clinical stage, and lymph node metastasis were possible prognostic indicators for both OS and DFS (all $p < 0.05$, Table III). Moreover, the results of multivariate analysis suggested that HCG18 expressions were independently associated with the OS (HR=2.896, 95% CI: 1.265-4.772, $p = 0.012$, Table III). In addition, the similar results in

Table II. Correlations between HCG18 expression and clinicopathological characteristics in NPC.

Variable	No. of patients (n)	HCG18 expression		*p*-value
		High	Low	
Gender				0.483
Male	65	31	34	
Female	44	24	20	
Age				0.387
< 60	56	26	30	
≥ 60	53	29	24	
Clinical stage				0.019
I-II	68	28	40	
III-IV	41	27	14	
Lymph node metastasis				0.009
Positive	74	31	43	
Negative	35	24	11	
EBV infection				0.502
Yes	54	29	25	
No	55	26	29	

Table III. Univariate and multivariate analyses of prognostic variables of DFS and OS in glioma patients.

Parameter	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Disease-free survival				
Gender	1.237 (0.872-1.899)	0.127	–	–
Age	1.462 (0.627-2.327)	0.118	–	–
Clinical stage	3.126 (1.347-4.886)	0.007	2.976 (1.158-4.572)	0.013
Lymph node metastasis	3.265 (1.339-4.627)	0.009	3.018 (1.258-4.362)	0.015
EBV infection	1.185 (0.882-1.985)	0.145	–	–
HCG18 expression	2.986 (1.416-4.776)	0.011	2.786 (1.277-4.562)	0.016
Overall survival				
Gender	1.375 (0.927-2.127)	0.164	–	–
Age	1.391 (0.875-1.896)	0.248	–	–
Clinical stage	3.257 (1.472-5.018)	0.004	3.117 (1.328-4.772)	0.007
Lymph node metastasis	3.362 (1.352-4.872)	0.007	3.167 (1.271-4.626)	0.009
EBV infection	1.264 (0.987-2.018)	0.117	–	–
HCG18 expression	3.174 (1.385-5.126)	0.006	2.896 (1.265-4.772)	0.012

DFS (HR=2.786, 95% CI: 1.277-4.562, $p = 0.016$) was also observed (Table III). Overall, our findings highlighted the clinical potential of HCG18 used as a novel biomarker

HCG18 Knockdown Inhibited NPC Cellular Growth and Promoted Cell Apoptosis

To explore the biological functions of HCG18 in NPC cells, we first knocked down HCG18 in SUNE-1 and CNE-2 cells by transfecting siRNAs by targeting HCG18 (si-HCG18-1, si-HCG18-2). The knockdown efficiency of these siRNAs was determined by qPCR assays (Figure 2A). Subsequently, the growth curves generated from CCK-8 assays revealed that the cellular growth of NPC cells was remarkably suppressed by HCG18 knockdown (Figure 2B). Thereafter, EdU assays were also performed to evaluate the NPC cell proliferation and we found that silencing HCG18 markedly depressed the proliferative rates of NCP cells, which was consistent with the results of CCK-8 assays (Figure 2C). Similarly, the clonogenic formation assays demonstrated that the clonogenic survivals of NPC cells were remarkably impaired by HCG18 depletion (Figure 2D). Since increased apoptosis is one of the major factors that contribute to cancer cell proliferation, we next thereby sought to detect whether the NPC cell apoptosis was affected by transfecting with HCG18 siRNAs. We performed flow cytometry analysis and found that NPC cells with HCG18 depletion had higher apoptotic rates than cells treated with control siRNAs (Figure 2E). Afterward, we examined the activities of caspase 3/9 in HCG18-depleted NPC cells and found that repressing HCG18 expression significantly increased the caspase 3/9 activities (Figure 2F). In summary, these findings indicated that HCG18 behaved as an oncogene to promote NPC cell proliferation.

Repressing HCG18 Expression Impeded the Metastatic Potentials of NPC Cells

Next, we wondered whether HCG18 depletion was able to regulate the mobility of NPC cells. To achieve that, we first measured the migration potentials of NPC cells upon HCG18 siRNAs treatment by wound-healing assays. As the data presented in Figure 3A, the wounded areas in HCG18 siRNAs-treated group were markedly wider than that of the control group, which indicated that the depression of HCG18 caused dramatical inhibitory effects on cellular migration capacities. Then, we carried out the transwell assays to determine the influence of HCG18 deficiency on cellular invasive abilities. The results validated that the depression of HCG18 contributed to notably reduced invasive cell number of NPC cells (Figure 3B). Collectively, these data suggested that HCG18 served as an important player in regulating the metastasis of NPC cells.

Reciprocal Repression Between HCG18 and MiR-140 in NPC Cells

Previous reports demonstrated that lncRNAs, particularly exhibiting in the cytoplasm, were capable of acting as ceRNAs to regulate gene

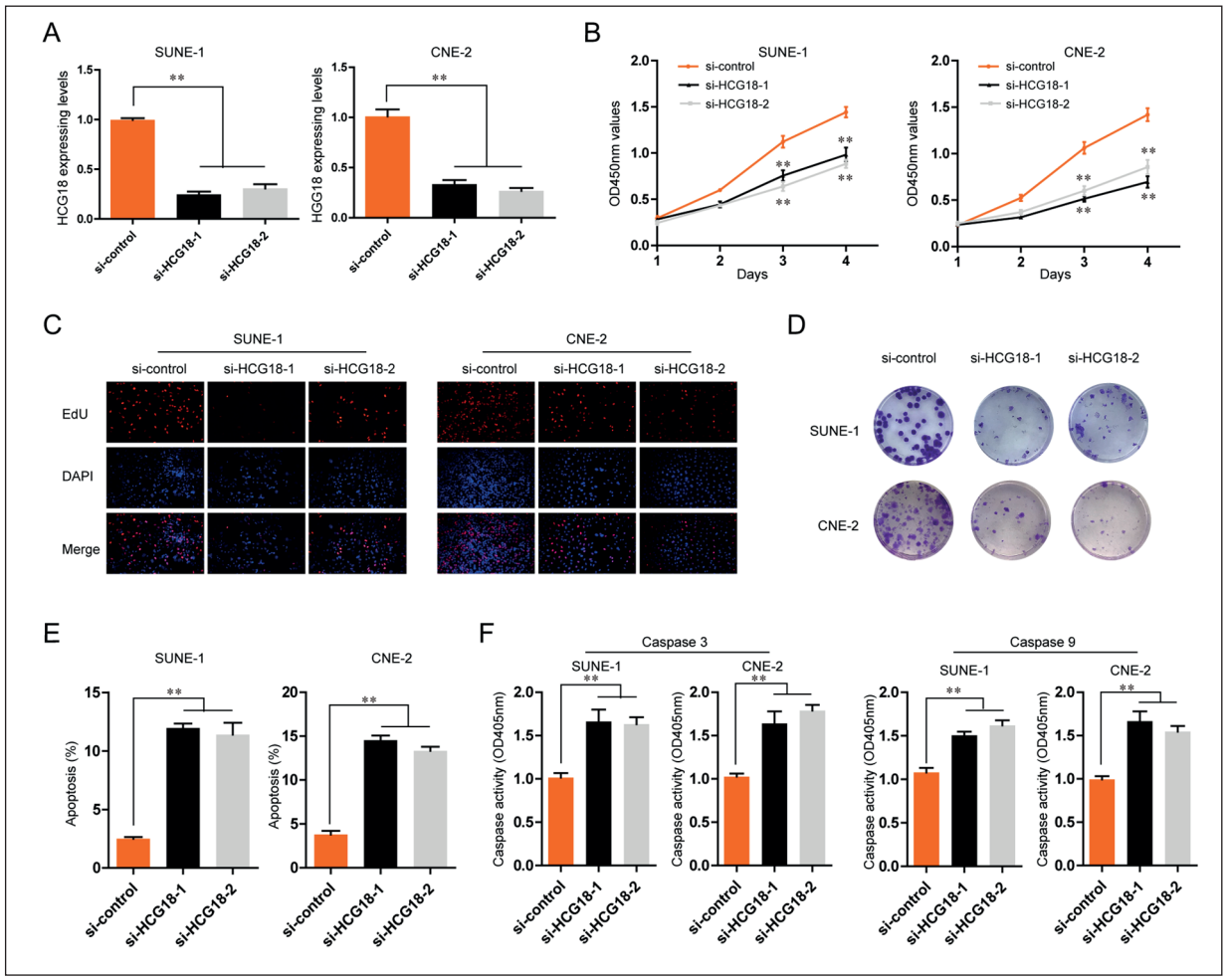

Figure 2. The effects of HCG18 on the proliferation and apoptosis of NPC cells. **A**, Real Time-PCR analyses detected the expressing levels of HCG18. **B**, CCK-8 assays. **C**, EdU assays detected NPC cell proliferation. The red cells represented the positive cells (proliferative cells). The blue fluorescence represented the cell nuclei which were stained by DAPI (magnification: × 100). **D**, Colony formation assays (magnification: × 10). **E**, The apoptotic cells were analyzed by flow cytometry. **F**, Caspase 3/9-activity detection. *$p < 0.05$, **$p < 0.01$.

expression[19]. Therefore, to further uncover the mechanisms by which HCG18 modulated NPC tumorigeneses, we first assessed the distribution of HCG18 in NPC cells. By using the subcellular fractionation location assays, we found that HCG18 was mainly expressed in the cytoplasm, which indicated that HCG18 might exert its functions *via* sponging specific miRNAs (Figure 4A). Hence, we next sought to find the downstream target miRNA of HCG18. To achieve that, we first conducted bioinformatics analyses using GSE32960 data and obtained the downregulated miRNAs in NPC tumor samples. The volcano map of GSE32960 data was shown in Figure 4B. Among these downregulated miRNAs, miR-140, a widely reported tumor suppresser attracted our attention and its expression in NPC tumor samples was also downregulated using bioinformatics analyses (Figure 4C). The results of qPCR assays also certified that miR-140 was downregulated in 109 NPC tumor specimens (Figure 4D). Moreover, by using "starBase" algorithm, we found that HCG18 formed complementary base pairing with miR-140 (Figure 4E). Next, we performed the Dual-Luciferase reporter assays to certify whether miR-140 was the exact target of HCG18. The data verified that the co-transfection of HCG18 wild-type (wt) reporters and miR-140 mimics resulted in notably decreased Luciferase activities in NPC cells, while the Luciferase activities were not changed in the cells co-transfected with HCG18 mutant-type (mut) reporters

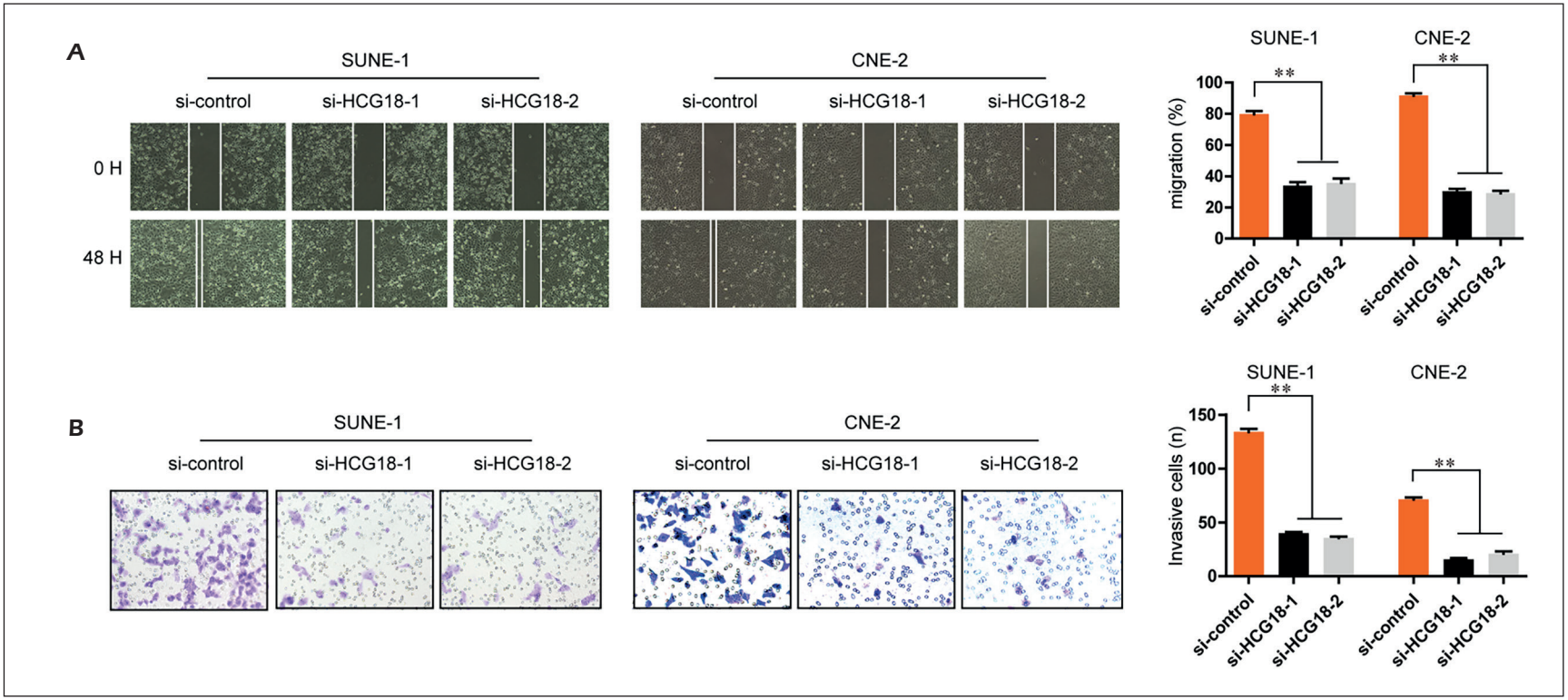

Figure 3. The influences of HCG18 on the migration and invasion of NPC cells. **A**, Wound-healing assays detected the migration of HCG18-silenced NPC cells (magnification: × 10). **B**, Transwell assays were conducted to determine the invasive abilities of NPC cells (magnification: × 40). *$p < 0.05$, **$p < 0.01$.

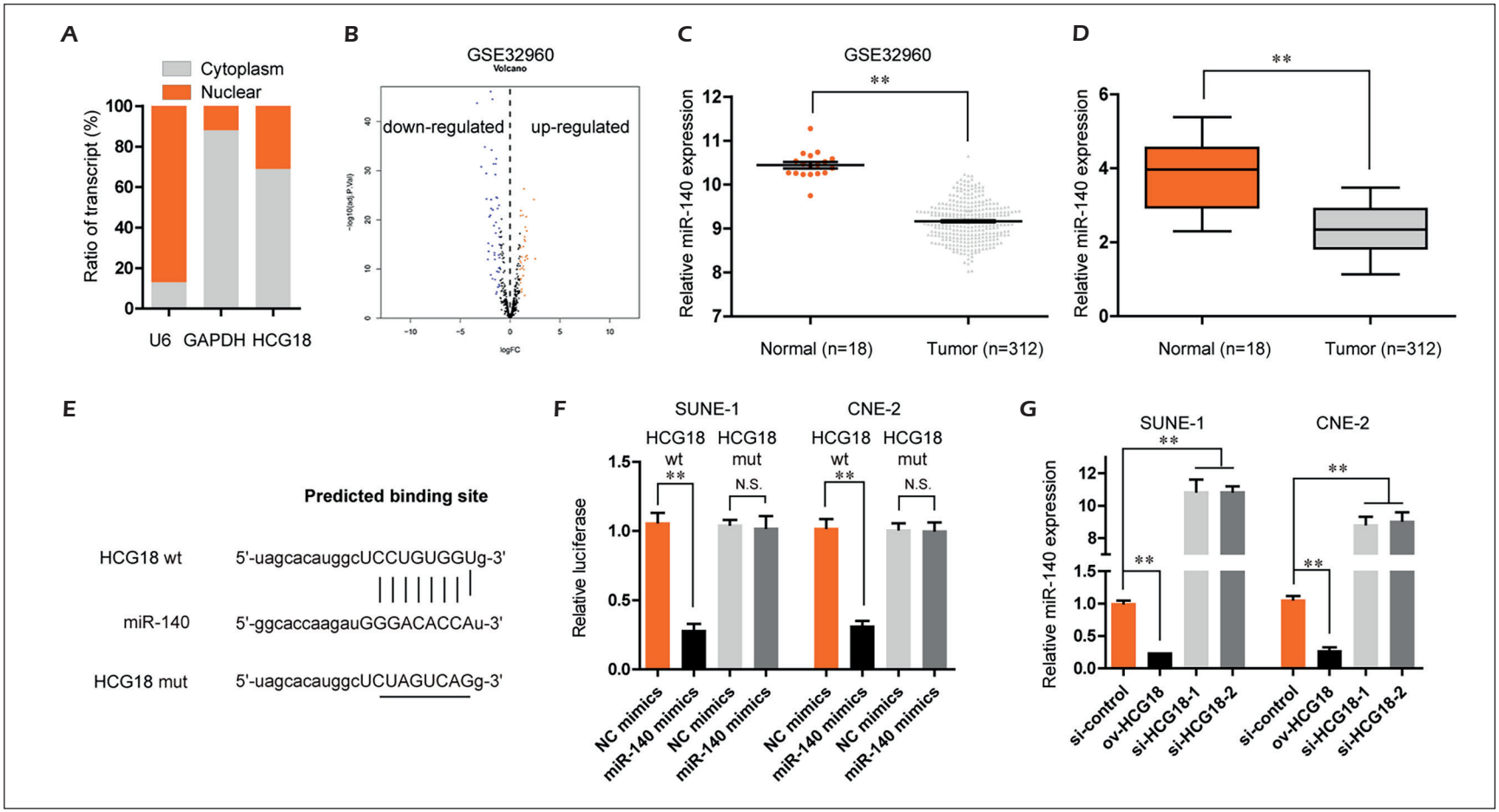

Figure 4. MiR-140 was directly targeted by HCG18 in NPC cells. **A**, Subcellular fractionation location assays. **B**, Volcano map of GSE32960 data. **C**, MiR-140 expression in GSE32960 data. **D**, MiR-140 expression in 109 NPC tissue samples. **E**, The "starBase" algorithm predicted the binding site. **F**, The Luciferase activity detection assays. **G**, Relative miR-140 levels in NPC cells after transfection with HCG18 siRNAs or overexpressing plasmids (ov-HCG18). *$p < 0.05$, **$p < 0.01$.

and miR-140 mimics (Figure 4F). In addition, the overexpression of HCG18 (ov-HCG18) caused significantly decreased miR-140 levels, while the depression of HCG18 led to markedly elevated miR-140 levels in NPC cells (Figure 4G). To sum up, these data validated that miR-140 was a target of HCG18 in NPC cells.

CCND1 Was a Target of MiR-140 and HCG18 Modulated Hedgehog Pathway and Wnt/β-Catenin Signaling

Many investigations revealed that miRNAs exerted their functions *via* targeting corresponding genes, and we thereby sought to discover the gene that potentially targeted by miR-140 in NPC cells. By using "miRDB" algorithm, we found that CCND1 was a possible candidate. As presented in Figure 5A, there was a potential binding site of miR-140 in the 3'UTR of CCND1. Therefore, the Luciferase reporter assays were conducted to validate whether CCND1 was the exact target of miR-140. The results demonstrated that ectopic expression of miR-140 could significantly suppress the Luciferase intensity of CCND1-WT plasmids, but not the mutant one, in NPC cells (Figure 5B). Furthermore, qPCR analyses revealed that miR-140 overexpression notably reduced the levels of both HCG18 and CCND1, whereas the inhibition of miR-140 markedly elevated HCG18 and CCND1 expression

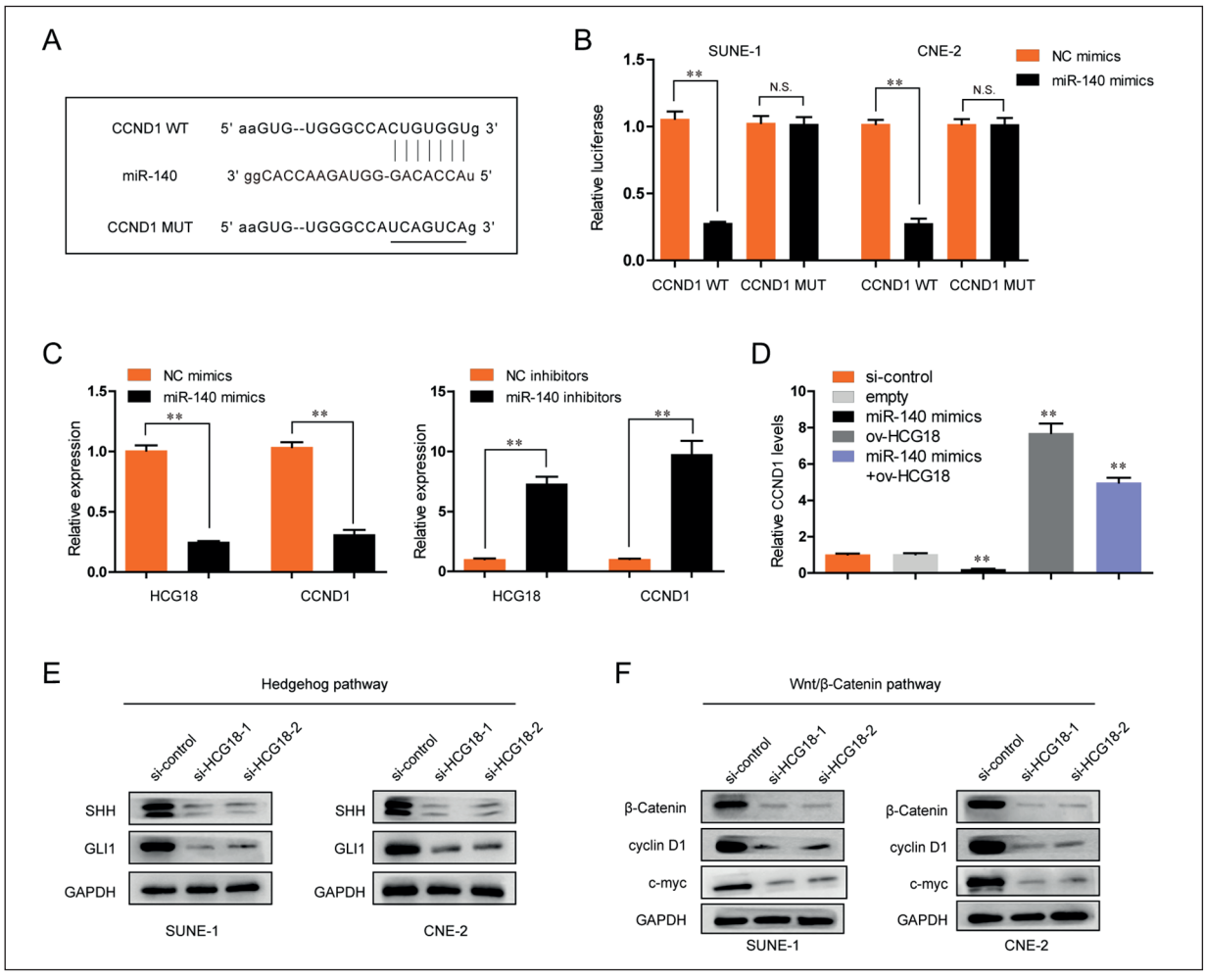

Figure 5. CCND1 was a target of miR-140 and HCG18 modulated Hedgehog signaling in NPC cells. **A**, The "miRDB" algorithm predicted the binding site between CCND1 and miR-140. **B**, The Luciferase activity detection assays. **C**, qPCR analyses detected the expression of HCG18 and CCND1 in SUNE-1 cells after miR-140 was enhanced expression or knocked down. **D**, Real Time-PCR assays detected the CCND1 levels under various conditions. **E**, Western blot assays detected protein levels of SHH and GLI1 in NPC cells. **F**, Western blot assays detected protein levels of β-Catenin, cyclin D1, and c-myc in NPC cells. *$p < 0.05$, **$p < 0.01$.

(Figure 5C). In addition, we also found that the overexpression of HCG18 significantly abrogated the inhibitory effects of miR-140 on CCND1 expression (Figure 5D). Therefore, the above data demonstrated that HCG18 was able to regulate CCND1 expression *via* targeting miR-140. Considering that CCND1 was involved in Hedgehog signaling and Wnt/β-Catenin pathway, we thereby wondered whether HCG18 could also modulate these two signaling pathways in NPC cells. Therefore, Western blot was conducted to determine the protein levels of several key factors involved in Hedgehog pathway and Wnt/β-Catenin signaling. The results suggested that the repression of HCG18 expression could remarkably suppress the protein levels of Hedgehog pathway and Wnt/β-Catenin signaling in NPC cells (Figures 5E and F). Taken together, we demonstrated that CCND1 was a target of miR-140 and HCG18 could regulate Hedgehog pathway and Wnt/β-Catenin signaling in NPC cells.

Discussion

More and more key molecular events involved in tumor progression were identified with the great improvement in the studies of genomics and proteomics[20]. These advancements resulted in novel breakthrough of finding novel NPC-related biomarkers, such as mRNAs, non-coding RNAs, and epigenetic alterations mutations[21,22]. Among these different biomarkers, lncRNAs are believed to be promising biomarkers due to the easy detection and their strong associations with clinical prognosis[23,24]. In this study, we firstly identified a novel NPC-associated lncRNA HCG18 whose overexpression was firstly demonstrated in our RT-PCR assays using 109 pairs of NPC tissues and matched normal specimens. Then, clinical assays indicated that the overexpression of HCG18 in NPC samples was associated with advanced clinical stage, positively lymph node metastasis, and shorter five-year survival of NPC patients. Moreover, for the preliminary determination of the clinical application of HCG18 as a novel biomarker in NPC patients, the multivariate analysis was performed and the results showed that HCG18 were independently associated with both OS and DFS, which highlighted the potential of HCG18 used as a novel prognostic marker.

Previously, the abnormal expressions of HCG18 in several tumors have been reported. However, its potential functional are rarely reported. Xu et al[16] firstly reported that HCG18 could influence the tumor proliferation and metastasis of bladder cancer cells. In the present study, we firstly decreased the levels of HCG18 in NPC cells for the determination of HCG18 function in NPC, finding that the knockdown of HCG18 distinctly suppressed the proliferation, migration, and invasion of NPC cells. Our findings revealed that HCG18 acted as a tumor promoter in NPC progression. To further explore NPC pathogenesis, our group focused on the molecular mechanism of HCG18 as a ceRNA using bioinformatics assays. Salmena et al[25] firstly proposed a novel regulatory mechanism named as ceRNA which showed that lncRNAs can antagonize miRNA functions *via* sponging miRNAs through a competing endogenous mechanism, which has been demonstrated in many studies[26]. By analyzing the expressions of HCG18 in cytoplasm and nucleus, we found that most of HCG18 expressed in cytoplasm of NPC cells, suggested that HCG18 may exhibit functional roles by acting as a ceRNA. Using Bioinformatics assays of miRNA recognition sequences on HCG18 indicated that miRNA-140 was a potential candidate. Previously, miRNA-140 has been indicated to exhibit anti-cancer roles in NPC[27,28]. Hence, we also revealed that miRNA-140 was a downregulated miRNA in NPC tissues. Of note, in our experimental assays, we provided evidence that supported the interaction of HCG18 miRNA-140 activity. Our findings suggested that HCG18 may act as a molecular sponge for miRNA-140. In addition, we identified the cyclin D1 (CCND1) gene as a novel direct target of miRNA-140. Previously, CCND1 has been demonstrated to be highly expressed in several tumors, including NPC and act as a tumor promoter[29,30]. Hence, we performed RT-PCR, finding that the overexpression of miRNA-140 resulted in the suppression of CCND1 levels. However, this suppression was reversed by the upregulation of HCG18. Thus, these results revealed that we discovered that HCG18 promotes NPC progression by functioning as a miRNA-140 sponge to increase the levels of CCND1.

The molecular mechanisms involved in the development and progression of tumors are very complex. In recent years, several tumor-related signaling pathways have been identified, such as PI3K/Akt pathway, Hippo-YAP1 pathway, Wnt/β-catenin signaling, and Hedgehog path-

way[31-33]. Increasing reports have disclosed that the dysregulation of these pathways may contribute to the malignant progression of various tumors. Researches[34,35] indicated that some tumor-associated lncRNAs could exhibit their regulatory effects by modulating the above pathways. In this study, we wondered whether abnormally expressed HCG18 may influence the activities of Wnt/β-catenin pathway and Hedgehog pathway. The results of Western blot indicated that the downregulation of HCG18 suppressed the expressions of SHH and GLI1 at protein levels, which revealed that the activity of Hedgehog pathway was inhibited. In addition, we also observed that the knockdown of HCG18 in SUNE-1 and CNE-2 significantly suppressed the expressions of β-Catenin, cyclin D1, and c-myc. Thus, our findings indicated that HCG18 may affect NPC cells by modulating the Wnt/β-catenin pathway and Hedgehog pathway. In the future, the detailed mechanism involved in the regulation between HCG18 and the above two pathways remained to be further explored.

There are still some limitations in our study. Firstly, the small sample size of our study might likely result in a weak conclusion and the clinical significance of HCG18 needed to be demonstrated in another independent cohort. Secondly, more experiments with *in vivo* assays were necessary for the supplement of more robust evidence for our results. Thirdly, additional molecular mechanisms of HCG18 in the modulation of NPC progression require further study.

Conclusions

We identified a previously unknown NPC-related lncRNA HCG18, which was highly expressed in NPC and associated with an unfavorable outcome of NPC patients. HCG18 acted as a tumor promoter by modulating miR-140/CCND1 axis and Wnt/β-catenin and Hedgehog pathway. Our data might provide novel research ideas for NPC treatments and prognosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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