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Original Article

LncRNA HCG18 facilitates melanoma progression by modulating miR-324-5p/CDK16 axis

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Abstract: Objective: LncRNA HCG18 has been reported to act as a tumor promoter in gastric cancer, hepatocellular carcinoma and nasopharyngeal carcinoma. However, the role of HCG18 in melanoma is still not clear. In this study, we detected the expression and molecular function of HCG18 in melanoma. Methods: The expression of HCG18 in melanoma cell lines and 50 pairs of melanoma and corresponding non-cancer tissues was detected by RT-qPCR. The relationship between HCG18 and clinicopathology was analyzed. We used HCG18 overexpressing melanoma cell lines A375 and M14, and si-HCG18 to knock down HCG18 expression. CCK-8, clone formation, Transwell assay and FCM were used to explore the effect of HCG18 knockdown on cell proliferation, migration, invasion and apoptosis in melanoma cells. Bioinformatics software was used to predict the downstream miRNA regulated by HCG18, and the downstream target genes regulated by miR-324-5p. Dual-luciferase reporter assay and RNA pull-down assay were used to verify whether miR-324-5p was related to the predicted sequence of HCG18. Results: HCG18 was highly expressed in melanoma tissues and cells. Besides, we found that HCG18 was closely correlated with thickness, TNM stage and metastasis. Functional experiments discovered that HCG18 knockdown restrained cell proliferation, migration and invasion, while promoted cell apoptosis in melanoma cells. HCG18 was confirmed to be a sponge of miR-324-5p, and CDK16 might be a downstream gene of miR-324-5p. HCG18 was found to reverse the effect of miR-324-5p by upregulating CDK16 expression in melanoma cell proliferation, apoptosis, migration and invasion *in vitro*. Conclusion: This study indicated that HCG18 played an essential role in the pathogenesis of melanoma and suggested that HCG18 might be a potential target for the treatment and diagnosis of melanoma.

Keywords: LncRNA HCG18, miR-324-5p, CDK16, melanoma, proliferation, migration, invasion

Introduction

Melanoma is a kind of malignant epithelial tumors derived from melanocytes of spinal nerve. Recently, the morbidity and mortality of melanoma have been on the rise globally, accounting for 1-3% of all malignancies, but it is increasing at an annual rate of 6-7% [1]. Melanoma in Europe and United States mostly occurs in the face or body surface, and is induced by overexposure to ultraviolet rays caused by sun exposure [2]. In China and Asia, melanoma tends to occur in parts of the body that are not exposed to the sun, such as the feet, which is more closely associated with genetic mutations [3]. Clinical studies have shown that melanoma can occur in any anatomical site where melanocytes exist. Early detection and surgical resection are still the best choices for curing melanoma [4]. At the same time, radiation therapy and chemical therapy are also the commonly used treatment methods. The development of malignant melanomas is always associated with some gene mutations and changes in expression level [5, 6]. Studying the mechanisms of melanoma at the molecular level can provide potential basis for new approaches and methods for the early diagnosis and treatment of malignant melanoma.

Long non-coding RNAs (LncRNAs) are a group of regulatory RNAs with a length of over 200 nucleotides. Increasing evidence has displayed

that abnormally expressed lncRNAs play a role in the progression of human cancers. For instance, HOXA11-AS was abnormally expressed in different cancers (glioma, lung cancer, melanoma, gastric cancer and colorectal cancer etc.), and was considered to be crucial in the tumorigenesis and development of tumors [7]. LncRNA HCG18 is a member of the HLA complex group. HCG18 has been found to be abnormally expressed in various malignant diseases. HCG18 was found to be overexpressed in intervertebral disc degeneration, and might be a new early diagnostic marker for patients with intervertebral disc degeneration [8]. HCG18 was reported to be upregulated in hepatocellular carcinoma, and overexpression of HCG18 is associated with poor prognosis [9]. Liu et al. found that HCG18 was upregulated and involved in progression of gastric cancer [10]. However, the regulatory mechanism of HCG18 in melanoma progression remains unknown.

As a class of important regulatory molecules in the human genome, lncRNAs exert the biological functions in various ways. LncRNAs can act as a sponge to interact with miRNAs to participate in the expression regulation of target genes [11, 12]. MiR-324-5p has been shown to be involved in various cancer progression, such as papillary thyroid carcinoma [13] and gallbladder carcinoma [14]. In the current study, we verified that HCG18 was a sponge of miR-324-5p, and HCG18 regulated melanoma progression by inhibiting miR-324-5p to upregulate CDK16 expression. Hence, our study indicated that HCG18 might be a possible target for diagnosing of patients with melanoma.

Materials and methods

Human tissues

Melanoma tissues were collected from 50 patients diagnosed with malignant melanoma at Jinan Central Hospital, and 50 normal skin tissues were used as controls. Among the patients, there were 26 males and 24 females, 28 patients older than 50 years and 22 younger than 50 years in age, with 33 patients in stage I+II and 17 patients in stage III+IV. The included patients had not been subjected to radiotherapy or chemotherapy. All experiments in this study were approved by the Ethics Committee of Jinan Central Hospital (Approval No. ML2016-03-27). All patients have signed the written informed consent.

Melanoma cell lines

Melanoma cell lines (SK-MEL-1, A375, M14 and A2058) and normal epidermal melanocytes HEMn-LP were obtained from Mini Jing Biology (Shanghai, China). Cells were placed in a high-sugar DMEM medium with 10% FBS, and 1% penicillin and streptomycin in an incubator at 37°C and 5% CO_2. The cells in the logarithmic growth phase were digested with trypsin (0.25%), and then collected for functional testing. Cells (3×10^5 cells/mL) were cultured in a 6-well plate. When the cell confluence reached 90%, the cells were randomly divided into the control group and si-HCG18 group. HCG18 expression was examined by RT-qPCR, and the siRNA with the highest transfection efficiency was selected for subsequent experiments.

Dual luciferase reporter assay

HCG18-Wt, HCG18-Mut, CDK16-Wt, CDK16-Mut and miR-324-5p mimic were transfected into A375 and M14 cells using Lipofectamine™ 2000. After transfection for 48 h, the luciferase activities were detected according to the dual luciferase reporter assay kit.

RNA pull-down assay

RNA pull-down assay was performed to detect whether HCG18 can be pulled down by miR-324-5p. Biotinylated miR-324-5p-Wt, biotinylated miR-324-5p-Mut, and biotinylated NC were synthesized by GenePharma (Shanghai, China). After transfection for 48 h, cells were incubated with Dynabeads M-280 streptavidin and biotinylated miRNA at room temperature for 30 min. Finally, HCG18 enrichment was examined by RT-qPCR.

CCK-8 assay

100 μL cell suspension was cultured in a 96-well plate and then incubated in a CO_2 incubator at 37°C. CCK-8 reagent (10 μL) was added to each well for 0, 24, 48 and 72 h, respectively. After being cultured for another 2 h, the OD value of cells at 450 nm was detected by a microplate analyzer.

Colony formation assay

A375 and M14 cells were inoculated in 6-well plates. When clones were visible in the dish,

cells were fixed with methanol, and stained with crystal violet. Finally, the plate was turned upside and covered with a transparent film with a grid for clone counting.

Cell migration and invasion assays

Transwell assay was adopted to detect melanoma cell migration and invasion. 8.0 μm Transwell chamber was used for the test. Different from the migration experiment, invasion experiment was to apply a layer of Matrigel (50 mg/L, 1:8) to the upper chamber. Cells were cultured without serum starvation for 12-24 hours and were digested with trypsin. Cells were suspended by a serum-free medium containing BSA. Cell suspension (100 μL, 1×10^5/100 μL) was added into the upper chamber, and DMEM with 20% FBS was added into the lower chamber. Cells were conventionally cultured at 37°C and 5% CO_2 for 12 h. Then, cells were stained with 1% crystal violet for 20 min. The number of invaded and migrated cells in random 5 fields was calculated by a microscope.

Cell apoptosis rate

Cell apoptosis rate was detected by flow cytometry (FCM). A375 and M14 cells were washed with cold phosphate buffer saline and then stained with Annexin V-FITC and propidium iodide (PI) at room temperature in the dark. Finally, cell apoptosis rate was analyzed by flow cytometer (ABI, USA).

RNA isolation and quantitative real-time PCR

Total RNA was isolated by Trizol reagent and detected by ultraviolet spectrophotometer. cDNA was synthesized by using a miRNA reverse transcription kit (TaKaRa). SYBR-Green qPCR Mix was used for PCR reaction. GAPDH served as an internal reference for HCG18 and CDK16, while U6 served as the internal reference for miR-324-5p. The primers were as follows: HCG18 forward: 5'-GCTAGGTCCTCTACTTTCTG-3', reverse: 5'-CAGAAAGTAGAGGACCTAGC-3'; miR-324-5p forward: 5'-GAGGCCAAGCCCTGGTATG-3', reverse: 5'-CGGGCCGATTGATCTCAGC-3'; CDK16 forward: 5'-TTGGGCCGTTGGCTGTTC-3', reverse: 5'-GTGCTCACGGCGGCTC-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH forward: 5'-CAGTGCCAGCCTCGTCTAT-3', reverse: 5'-AGGGGCCATCCACAGTCTTC-3'.

The relative expressions of HCG18, miR-324-5p and CDK16 were calculated by $2^{-\Delta\Delta Ct}$ method.

Subcellular localization assay

Cytoplasmic and nuclear RNAs were extracted by the Nuclear/Cytosol Fractionation kit (Biovision, USA). RT-qPCR assay was used to detect the expression of HCG18 in cytoplasm and nuclear fractions of melanoma cells. GAPDH was used as the cytoplasm control, and U6 as the nucleus control.

Western blot

Total protein was obtained by RIPA cell lysate. The protein concentration and purity were detected by BCA kit. Proteins were isolated by 10% SDS-PAGE and transferred onto PVDF membrane. After blocked with 5% skim milk, the membrane was incubated with the primary antibody (CDK16, 1:1000; β-actin, 1:1000) overnight. After washing with TBST, the corresponding goat anti-rabbit secondary antibody (1:1000) was added to block for 1 h. Finally, the images were collected by the gel imaging system, and protein bands were analyzed by Image J.

Statistical analysis

The data were presented as mean ± standard deviation ($\bar{x}$ ± sd) and analyzed by SPSS 21.0 software. The differences between the two groups were compared by t test. The differences among multiple groups were compared by one-way ANOVA with Tukey post hoc test. Pearson coefficient method was used to detect the correlation among HCG18, miR-324-5p and CDK16. $P<0.05$ was considered statistically significant.

Results

HCG18 was upregulated in melanoma

LncRNA HCG18 has been shown to play a vital role in a variety of tumors; however, its role in melanoma has not been fully illustrated. In order to probe the function of HCG18 in melanoma progression, RT-qPCR was performed to measure the expression level of HCG18 in melanoma tissues and cells. The RT-qPCR analysis data displayed that the expression of HCG18 was dramatically higher in melanoma tissues

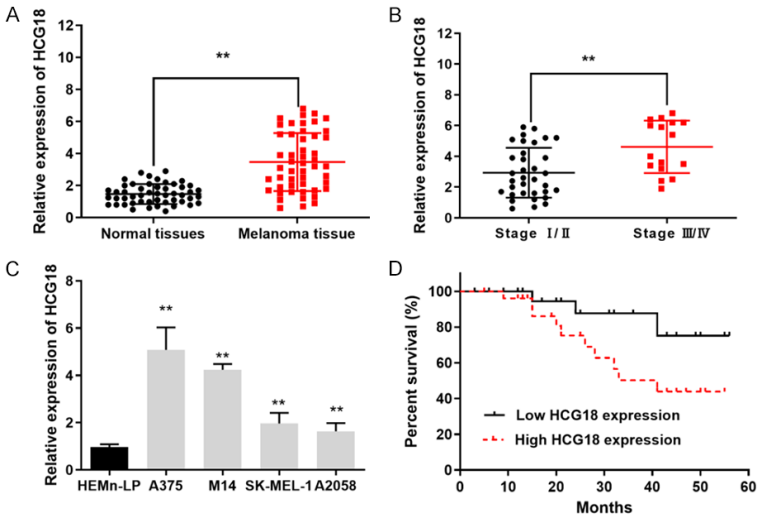

Figure 1. HCG18 was upregulated in melanoma. A: The expression of HCG18 in melanoma tissues and normal tissues was detected by RT-qPCR; B: The expression of HCG18 in melanoma patients with I/II and III/IV stage; C: The expression of HCG18 in melanoma cell lines (A375, SK-MEL-1, M14 and A875) and normal human epidermal melanin cells HEMa-LP; D: The survival rate in melanoma patients with high HCG18 expression and low HCG18 expression. **P<0.01.

Table 1. Association between HCG18 expression and clinicopathological characteristics of patients with melanoma

Clinical characteristics	Number of cases	HCG18 expression		P
		High (n=27)	Low (n=23)	
Age (years)				0.945
<50	22	12	10	
≥50	28	15	13	
Gender				0.093
Female	24	10	14	
Male	26	17	9	
Thickness				0.035*
<2 mm	29	12	17	
≥7 mm	21	15	6	
TNM stage				0.022*
I-II	33	14	19	
III-IV	17	13	4	
Metastasis				0.013*
Present	19	6	13	
Absent	31	21	10	

Note: *P<0.05.

than in the normal tissues (**Figure 1A**). In addition, the expression level of HCG18 in III/IV stage was higher compared with that in I/II stage (**Figure 1B**). Similarly, HCG18 was found to be upregulated in melanoma cells (A375, SK-MEL-1, M14 and A875) compared with HEMa-LP cells (**Figure 1C**). Furthermore, 50 patients with melanoma were divided into two subgroups according to the median value of HCG18 expression. The Kaplan-Meier curve showed that the overall survival time of patients with high expression of HCG18 was shorter than those with low HCG18 expression (**Figure 1D**). Additionally, the correlation between HCG18 expression and clinicopathological factors of melanoma patients was analyzed. We found that the expression of HCG18 was closely correlated with thickness (P=0.035), TNM stage (P=0.022) and metastasis (P=0.013; **Table 1**). Therefore, HCG18 was identified to be involved in melanoma progression.

HCG18 knockdown suppressed tumor development and promoted apoptosis of melanoma cells

To investigate whether HCG18 affects the progression of melanoma, HCG18 expression was decreased by HCG18 si-RNA. The expression of HCG18 was notably reduced in three specific si-RNA transfection groups compared with NC group, and the interference effect of si-HCG18 II was the most obvious (**Figure 2A**). Therefore, si-HCG18 II was selected for subsequent experiments. The proliferation ability of melanoma cells was measured by CCK-8 and clone formation assay. We found that HCG18 knockdown notably restricted proliferation in A375 cells and M14 cells (**Figure 2B**, **2C**). Likewise, the clone number of A375 cells and M14 cells was significantly decreased when HCG18 expression was downregulated (**Figure 2D**). FCM was performed to detect the function of HCG18

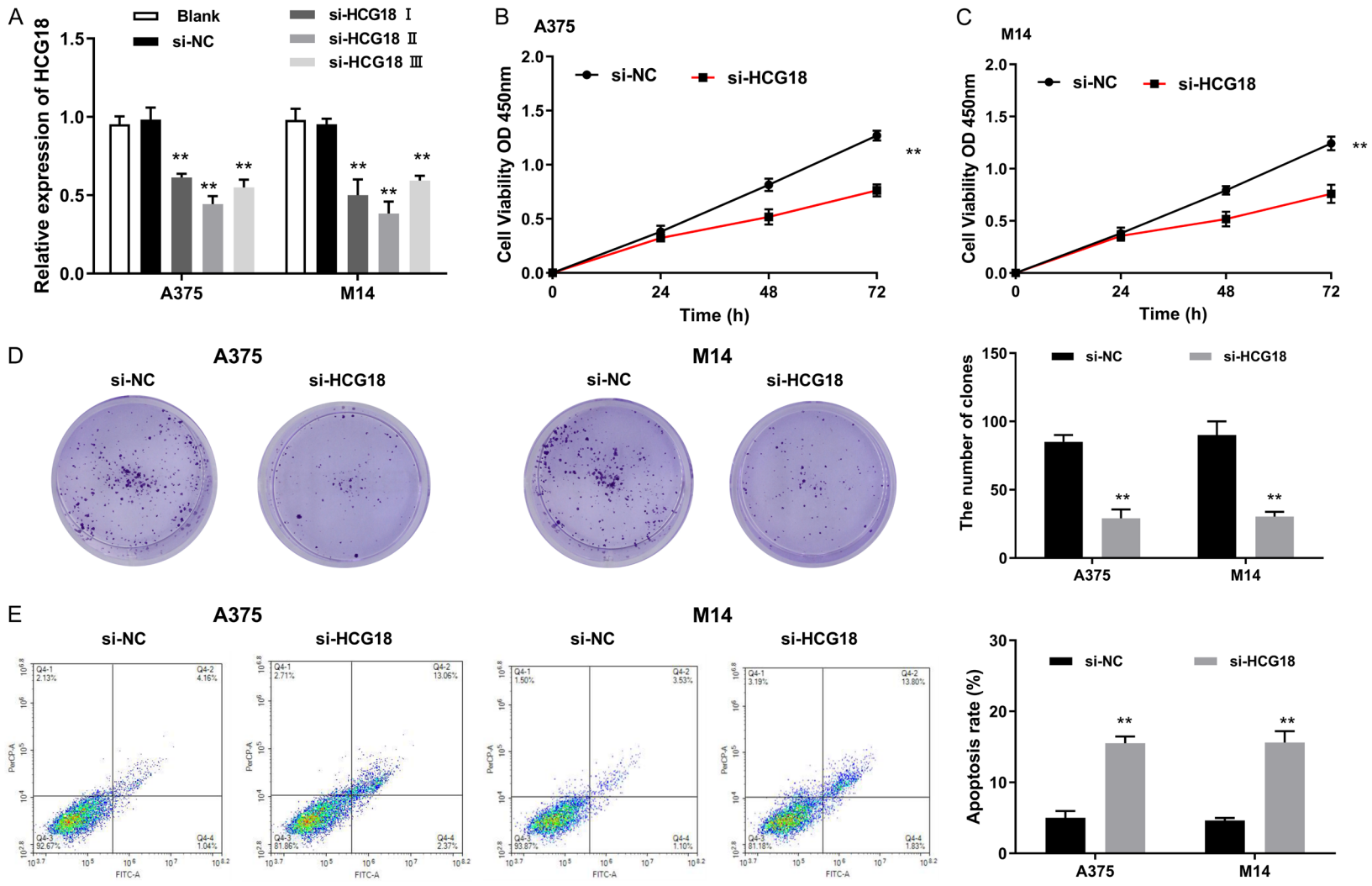

Figure 2. HCG18 knockdown suppressed growth and promoted apoptosis of melanoma cells. A: The expression of HCG18 in melanoma cells transfected with HCG18 si-RNAs; B and C: HCG18 knockdown notably suppressed proliferation of A375 and M14 cells; D: HCG18 knockdown reduced the number of clones; E: HCG18 promoted apoptosis in melanoma cells. **P<0.01.

silencing in cell apoptosis in melanoma cells. We noticed that HCG18 knockdown obviously increased cell apoptosis rate in melanoma cells (**Figure 2E**). Furthermore, the migration and invasion of melanoma cells were tested by Transwell assay. HCG18 knockdown was found to block melanoma cell migration (**Figure 3A**, **3B**). Similarly, cell invasion was suppressed by HCG18 knockdown (**Figure 3C**, **3D**).

HCG18 sponged miR-324-5p in melanoma cells

To detect the mechanism of HCG18 in the progression of melanoma, the distribution of HCG18 in melanoma cells was first detected. We noticed that HCG18 was mainly expressed in the cytoplasm, suggesting that HCG18 might function by sponging miRNAs (**Figure 4A**). Bioinformatics analysis was used to predict the potential target miRNAs of HCG18. There were binding sites between HCG18 and miR-324-5p predicted by StarBase (**Figure 4B**). Then, the paired binding sites were verified by using the dual-luciferase reporter assay and RNA pull-down. The relative luciferase activity of HCG18-WT was obviously declined under the influence of miR-324-5p, while HCG18-MUT remained at the same level (**Figure 4C**, **4D**). Moreover, we found that HCG18 was pulled down by biotin-labeled miR-324-5p oligos (**Figure 4E**, **4F**). Next, we found that miR-324-5p was downregulated in melanoma tissues (**Figure 4G**). In addition, we found no statistical significance between miR-324-5p expression and the survival time of patients (**Figure 4H**). Simultaneously, Pearson's correlation illustrated that HCG18 was inversely related to miR-324-5p expression in melanoma (**Figure 4I**). All data strongly proposed that HCG18 acted as a sponge of miR-324-5p in melanoma.

HCG18 acted as a sponge of miR-324-5p to increase CDK16 expression in melanoma

TargetScan showed that there were binding sits between CDK16 and miR-324-5p (**Figure 5A**). MiR-324-5p reduced the luciferase activity of CDK16-WT but had no effect on CDK16-MUT (**Figure 5B**, **5C**). The luciferase activity of CDK16-WT was reduced by HCG18 knockdown and rescued by miR-324-5p inhibitor (**Figure 5D**, **5E**). We noticed that CDK16 was overexpressed in melanoma tissues (**Figure 5F**). Western blot assay indicated that the protein expression of CDK16 was upregulated compared with normal tissues (**Figure 5G**). Moreover, CDK16 expression was not associated with the overall survival time in patients with melanoma (**Figure 5H**). Pearson's correlation illustrated that HCG18 was positively related to CDK16 expression in melanoma (**Figure 5I**). Therefore, our results indicated that HCG18 increased CDK16 expression by sponging miR-324-5p in melanoma.

HCG18/miR-324-5p regulated cell progression by upregulating CDK16 expression in melanoma

Furthermore, CDK16 expression was notably decreased by miR-324-5p mimic, while HCG18 reversed the effect of miR-324-5p mimic (**Figure 6A**). CCK-8 assay and clone formation assay illustrated that miR-324-5p mimic suppressed cell proliferation, while HCG18 overexpression attenuated the effect of miR-324-5p mimic (**Figure 6B-D**). Whereas, apoptosis rate of miR324-5p-elevated cells was inhibited by HCG18 vector (**Figure 6E**). Moreover, miR-324-5p mimic blocked cell migration and invasion, but the effect was attenuated by HCG18 overexpression (**Figure 6F**, **6G**). These results suggested that HCG18 facilitated the progression of melanoma by sponging miR-324-5p to upregulate CDK16 expression.

Discussion

Melanoma is the deadliest type of skin cancer, with a high degree of malignancy, and its incidence is increasing year by year. The cause of melanoma is unclear, and may be related to race, heredity, burns and traumatic stimuli. The carcinogenesis and pathophysiology of melanoma have been studied extensively in the past few decades. However, the molecular mechanism of melanoma development remains unclear. Therefore, exploring the pathogenesis of melanoma will help to develop new therapeutic targets and treatment approaches. In the study of the mechanism of melanoma, it was found that lncRNAs were involved in the regulation of the development of melanoma. For example, TUG1, NEAT1, and PVT1 influenced the occurrence and development of melanoma by regulating cell proliferation, migration, invasion, apoptosis and autophagy [15-18].

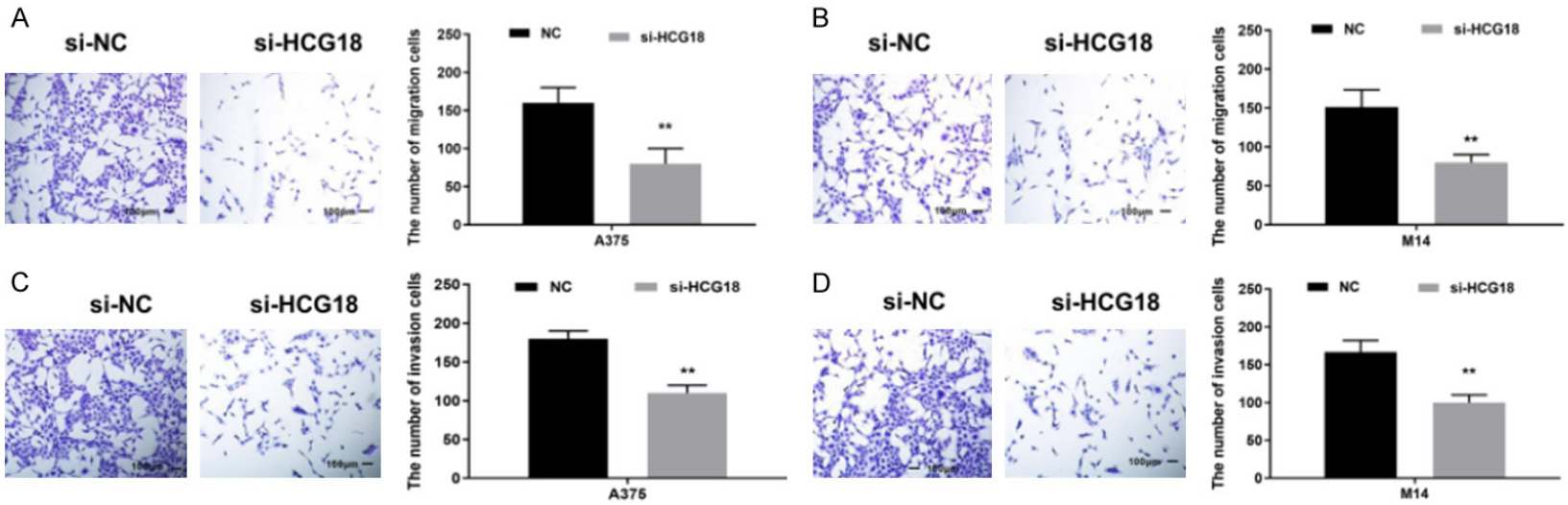

Figure 3. HCG18 knockdown suppressed migration and invasion of melanoma cells. A: HCG18 knockdown suppressed migration of A375 cells; B: HCG18 knockdown suppressed migration of M14 cells; C: HCG18 knockdown suppressed invasion of A375 cells; D: HCG18 knockdown suppressed invasion of M14 cells. $^{**}P<0.01$.

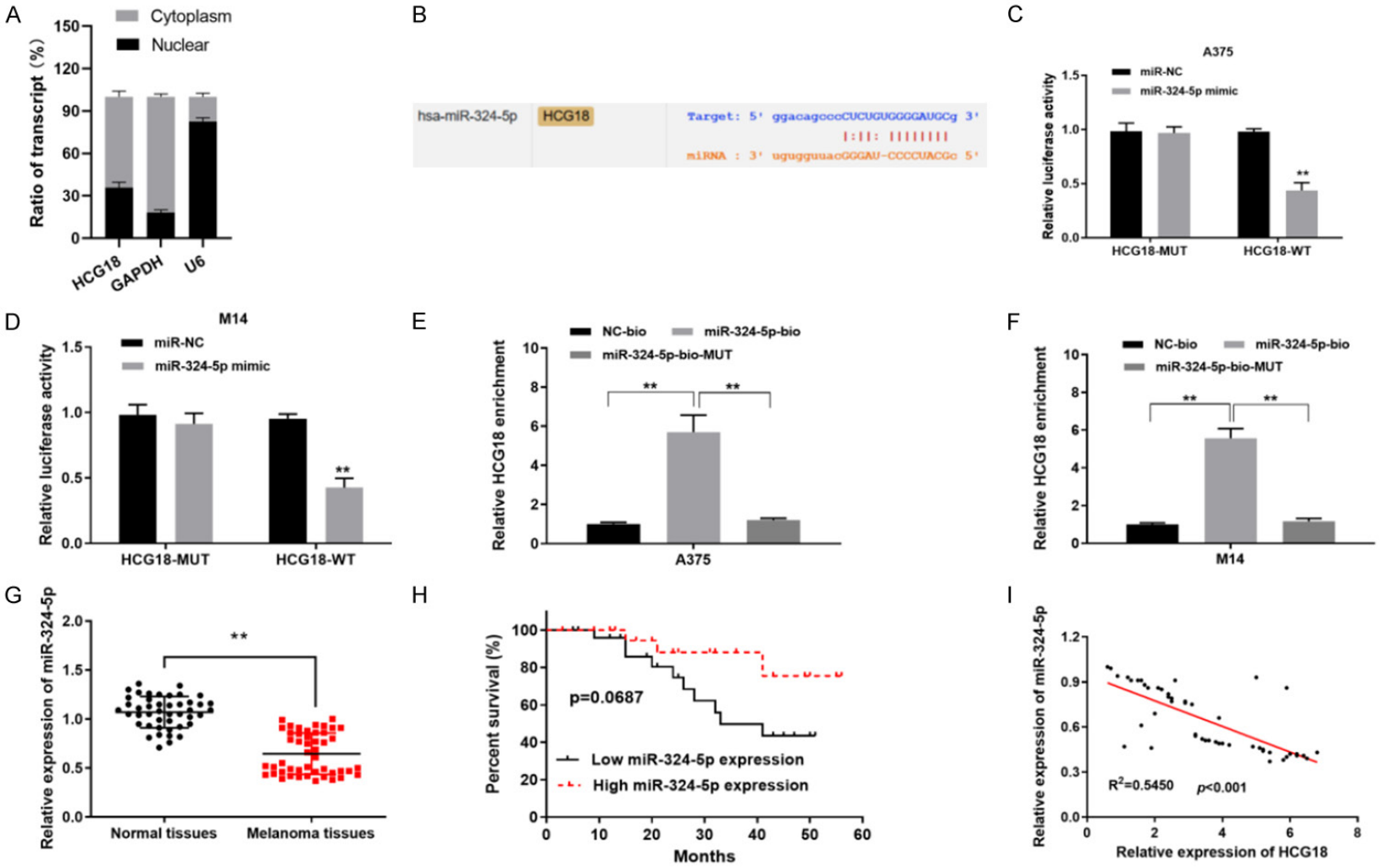

Figure 4. HCG18 sponged miR-324-5p in melanoma cells. A: Subcellular fractionation location assay; B: Base pairing region of miR-324-5p in WT-HCG18 was shown by StarBase; C and D: Luciferase activity of WT-HCG18 or MUT-HCG18 was evaluated in A375 and M14 cells co-transfected with miR-324-5p mimic; E and F: RNA pull down assay showed that HCG18 was pulled down by biotin-labeled miR-324-5p oligos; G: The expression of miR-324-5p in melanoma tissues; H: The survival rate in melanoma patients with high miR-324-5p expression and low miR-324-5p expression; I: Pearson correlation analysis identified a negative correlation between HCG18 and miR-324-5p expression in melanoma tissues. $^{**}P<0.01$.

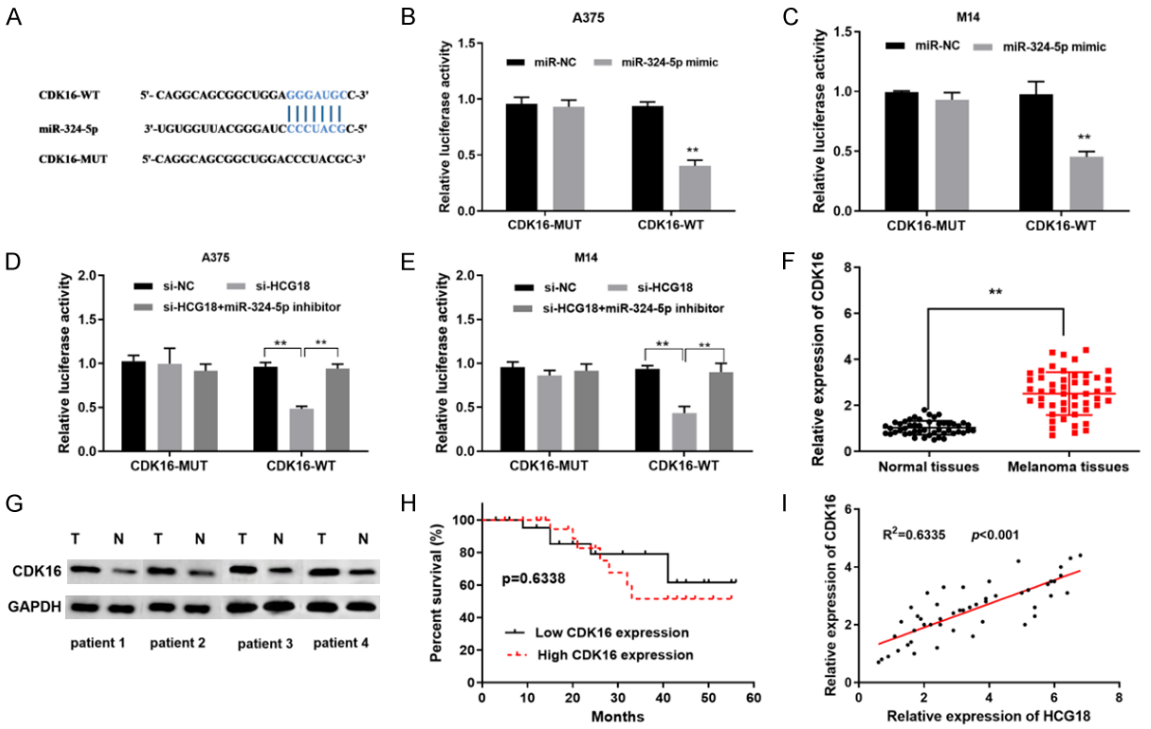

Figure 5. HCG18 acted as a sponge of miR-324-5p to increase CDK16 expression in melanoma. A: TargetScan showed that there were binding sites between CDK16 and miR-324-5p; B and C: Luciferase activity of WT-CDK16 or MUT-CDK16 was evaluated in A375 and M14 cells co-transfected with miR-324-5p mimic; D and E: Luciferase activity of indicated groups in melanoma cells; F: The mRNA expression of CDK16 in melanoma tissues; G: The protein expression of CDK16; H: The survival rate in melanoma patients with high CDK16 expression and low CDK16 expression; I: Pearson correlation analysis identified a positive correlation between HCG18 and CDK16 expression in melanoma tissues. $^{**}P<0.01$.

In the current study, we explored the expression and mechanism of HCG18 in the progression of melanoma. HCG18 is a recently discovered lncRNA that has been reported to play a key role in many types of cancers. HCG18 was upregulated in clear cell renal cell carcinoma, and acted as a tumor prompter by inhibiting miR-152-3p and increasing RAB14 [19]. In our work, we displayed that HCG18 was notably overexpressed in melanoma tissues. Additionally, HCG18 was discovered to be expressed with higher level in melanoma cells than the normal melanocytes. Hence, we suggested that HCG18 might be a tumor promoter in melanoma. Consistent with our findings, HCG18 was also overexpressed in gastric cancer [20]. Furthermore, we knocked down HCG18 expression in A375 and M14 cells. CCK-8, clone formation, Transwell and FCM were used to examine the role of HCG18 knockdown on melanoma cell progression. We found that HCG18 knockdown notably inhibited cell proliferation and mobility, while induced cell apoptosis in melanoma cells. Contrary to our findings, HCG18 was downregulated in papillary thyroid cancer, and it suppressed cell progression and induced cell apoptosis by regulating miR-106a-5p/PPP2R2A axis [21].

LncRNAs can act as a 'molecular sponge' to block the post-transcriptional inhibition of miRNAs in downstream target genes to restore the function of target genes [22]. Therefore, lncRNAs are called competitive endogenous RNAs (ceRNAs). HCG18 promoted tumor progression by sponging miR-214-3p to increase CENPM expression in hepatocellular carcinoma [23]. Liu et al. found that HCG18 regulated gastric cancer development by suppressing miR-141-3p expression [10]. Moreover, HCG18 was reported to facilitate colorectal cancer progression by inhibiting miR-1271 to facilitate MTDH expression [24]. In our study, HCG18 was confirmed to be a sponge of miR-324-5p by dual-luciferase reporter assay and RNA pull-down. Furthermore, HCG18 expression was

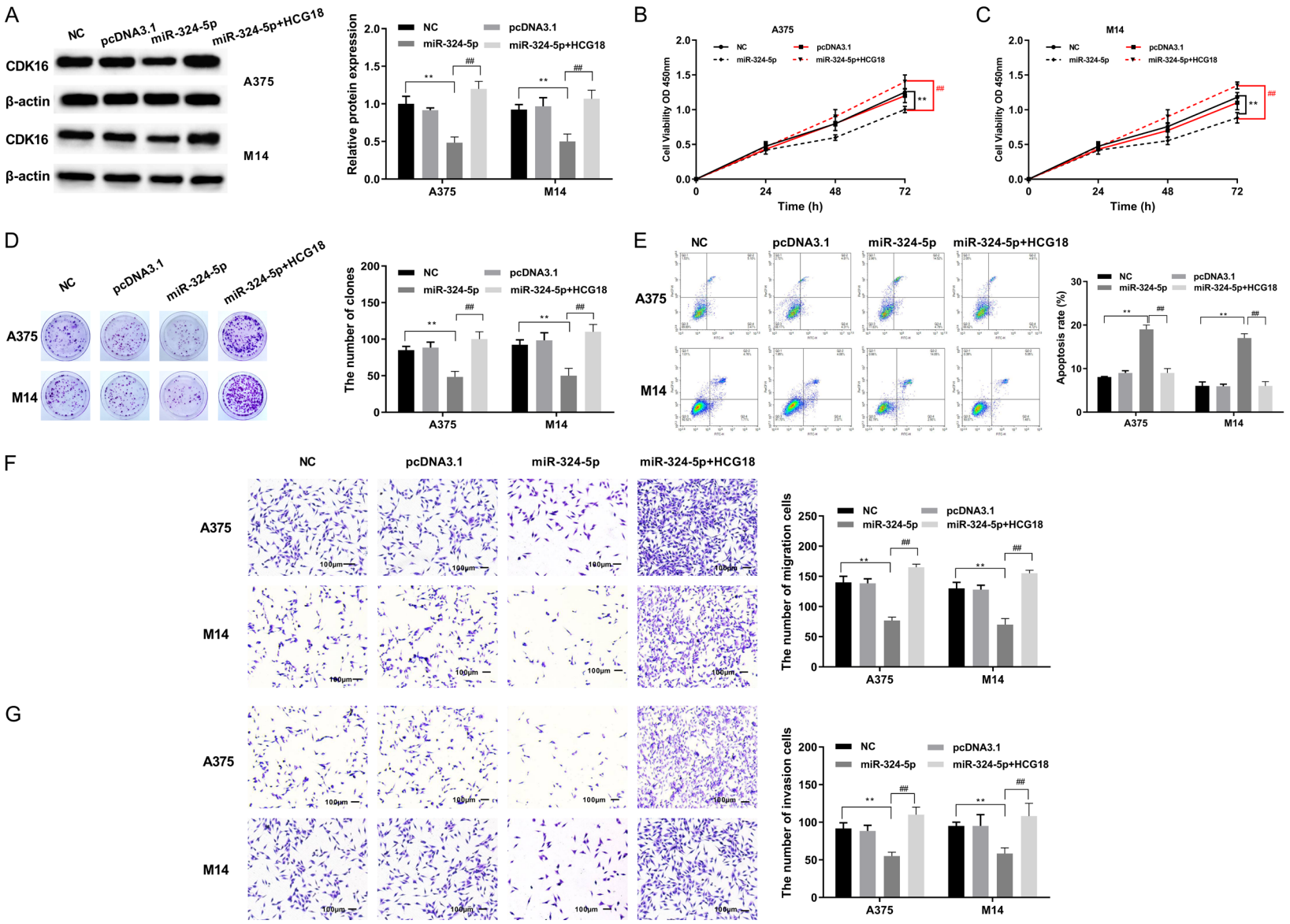

Figure 6. HCG18/miR-324-5p regulated cell progression by upregulating CDK16 expression in melanoma. A: The expression of CDK16 in indicated groups; B-D: The cell proliferation was detected by CCK-8 assay and colony formation assay; E: Cell apoptosis was detected by FCM; F and G: Cell migration and invasion were detected by Transwell assay. **P<0.01, compared to the NC group; ##P<0.01, compared to the miR-324-5p group.

negatively related to miR-324-5p expression in melanoma.

Cyclin-dependent kinase 16 (CDK16), located on chromosome Xp11.23, is a serine/threonine protein kinase. CDK16 can play a vital role in the signal transduction pathways of terminally differentiated cells, and in exocytosis and the transport of secretory proteins from the endoplasmic reticulum [25]. Numerous studies have reported that CDK16 is overexpressed in various tumors, and plays a vital role in tumor cell proliferation, migration, invasion and apoptosis [26, 27]. In this work, CDK16 was confirmed to be the target gene of miR-324-5p. CDK16 was upregulated in non-small lung cancer, and acted as an oncogene by regulating p27 expression [28]. We noticed that CDK16 was notably overexpressed in melanoma tissues. Similar to our results, Yanagi et al. found that CDK16 was overexpressed in melanoma [29]. Our results verified that HCG18 facilitated cell progression by sponging miR-324-5p to upregulate CDK16 expression in melanoma. However, our study still has some limitations. *In vivo* studies are needed to verify the role of HCG18 in melanoma. In addition, other mechanisms of HCG18 in the progression of melanoma need to be further studied.

In summary, HCG18 acts as a cancerogen in melanoma, and it facilitates melanoma cell progression by regulating miR-324-5p/CDK16 axis. Our findings suggest that HCG18 may be a target for the diagnosis and treatment of melanoma.

Disclosure of conflict of interest

None.

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