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*Original Article*

# LncRNA HCG18 promotes cell multiplication and metastasis by miR-148b/ETV5 regulation in osteosarcoma

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**Abstract:** Objective: To determine the effects of long non-coding RNA (LncRNA) HCG18 on cell multiplication and invasion of osteosarcoma. Methods: MTT assay and transwell assay were used for cell multiplication and invasion, respectively. Real-time quantitative polymerase chain reaction (RT-qPCR) and western blot were used to determine transcriptional and translational expression. Luciferase reporter assay was used to identify the specific target relationships. Results: The expression of LncRNA HCG18 was dramatically increased in osteosarcoma cells compared to the normal tissues. LncRNA-HCG18 accelerated cell multiplication and invasion *in vitro*, which was achieved by down-regulating the overexpression of miR-148b, and down-regulating ETV5, indicating combination of ETV5 and miR-148b in osteosarcoma. Overexpression of ETV5 could reverse the inhibitory effect of knockout of lncRNA HCG18 on cell multiplication and invasion. Conclusion: LncRNA HCG18 acted as a sponge of miR-148b and played an oncogenic role in osteosarcoma, providing therapeutic targets for osteosarcoma.

**Keywords:** LncRNA HCG18, osteosarcoma, cell multiplication, invasion

## Introduction

Osteosarcoma, a malignant bone tumor, accounts for about 3-5% of malignant tumors [1, 2]. Osteosarcoma has a very poor prognosis [3-5]. Although many environmental carcinogenic factors and oncogenes have been confirmed, the cause of osteosarcoma remains unclear. The current treatment for osteosarcoma includes surgical resection supplemented by chemotherapy. However, the current treatment regimen is only effective for patients who have not yet metastasized [6-8]. Therefore, it is urgent to the diagnosis biomarkers and treatment targets for osteosarcoma.

Long non-coding RNAs (LncRNAs) plays an important role in many life activities, such as dose compensation effect, epigenetic regulation, cell cycle regulation, and cell differentiation regulation [9]. The oncogenic role of LncRNA HCG18 (herein referred to HCG18) has been verified in various cancers, such as gastric cancer [10, 11], hepatocellular carcinoma [12], as well as colorectal cancer [13]. In Hodgkin lymphoma, HCG18 is a key LncRNA in disease relapse [14]. The regulation of many kinds of LncRNAs in osteosarcoma was also verified [15, 16], while the HCG18 has been rarely investigated. MicroRNAs (miRNAs) play an important role in cell growth and apoptosis, blood cell differentiation, homeobox gene regulation, neuronal polarity, insulin secretion, brain morphogenesis, cardiogenesis, and post embryonic development [17]. Recently, a study showed that the decline of miR-148b in osteosarcoma is related to a poor clinical prognosis [18]. Notice, as a competitive endogenous RNA (ceRNA), lncRNA interacts with miRNA, participates in the expression regulation of target genes, and plays an important role in tumorigenesis and development [19]. Besides, LncRNA-miRNA interaction network contributes to further experimental studies and can be used to improve biomarker prediction to develop new treatments for cancer [20].

ETV5, a transcription factor of the ETS family, has been shown to contribute to tumor growth and progression in many types of cancer [21, 22]. The function of ETV5 in osteosarcoma remains largely unknown. The combination of miR-148b and ETV5 was predicted by bioinformatics website ENCORI (http://starbase.sysu.edu.cn/). The overexpression of ETV5 has been implicated in some malignancies, including thyroid cancer [21], ovarian cancer [23], and non-small cell lung cancer (NSCLC) [24], and is validated to modulate cell multiplication, migration, and invasion. Nonetheless, the role of ETV5 and miR-148b in osteosarcoma and the specific mechanisms need to be further verified.

The research aimed at investigating the expression and function of HCG18 in osteosarcoma, and the downstream mechanism was investigated. Here, we found that HCG18 was up-regulated in osteosarcoma, promoted cell multiplication and invasion by targeting miR-148b negatively, and then enhanced the expression of ETV5.

## Material and methods

### *Cell culture*

Cells lines of osteosarcoma including U2OS, 143B, MG-63, Saos-2 and HOS cells, and hFOB1.19 cells, a human normal osteoblastic cell line, were collected from American Type Culture Collection (ATCC) and incubated with DMEM culture (Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS) (Gibco) in 37°C with 5% $CO_2$. Specimens of clinical tumor tissues and paracancerous tissues of osteosarcoma were derived from newly diagnosed untreated patients.

This study was approved by the Ethics Committee of the people's Hospital of Hanchuan City, Hubei Province. Before participating in the experiment, each subject was required to obtain written informed consent.

### *Luciferase reporter assay*

The binding sites of miR-148b with HCG18 and ETV5 were predicted with the tool of Starbase 2.0 (http://starbase.sysu.edu.cn). To examine whether miR-148b targeted HCG18 and ETV5 directly, HCG18 WT reporter plasmid, mutated type (mut) HCG18 reporter plasmid (mut HCG18), ETV5 WT reporter plasmid, and mut ETV5 reporter plasmid were established into the pmiR-GLO vector (Promega, Madison, WI, USA). The assays were described previously [25].

### *Western blot*

In short, total cellular proteins were extracted with RIPA buffer. The protein concentration was determined by BCA method. The same amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millikon, Boston, USA). 3% bovine serum albumin (BSA) was sealed in PBST at room temperature for 1 hour, then incubated with the first antibody at 4°C overnight.

Main antibodies: ETV5 (1:1000, Cell Signaling Technology, USA) and GAPDH (diluted 1:2000, Cell Signaling Technology). The membrane and the second antibody were incubated at room temperature for 2 hours, and the protein bands were displayed by ECL system (Amersham Pharmacia, Piscataway, NJ, USA).

### *Real-time quantitative polymerase chain reaction (RT-qPCR)*

Total RNA was extracted (TRIzol reagent, Takara, Otsu, Japan), reversed to cDNA (RevertAid cDNA Synthesis Kit, ThermoFisher Scientific), and amplified (ABI Prism 7900 HT platform, Applied Biosystems, Foster City, CA, USA). GAPDH was used as endogenous control. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative gene expression. The primers used in this study were as follows: HCG18-F: 5'-TGAAGTCGACGAGAGGAGC-3'; HCG18-R: 5'-ACTAGTCGAGAGTGAGGTGC-3'. GAPDH-F: 5'-TCCGTGGTCCACGAGAACT-3; GAPDH-R: 5'-GAAGCATTTGCGGTGGACGAT-3'. miR-148b-F: 5'-CCAAATTTCCCAATCAGGCATCACAG-3'; miR-148b-R: 5'-TAACCATTAGCAGGGTCCGAGGTATTC-3'. TV5-F: 5'-ACTGGAAGGC AAAGTCAAAC A-3'; TV5-R: 5'-GCTGGGTCAT CAAGAAGGGT GA-3'.

### *Cell transfection*

HCG18 short hairpin RNA (ShRNA), miRNA mimic and inhibitor (GenePharma, Shanghai, China), were used for the transfection of miRNAs mimics or inhibitors. The transfection materials were diluted with serum-free medium

and mixed with Liposome 2000 reagent (Invitgen, Carlsad, CA, USA). After 48 hours, the cells were used in functional experiments.

*MTT assay*

The transfected cells were added to a 96-well plate and cultured at 37°C with 5% $CO_2$. Then we added 10 μL MTT solution (10 mg/mL), incubate for 2 hours, removed the culture medium, and dissolved formazine with 100 μL dimethyl sulfoxide solution. The OD was measured at 490 nm with a micro flat panel reader (BioTek, Winooski, VT, USA).

*Transwell assay*

This was used for determining the cell invasion. The upper chambers were precoated with Matrigel (BD Biosciences, USA). Then the cells were diluted to 2 × $10^5$/well and suspended into the upper chamber. DMEM medium (with 12% FBS) was added into the lower chamber. After cells passed through the membrane, paraformaldehyde was used to fix cells and the cells were dyed with 0.1% crystal violet. The cells were observed by microscope and counted. The cell invasion test was the same as the cell migration test, except that a certain proportion of the Matrigel (BD Biosciences, CA, USA) (serum-free medium:Matrigel =7:1) was added to the Transwell chamber.

*RIP assay*

RIP was performed using Magna RIP™ kit (Millipore, Billerica, MA, USA). The harvested cells were lysed and incubated with magnetic beads coupled with anti-Argonaute 2 (micropores), with IgG (micropores) as negative control. Proteinase K was used to remove the protein, immunoprecipitated RNA was purified, and qRT-PCR analysis was carried out.

*RNA pull-down assay*

Biotin-labeled probe (Bio-miR-148b) and its negative control against miR-148b were from Shanghai, China. Transfected cells were lysed and incubated with streptavidin coupled microspheres (Sangon). After treatment with protease K, HCG18 was detected using qRT-PCR.

*Statistical analysis*

Results are shown as the mean ± SD and analyzed by SPSS software, 16.0 (SPSS, Chicago, USA). The statistical approaches mainly included a two-tailed Student's *t* test. A difference with $P<0.05$ was regarded to be significant. Graphs were mainly made by GraphPad Prism 6 (GraphPad, San Diego, USA).

## Results

*Expression of LncRNA HCG18, miR-148b, and ETV5 in osteosarcoma*

To detect the levels of HCG18, miR-148b, and ETV5, RT-qPCR assay was used to detect the osteosarcoma tissues (n=20) and adjacent tissues (n=20). The expressions of HCG18 and ETV5 were increased in tumor cells, while the expression of miR-148b was decreased (**Figure 1A**). In order to select the proper cell model with high expression of HCG18 for further investigation, the cell lines of osteosarcoma including U2OS, 143B, MG-63, Saos-2, and HOS were screened. As **Figure 1B**, HCG18 expression was increased in the cell lines of osteosarcoma and Saos-2 cells had the most obvious elevation of expression of HCG18. In addition, the level of miR-148b was reduced and the expression of ETV5 was revised in Saos-2 cells, respectively.

*The decreased expression of HCG18 suppressed the cell multiplication and invasion in osteosarcoma cells*

To determine the part that HCG18 played in cell multiplication and invasion of osteosarcoma cells, *HCG18* shRNA was used to silence the level of HCG18. The expression of HCG18 was suppressed in Saos-2 cells transfected with *HCG18* shRNA (**Figure 2A**). The cells were cultured for 24~96 h, and the viability of the cells with decreased HCG18 level was reduced, compared to the Saos-2 cells transfected with shRNA vector (**Figure 2B**). Furthermore, the migration and invasion ability of Saos-2 cells was also reduced after HCG18 knockdown (**Figure 2C**). The results showed that HCG18 encouraged cell multiplication and invasion in osteosarcoma cells.

*HCG18 regulated cell multiplication and invasion by targeting miR-148b*

We delved into the mechanism of HCG18 on regulation of cell multiplication and invasion in osteosarcoma. RT-qPCR was performed and showed that knockdown of HCG18 enhanced

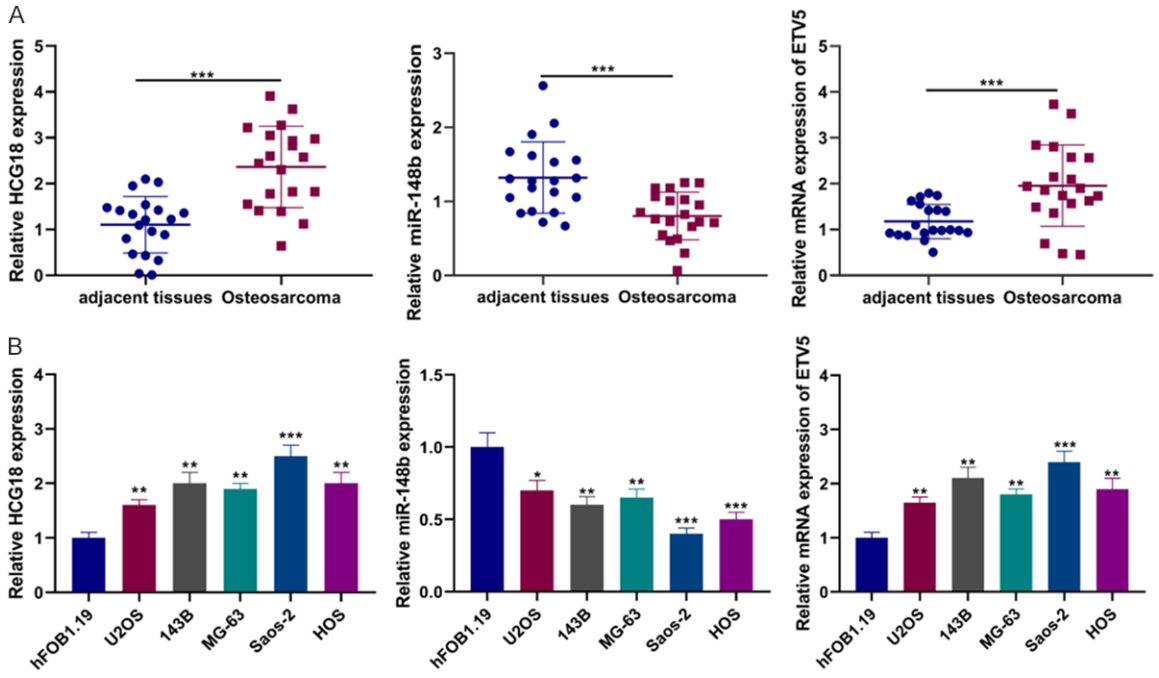


**Figure 1.** Expression of HCG18, ETV5, and miR-148b in osteosarcoma. A. The expression of HCG18, ETV5, and miR-148b in tumor tissues and normal tissues were determined by RT-qPCR (n=10). ***$P$<0.001, compared with adjacent tissues. B. The expression of HCG18, ETV5, and miR-148b in cell lines were determined by RT-qPCR. *$P$<0.05, **$P$<0.01, ***$P$<0.001, compared with hFOB1.19 group.

the expression of miR-148b in Saos-2 cells (**Figure 3A**), showing HCG18 regulated the expression of miR-148b negatively. To explore the underlying mechanisms of HCG18, Starbase v2.0 forecasted the miRNA binding sites and found conserved binding sites of miR-148b (**Figure 3B**). Luciferase reporter analysis showed that mir-148b mimics and HCG18-WT transfection significantly inhibited the luciferase activity (**Figure 3B**), indicating that HCG18 targeted miR-148b. RIP and RNA pull-down experiments further revealed that HCG18 could bind to miR-148b in cells (**Figure 3C**, **3D**). Then, the function of miR-148b on the cell multiplication and invasion was verified. After raising the level of miR-148b by transfection of miR-148 mimics (**Figure 3E**), compared with mimic NC group, the cell vitality was reduced remarkably (**Figure 3F**), and the cell invasion and migration were suppressed (**Figure 3G**). Correlation analysis also indicated a negative correlation between the HCG18 and miR-148b (**Figure 3H**). It is suggested that high expression of miR-148 suppressed the cell multiplication and invasion in osteosarcoma.

*MiR-148b negatively regulates ETV5*

We further investigated the downstream mechanism of miR-148b regulation. First, after high expression of miR-148b, the level of ETV5 was reduced (**Figure 4A**, **4B**), suggesting a negative regulation of miR-148b on ETV5. It was predicted that miR-148b could bind ETV5 (**Figure 4C**). The luciferase reporter assay was used to determine the interaction of miR-148b and ETV5 and the results indicated that after transfection with miR-148b mimics and ETV5 wide type (WT), the relative luciferase activity decreased (**Figure 4C**). This verified that ETV5 was the direct target of miR-148b. RIP and RNA pull-down experiments further revealed that ETV5 could bind to miR-148b in cells (**Figure 4D**, **4E**).

*HCG18 targeted miR-148b and up-regulated ETV5 expression in osteosarcoma*

To make a thorough inquiry the regulation of HCG18/miR-148b/ETV5 on cell multiplication and invasion, Saos-2 cells were transfected with *HCG18* shRNA or miR-148a inhibitor. The

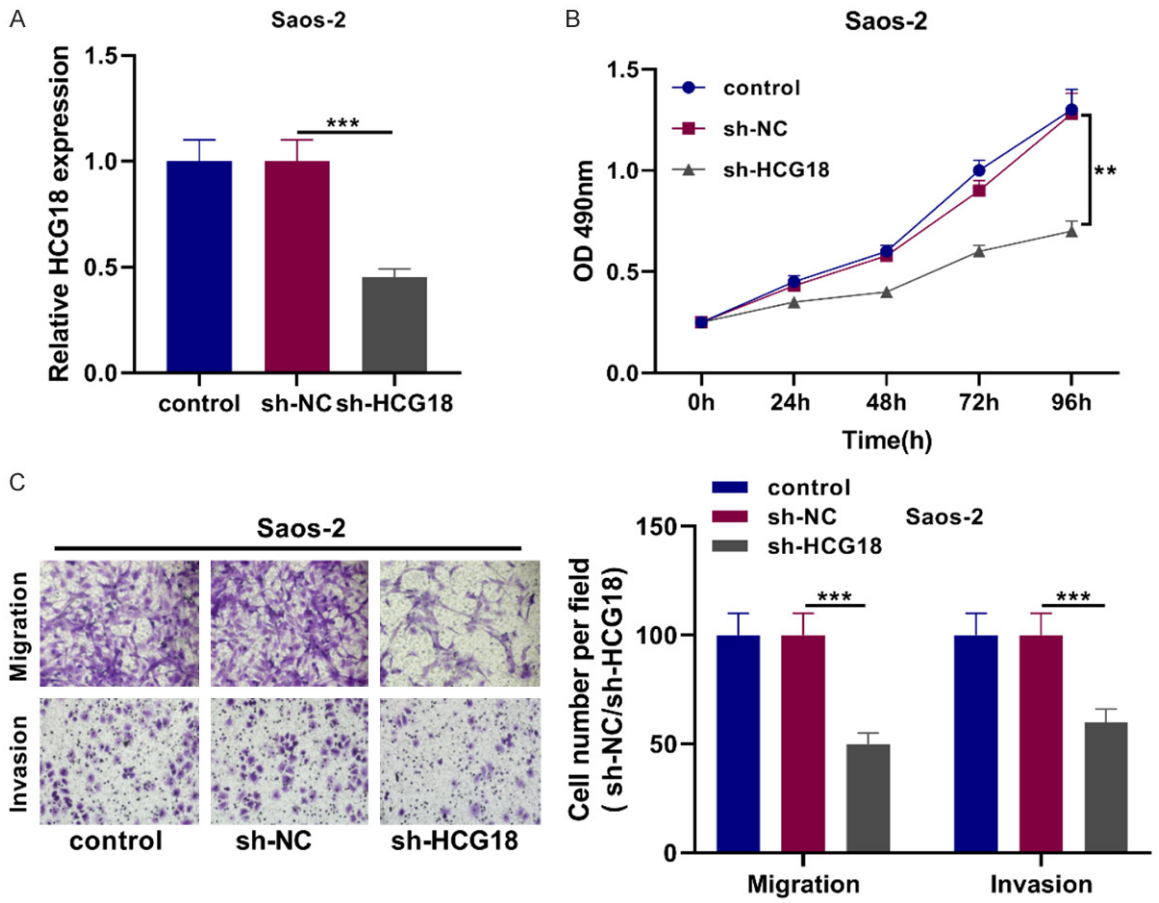


**Figure 2.** The regulation of HCG18 on cell multiplication and invasion in Saos-2 cells. A. The expression of HCG18 in Saos-2 cells transfected with *HCG18* shRNA. ***P*<0.01, compared with sh-NC. B. The cell viability was determined by MTT assay. ***P*<0.01, compared with sh-NC. C. Cell invasion and migration were determined by transwell assay. The scale was 20×. ****P*<0.001, compared with sh-NC.

expression of ETV5 was decreased after *HCG18* shRNA transfection, while it was increased when miR-148b knockdown (**Figure 5A**). As shown in **Figure 5B**, **5C**, both of cell multiplication, invasion and migration ability were decreased after HCG18 knockdown. However, the cell multiplication and invasion were promoted after miR-148b inhibitor transfection. This suggests that miR-148b was a downstream target of HCG18 and was involved in HCG18-regulated cell multiplication and invasion in osteosarcoma.

## Discussion

LncRNAs as an oncogene or tumor suppressor, and play an significant part in the process of oncogenesis and development [26, 27]. In our study, we explored the oncogenic role of HCG18 in osteosarcoma, showing the mechanism of HCG18 on cell multiplication and invasion. HCG18 was enhanced in clinical osteosarcoma tumor tissues, targeted miR-148b negatively, and enhanced the expression of ETV5. Knockdown of HCG18 lessened cell multiplication and invasion effectively, which could be blocked by high expression of ETV5.

In various cancers, previous studies have shown the tumorigenic effects of HCG18, whose oncogenic role was also verified in osteosarcoma presently [28, 29]. For example, HCG18 promoted progression of hepatocellular carcinoma by the miR-213-3p/CENPM axis [12], and facilitated gastric cancer progression by increasing DNAJB12 expression by sponging miR-152-3p [30]. In addition to cancers, HCG18 also promoted disc degeneration by inhibiting miR-146a-5p/TRAF6 axis [25]. Moreover, by regulating miR-146a/TRAF6 axis, M1 macrophage polarization was promoted and diabetic peripheral neuropathy was promoted [31]. In

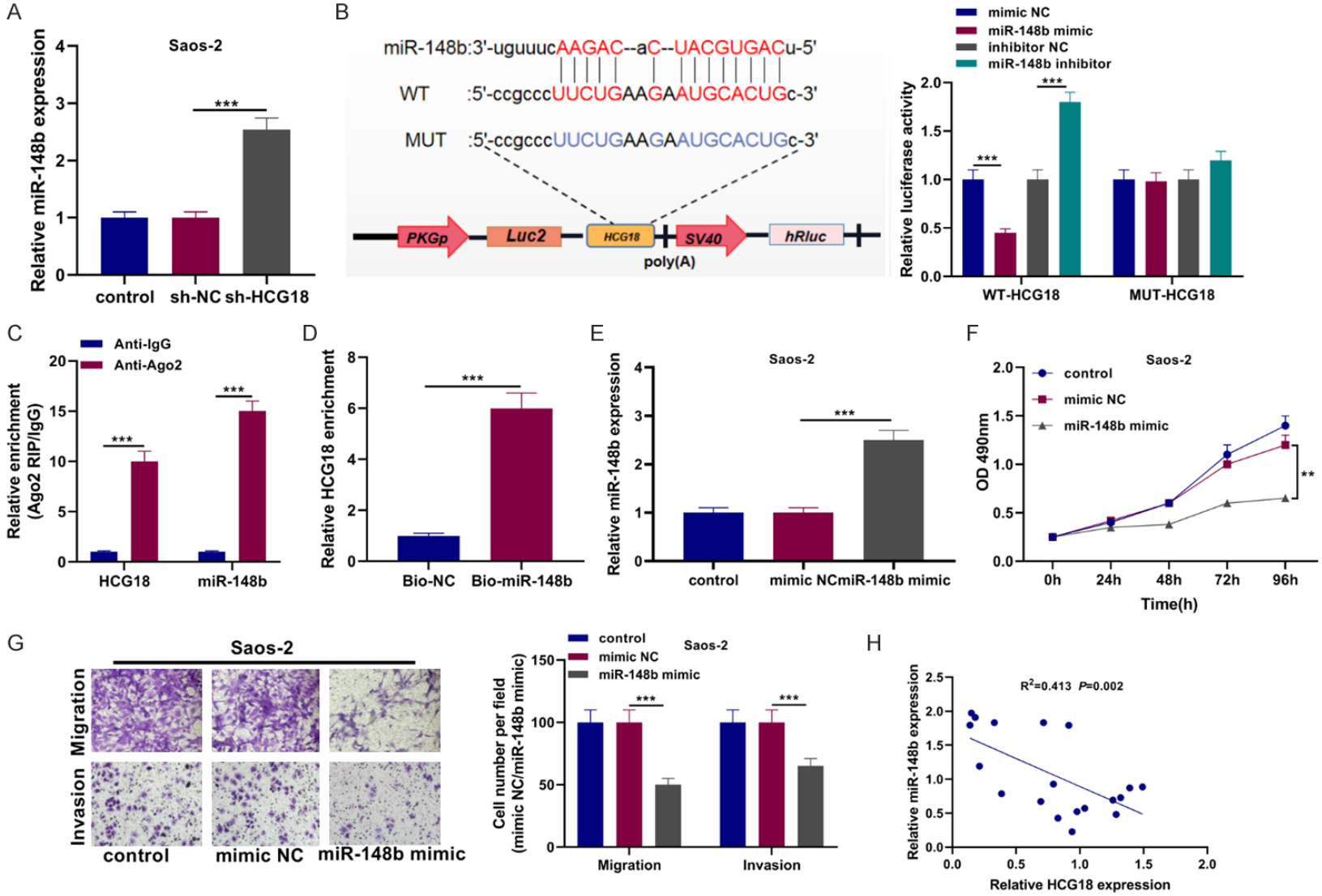


**Figure 3.** The regulation of miR-148b on cell multiplication and invasion in Saos-2 cells. A. The expression of miR-148b in Saos-2 cells transfected with *HCG18* shRNA. ***$P$<0.001, compared with sh-NC. B. Predictive binding sites and luciferase activity of HCG18 WT and HCG18 mut in cells treated with miR-148b mimic, inhibitor, or NC. ***$P$<0.001, compared with miR-148b mimic/inhibitor. C. RNA immunoprecipitation assay was used to detect the combination of HCG18 and miR-148b. ***$P$<0.001, compared with Anti-IgG. D. The relationship between HCG18 and miR-148b was evaluated by RNA pull-down assay using biotinylated HCG18 probe. ***$P$<0.001, compared with Bio-NC. E. The expression of miR-148b in Saos-2 cells transfected with miR-148b mimic. ***$P$<0.001, compared with mimic NC. F. The cell viability was determined by MTT assay. **$P$<0.01, compared with mimic NC. G. Cell invasion and migration were determined by transwell assay. The scale was 20×. ***$P$<0.001, compared with mimic NC. H. Correlation analysis of relative expression of HCG18 and miR-148b.

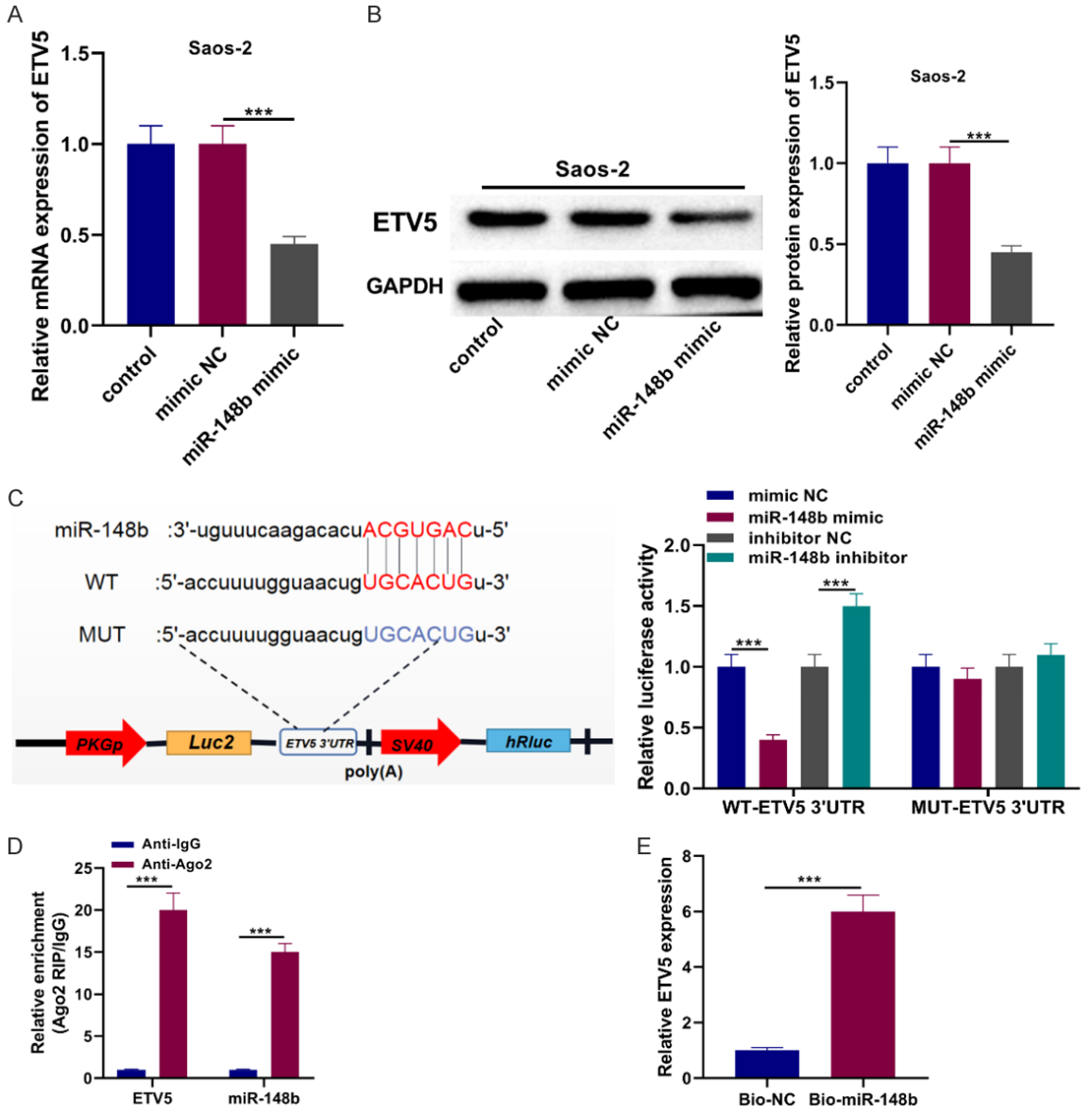


**Figure 4.** ETV5 is the target of miR-148b. A, B. The expression of ETV5 in Saos-2 cells transfected with miR-148b mimic. ***$P$<0.001, compared with mimic NC. C. Predictive binding sites and luciferase activity of ETV5 WT and ETV5 mut in cells treated with miR-148b mimic, inhibitor or NC. ***$P$<0.001, compared with miR-148b mimic/inhibitor. D. RNA immunoprecipitation assay was used to detect the combination of ETV5 and miR-148b. ***$P$<0.001, compared with Anti-IgG. E. The relationship between ETV5 and miR-148b was evaluated by RNA pull-down assay using biotinylated ETV5 probe. ***$P$<0.001, compared with Bio-NC.

these diseases, HCG18 was aberrantly up-regulated. Our results also showed an increased expression of HCG18 in clinical tumor tissues of osteosarcoma. This indicated that HCG18 expression might be a prognostic biomarker in cancers and related disease. However, only Saos-2 cells, which had higher expression of HCG18, were used for verification in vitro, and the sample size was small (only 10 patients participated). According to our results of screening of HCG18 expression, although there is an increasing tendency among the cell lines and tumor samples of osteosarcoma, there were some exceptions. HCG18 expression was not increased significantly in a few tumor samples (**Figure 1A**). This might result from individual differences. Therefore, the correlation between expression of HCG18 and malignant degree of cancer needs to be further investigated.

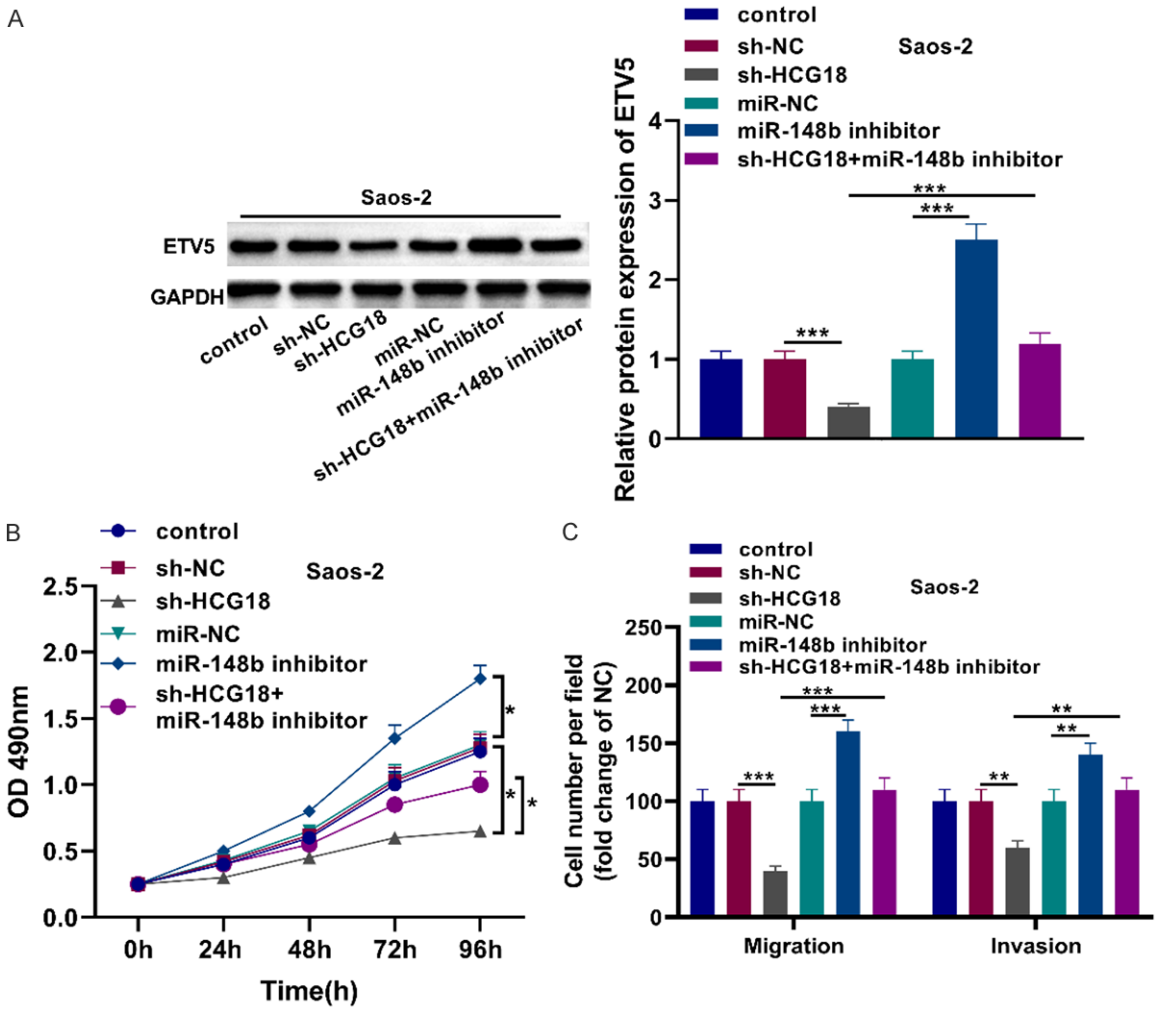


**Figure 5.** Regulation of HCG18/miR-148b/ETV5 on cell multiplication and invasion. A. The expression of ETV5 in Saos-2 cells transfected with *HCG18* shRNA and miR-148b inhibitor. ***$P$<0.001, compared with sh-NC/miR-NC/sh-HCG18. B. The cell viability was determined by MTT assay. *$P$<0.05, compared with sh-NC/miR-NC/sh-HCG18. C. The cell invasion and migration were determined by transwell assay. **$P$<0.01, ***$P$<0.001, compared with sh-NC/miR-NC/sh-HCG18.

miR-148b has been proposed to act as a tumor suppressor miRNA in gastrointestinal stromal tumor and endometrial cancer cells [32, 33], and shows inhibitory effects on cancer progression, including metastasis [34], cell growth, and multiplication [35]. Down-regulated miR-148b also increases resistance in diffuse large B-cell lymphoma cells [36]. This evidence indicates the antitumor role of miR-148b in cancer. Our results are consistent with the previous report. It was shown that expression of miR-148b was decreased in osteosarcoma, and was related to cell multiplication and invasion negatively. We also inferred that HCG18 was a ceRNA in osteosarcoma, suggesting that HCG18 contained a miR-148b binding site. The expression of HCG18 and miR-148b in osteosarcoma were negatively corelated, inferring that HCG18 could sponge miR-148b in osteosarcoma.

The ETS family of transcription factors plays an important role in cell multiplication, differentiation, lymphocyte development, transformation, angiogenesis, and apoptosis [37]. ETV5 is a pivotal member of the ETS family, and its up-regulation advanced to tumor growth and progression of thyroid cancer through PIK3CA [21]. In neuroblastoma, increased ETV5 expression significantly affects graft migration, invasion, and colony formation, and ETV5 inhibition reduces cell multiplication in the mouse xeno-

graft model [38]. These facts show that ETV5 has the characteristics of oncogenes and attach great importance to the occurrence and development of tumors. Our results showed that ETV5 up-regulation promoted the cell multiplication and invasion in osteosarcoma, and reversed the inhibitory effects of HCG18 knockdown. In this study, ETV5 was identified as a target gene for osteosarcoma, which was consistent with previous reports. Here, our results suggested that miR-148b up-regulation partially reverses HCG18 down-regulation and reduces the effect of ETV5. These data suggest that HCG18 exerts its biologic effects at least in part through the miR-148b/ETV5 pathway. In addition, previous studies have shown that ETV5 transcription is associated with the promotion of BDNF and EMT at the forefront of endometrial carcinoma infiltration [39]. Thus, the downstream mechanism of ETV5-regulated cell multiplication and invasion will be further studied.

## Conclusion

Our study found that HCG18 promoted ETV5 expression by sponging miR-148b, and then promoted cell multiplication and invasion in osteosarcoma. With the further development of relevant studies in the future, HCG18 may provide a new molecular target for the treatment of osteosarcoma.

## Acknowledgements

This study was approved by the Ethics Committee of Hubei Hanchuan people's Hospital. Written informed consent was obtained from each subject prior to participation.

## Disclosure of conflict of interest

None.

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