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LncRNA HCP5 promotes the development of cervical cancer by regulating MACC1 via suppression of microRNA-15a

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Abstract. – OBJECTIVE: To explore the role of long non-coding RNA (lncRNA) HCP5 in the development of cervical cancer and its underlying mechanism.

PATIENTS AND METHODS: Expression levels of HCP5, MACC1 and microRNA-15a in cervical cancer tissues and paracancerous tissues were detected. The relationship between HCP5 expression and prognosis of patients with cervical cancer was analyzed by Kaplan-Meier. Cell proliferation was detected by Cell Counting Kit-8 (CCK-8) assay after altering expressions of HCP5 and microRNA-15a by plasmids transfection. The binding condition of HCP5, MACC1 and microRNA-15a was evaluated by luciferase reporter gene assay. The regulatory effect of microRNA-15a on MACC1 expression was determined by Western blot.

RESULTS: HCP5 and MACC1 were overexpressed in cervical cancer tissues than those of paracancerous tissues. The survival rate of patients with cervical cancer was negatively correlated to HCP5 expression, but positively correlated to microRNA-15a expression. Luciferase reporter gene assay showed that microRNA-15a was directly bound to HCP5 and MACC1. Besides, overexpression of microRNA-15a could remarkably inhibited MACC1 expression. *In vitro* experiments showed that HCP5 promoted proliferation of cervical cancer cells, which was reversed by microRNA-15a knockdown.

CONCLUSIONS: Overexpressed HCP5 promoted the development of cervical cancer through increasing MACC1 expression by microRNA-15a adsorption.

Key Words:
HCP5, CeRNA, Proliferation, MicroRNA-15a, MACC1.

Introduction

Cervical cancer is a common malignancy in females that seriously threatens physical and mental health of women, especially in young women[1,2]. More than 270,000 women worldwide die of cervical cancer each year, of whom 85% are from developing countries. It is reported that 99.8% of patients with cervical cancer had a history of HPV (human papillomavirus) infection. However, HPV infection alone is not sufficient to cause cervical cancer[3,4]. The basic research on the pathogenesis of cervical cancer is of great significance for clinical treatment. Particularly, biological functions and regulatory effects of relative genes in cervical cancer need urgently to be explored.

Long non-coding RNA (lncRNA) is a type of endogenous non-coding RNA with over 200 nt in length. Although lncRNA itself does not transcribe and encode functional proteins, it is involved in the regulation of gene expressions. LncRNAs are widely involved in physiological processes, such as nuclear transport, alternative splicing and epigenetics[5]. In addition, lncRNA can also be served as a structural component that regulates the stability and attenuation of mRNA, and can even be used as a precursor to small non-coding RNA (sncRNA)[6,7]. Researches have found that lncRNAs are differentially expressed in cancer cells. For example, differentially expressed HOTAIR, MALAT1 and CCAT2 have been confirmed to be involved in the occurrence of cervical cancer[8].

MicroRNAs are a class of endogenous sncRNAs with approximately 22 nt in length. Functionally, microRNA could selectively regulate gene expressions by binding to corresponding mRNA, which exerts an important role in most biological processes.

MicroRNA response elements (MREs) are formed by mature microRNAs and their target RNAs. The expression of target RNA could be affected by multiple microRNAs[9]. Based on that, the hypothesis of competing endogenous RNAs (ceRNAs) is proposed. Functionally, ceRNAs can

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competitively bind to microRNAs through MREs, thereby influencing microRNAs functions and inhibiting gene expressions[10]. It has been reported that PTPNP1 regulates PTEN expression through ceRNAs and participates in the development of breast cancer[11]. However, lncRNAs which function as ceRNAs in cervical cancer still need to be further investigated.

Patients and Methods

Patients

A total of 48 patients with cervical cancer who were treated in The First People's Hospital of Xianyang City during 2014 to 2016 were selected. Enrolled patients did not receive any preoperative radiotherapy and chemotherapy. During the operation, cervical cancer tissues and paracancerous tissues (2 cm away from the tumor tissues) were surgically resected, and immediately preserved in liquid nitrogen. All tissues were pathologically confirmed. This study was approved by the Hospital Ethics Committee and patients signed the informed consent.

Cell Culture

HeLa, SiHa and HCerEpiC cell lines were provided by the American Type Collection Center (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 (Roswell Park Memorial Institute-1640, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The cells were maintained in a 5% CO_2 incubator at 37°C. Cells were passaged when cell confluence was up to 80%.

Cell Transfection

Cells were seeded in the 6-well plates at a density of 2.0-7.0×106 per well. Cell transfection was performed when the cell confluence was up to 70-90% according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids were purchased from GenePharma (Shanghai, China). The sequences of plasmids used in this experiment were as follows: MicroRNA-15a mimics: UAGCAGCACAUAAUGGUUUGUG; MACC1 siRNA, F:CACCAUAGCUUGCAAAGUATT, R: UACUUUGCAAGCUAUGGUGTT; HCP5 siRNA, F: CACGUGUUCUUCCUACUGATT, R: UCAGUAGGAAGAACACGUGTT.

Western Blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). Protein sample was separated by electrophoresis on 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered saline and Tween 20) and followed by the incubation of secondary antibody. The protein blot on the membrane was exposed by enhanced chemiluminescence (ECL).

Luciferase Reporter Gene Assay

Cells were seeded in the 24-well plates. After culturing for 24 h, cells were co-transfected with microRNA-15a mimics or microRNA negative control, HCP5 wild-type or HCP5 mutant-type and MACC1 wild-type or MACC1 mutant-type, respectively for 48 h. Dual-Glo luciferase assay system (Shanghai Qcbio Science & Technologies Co., Ltd, Shanghai, China) was used to detected luciferase activities of firefly and Renilla. The calculated formula was: relative luciferase activity=-firefly luciferase activity/Renilla luciferase activity. All reporter genes and RNA oligonucleotide sequences were all constructed by GenePharma (Shanghai, China).

Prediction of Target Genes

Target genes of microRNA-15a were predicted by RegRNA, TargetScan and PicTar. Besides, the binding condition of microRNA-15a and HCP5 was predicted by RNA22 version 2.0.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted in treated cells using TRIzol method (Invitrogen, Carlsbad, CA, USA). The mRNAs were reversely transcribed to cDNAs using PrimeScript RT reagent kit (TaKaRa, Otsu, Shiga, Japan), followed by the qRT-PCR reaction according to the instructions of miScript SYBR Green PCR kit (TaKaRa, Otsu, Shiga, Japan). The reaction conditions were as follows: denaturation at 94°C for 30 s, followed by annealing at 55°C for 30 s and extension at 72°C for 90 s, for a total of 40 cycles. Each sample was repeatedly performed for 3 times. Primers used in this study were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: ACCCACTCCTCCACCTT-

TGA, R: CTGTTGCTGTAGCCAAATTCGT; U6, F: CTCGCTTCGGCAGCACATATA, R: AAATATGGAACGCTTCACGA; MACC1, F: AACCCCAAACCTAAAAAGACTC, R: ACCCAGGACATCAGCTAAAACT; HCP5, F: CCACTATTGGCCATCAAAGG, R: ATACTGTCCAATTCCCCTGT; MicroRNA-15a, RT-primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAAC; QF: GCGGCTAGCAGCACATAATGG.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded in the 96-well plates at a density of 1.0-3.0×103 per well. After culturing for 24 h, serum-free RPMI-1640 was replaced. 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) were added into each well. Absorbance values at the wavelength of 450 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) for statistical analysis and GraphPad Prism 5 (La Jolla, CA, USA) for image editing. Kaplan-Meier was introduced to analyze the survival rate. The quantitative data were represented as mean ± standard deviation (`x±s). The t-test was used for comparing measurement data. $p<0.05$ was considered statistically significant.

Results

HCP5 was Overexpressed in Cervical Cancer Tissues

48 cervical cancer tissues and paracancerous tissues were surgically resected. The data showed that HCP5 was overexpressed in cervical cancer tissues than that of paracancerous tissues (Figure 1A). Patients with cervical cancer were further assigned into high expression group and low expression group based on their HCP5 expression levels. We found that the overall survival of patients with cervical cancer who had higher expression of HCP5 was shorter than those with lower expression (p=0.0448, Figure 1D). It is reported that microRNA-15a expression was closely related to development of prostate cancer and ovarian cancer[12,13]. By online prediction, HCP5 was found to bind to microRNA-15a. Previous researches[14] have demonstrated that cytoplasmic HCP5 participates in glioma development by regulating expressions of target genes as microRNA sponge. Furthermore, MACC1 was confirmed to be the target gene of microRNA-15a predicted by RegRNA, TargetScan and PicTar. Downregulated microRNA-15a (Figure 1B) and overexpressed MACC1 (Figure 1C) were found in cervical cancer tissues in comparison of paracancerous tissues, indicating that HCP5 may bind to MACC1 as ceRNA.

Downregulated HCP5 Inhibited Cell Proliferation

As shown in Figure 2A, HCP5 was overexpressed in cervical cancer cell lines (HeLa and SiHa) than that of normal cervical epithelial cell line (HCerEpiC). Transfection plasmids of microRNA-15a and HCP5 were constructed, and their transfection efficacies were verified by qRT-PCR (Figure 2B-D). CCK-8 assay elucidated that cell proliferation was decreased after HCP5 knockdown in a time-dependent manner, which could last for 96 h (Figure 2E and 2F). The above results indicated that HCP5 could participate in the development of cervical cancer via regulating proliferation of cervical cancer cells.

HCP5 regulated MACC1 expression by competing microRNA-15a as ceRNA

To further explore the mechanism of HCP5 in regulating cervical cancer, we predicted the binding site of HCP5 and microRNA-15a by RNA22, followed by the construction of corresponding reporter genes (Figure 3A). The data showed that microRNA-15a mimic and HCP5 wild-type presented the lowest luciferase activities, indicating that microRNA-15a could bind to HCP5 (Figure 3B and 3C). Luciferase reporter gene assay also demonstrated that microRNA-15a could bind to MACC1 (Figure 3E and 3F). Western blot results illustrated that microRNA-15a was capable of regulating MACC1 expression (Figure 3G).

Furthermore, rescue experiments were performed to verify the relationship between HCP5 and microRNA-15a. Decreased viabilities of HeLa and SiHa cells resulted from HCP5 knockdown were reversed by microRNA-15a knockdown (Figure 4A and 4B). The above data revealed that HCP5 promotes the development of cervical cancer through upregulating MACC1 expression by microRNA-15a adsorption.

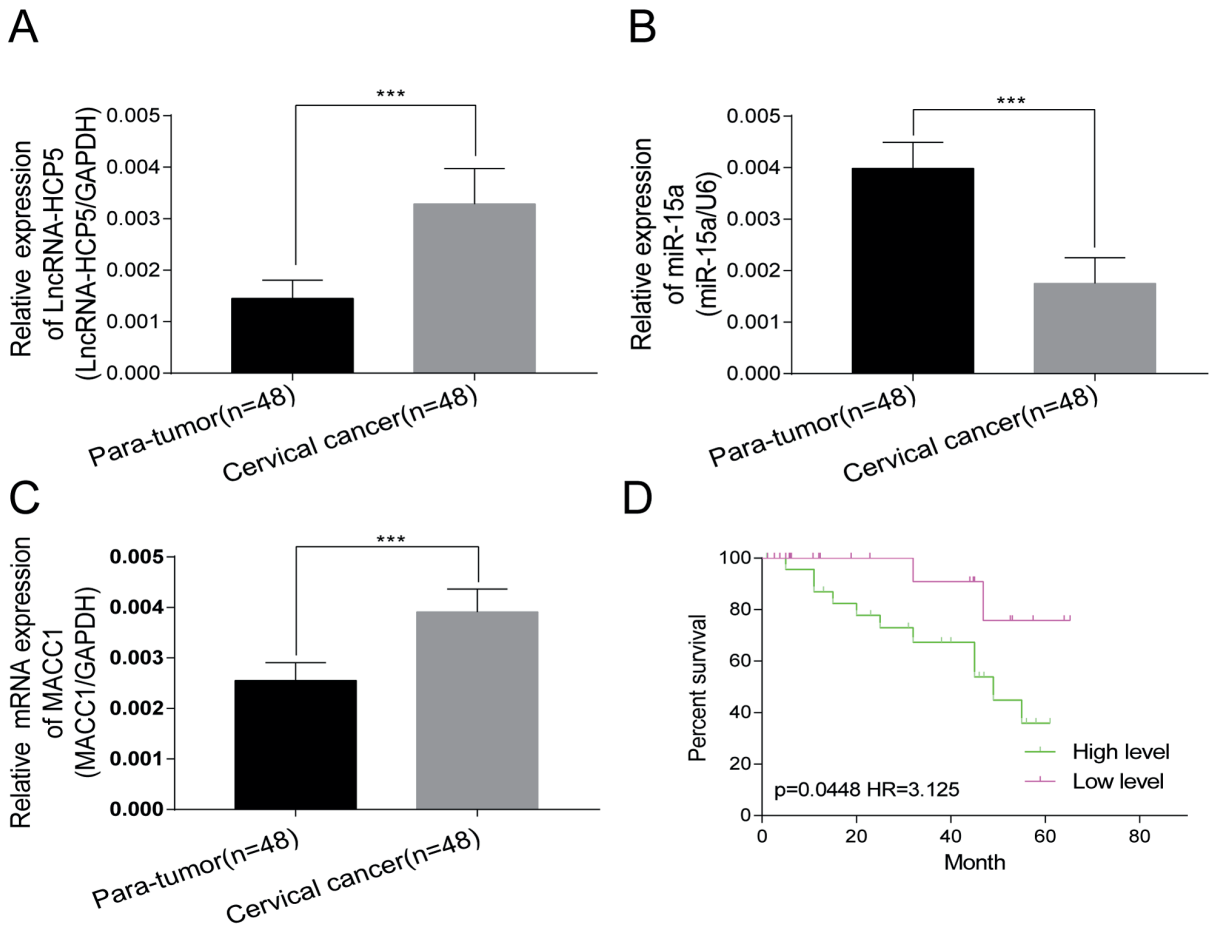

Figure 1. HCP5 was overexpressed in cervical cancer tissues. ***A,*** HCP5 was overexpressed in cervical cancer tissues than that of paracancerous tissues. ***B,*** MicroRNA-15a was downregulated in cervical cancer tissues than that of paracancerous tissues. ***C,*** MACC1 was overexpressed in cervical cancer tissues than that of paracancerous tissues. ***D,*** The overall survival of patients with cervical cancer who had higher expression of HCP5 was lower than those with lower expression.

Discussion

Cervical cancer is a common malignant cancer with difficult diagnosis and poor prognosis. The specific pathogenesis of cervical cancer, however, has not been fully elucidated. Accumulating researches[15-17] have demonstrated that differentially expressed lncRNAs are closely related to tumor development, such as gastric cancer, liver cancer, and lung cancer. Some certain lncRNAs have been found to be differentially expressed in cervical cancer. For example, Gibb et al[18] identified that there are 1056 lncRNAs expressed in cervix, of which 668 are expressed in non-neoplastic cervical tissues. Qin et al[19] found that MEG3 is overexpressed in cervical cancer tissues than that of paracancerous tissues. Moreover, overexpressed MEG3 could arrest cell cycle and apoptosis of HeLa cells. In addition, BC200, UCA1, and H19 have also been reported to be differentially expressed in cervical cancer[20].

Previous studies have reported that lncRNA HCP5 is involved in tumor development. In the present study, HCP5 expression was higher in cervical cancer tissues than that of paracancerous tissues. Overexpressed HCP5 was confirmed to be negatively related to prognosis of cervical cancer. For *in vitro* experiments, HCP5 elevated cell proliferation as an oncogene. HCP5 promoted the development of cervical cancer through upregulating MACC1 expression by microRNA-15a adsorption.

MACC1 is located on human chromosome 7 (7p21.1) that contains 7 exons and 6 introns.

The encoded protein sequence consists of 825 amino acids[21]. MACC1 mediates tyrosine kinase-dependent signaling pathways. It is worth noting that HGF and its receptor c-Met, which is the major factor in MACC1 pathway, is also located on chromosome 7 7q21.1 and 7q31.2, respectively. Researches have shown that MACC1 activates the HGF/c-Met pathway by binding to the c-Met promoter, thereafter elevating the expression level of c-Met pro-

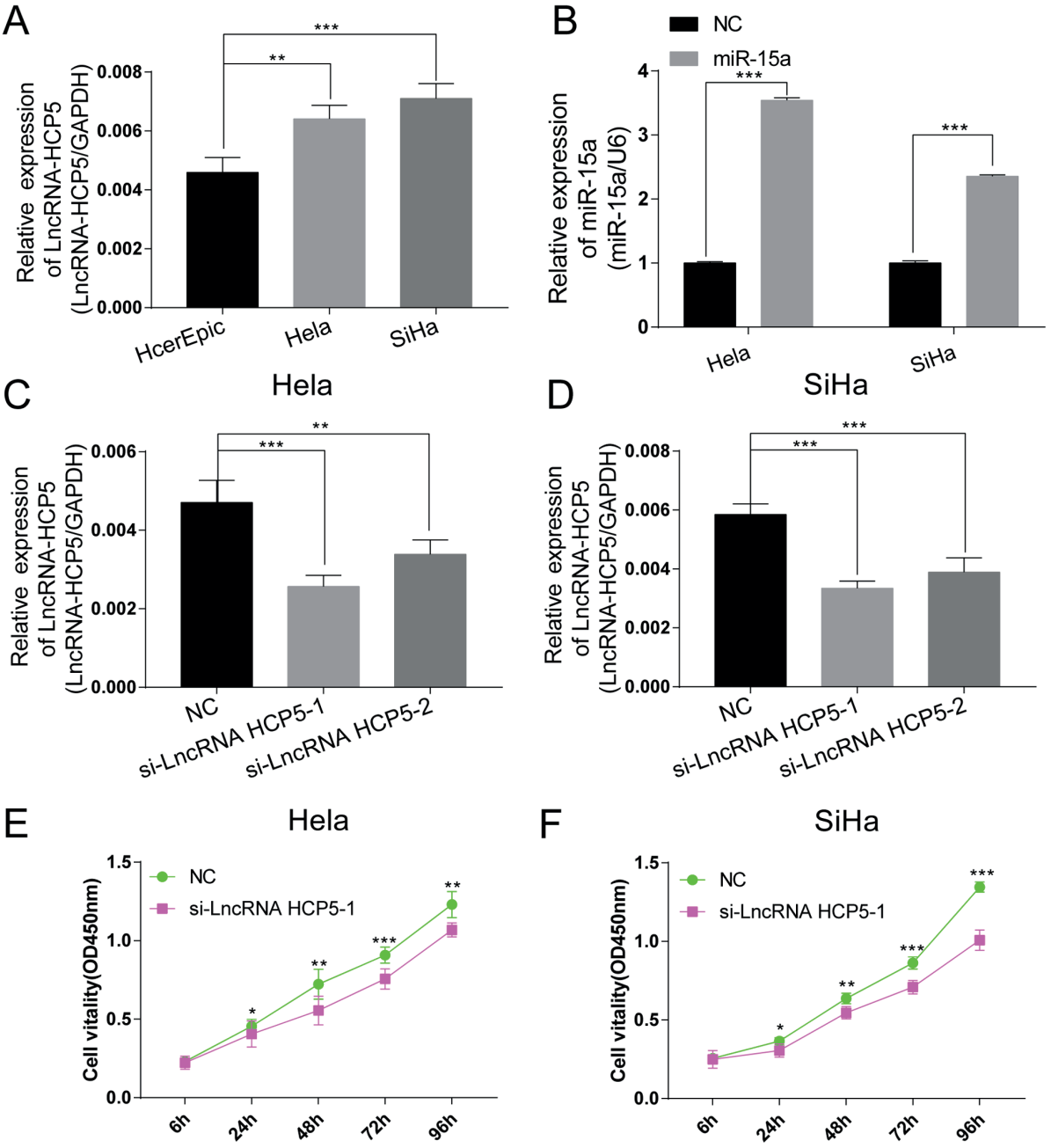

Figure 2. LncRNA HCP5 increased viabilities of HeLa and SiHa cells. ***A,*** HeLa and SiHa cells presented higher expression of lncRNA HCP5 than that of HCerEpiC cells. ***B,*** MicroRNA-15a expressions in HeLa and SiHa cells were increased after microRNA-15a overexpression. ***C-D,*** Transfection efficacy of small interference sequence of HCP5. ***E-F,*** Viabilities of HeLa and SiHa cells were decreased after HCP5 knockdown.

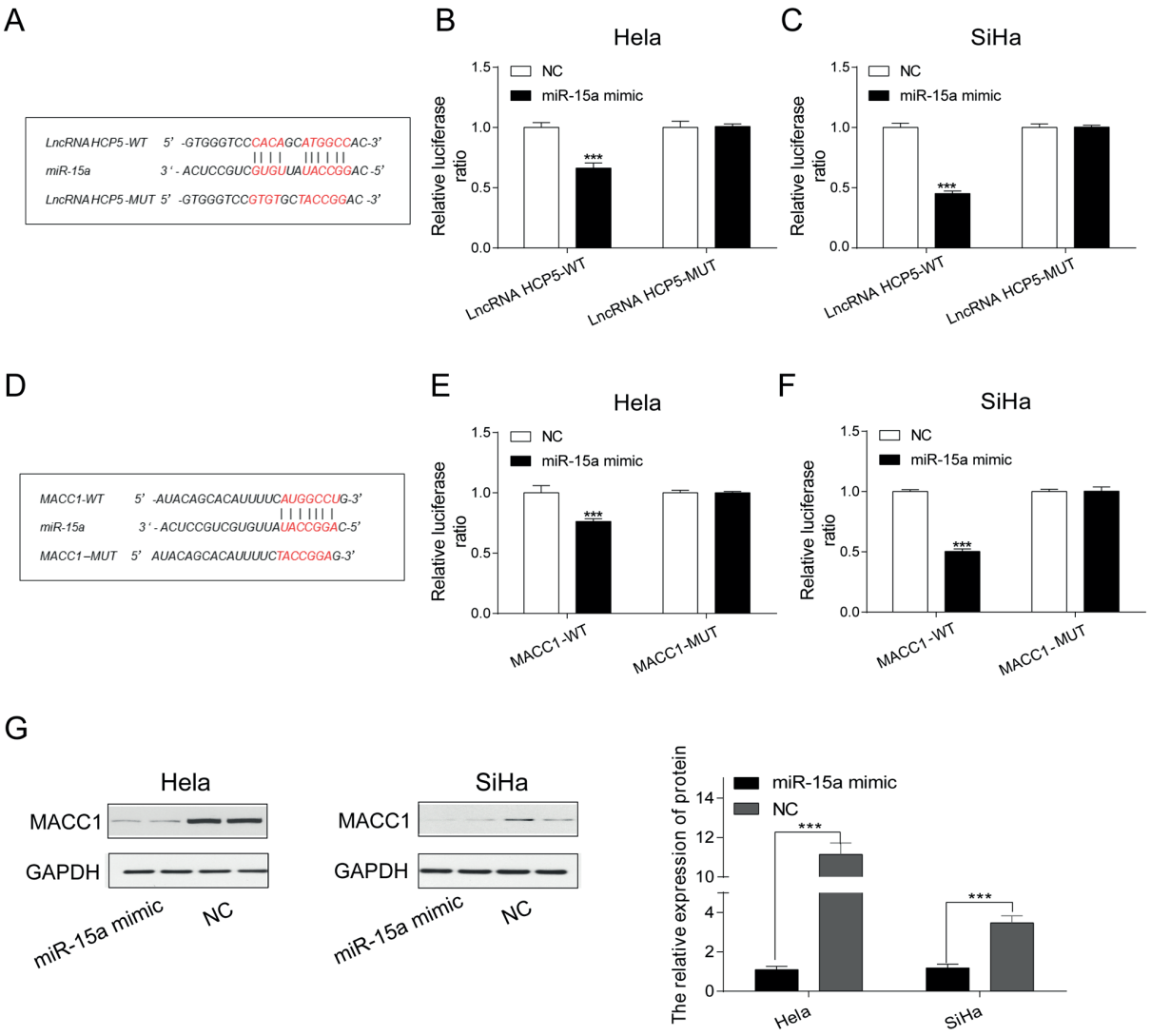

Figure 3. HCP5 regulated MACC1 expression by competing microRNA-15a as ceRNA. ***A,*** Sequences of HCP5 wild-type and HCP5 mutant-type. ***B-C,*** MicroRNA-15a mimic and HCP5 wild-type presented the lowest luciferase activities. ***D,*** Sequences of MACC1 wild-type and MACC1 mutant-type. ***E-F***, MACC1 wild-type and microRNA-15a mimic presented the lowest luciferase activities. G. Protein expression of MACC1 was increased in HeLa and SiHa cells after microRNA-15a overexpression.

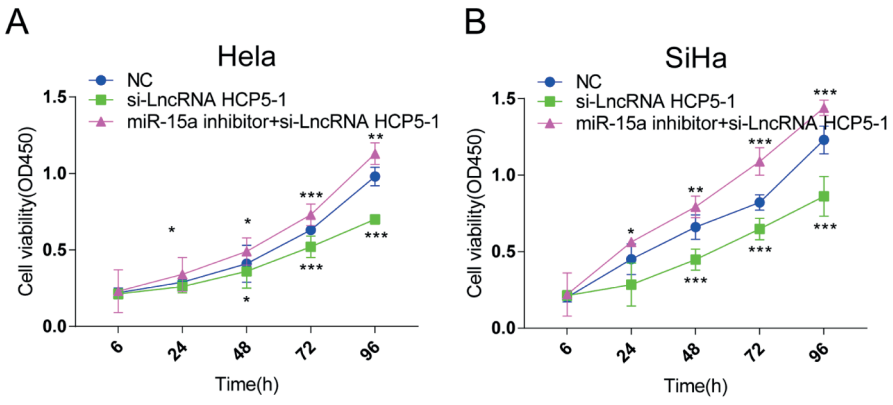

Figure 4. MicroRNA-15a reversed the cellular function of HCP5 in HeLa and SiHa cells. ***A-B***, Decreased viabilities of HeLa and SiHa resulted from HCP5 knockdown were reversed by microRNA-15a knockdown.

tein and binding to HGF. HGF further induces MACC1 to translocate in the cell nucleus from the cytoplasm and drive the positive feedback of MACC1. As a consequence, cell proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, cell movement, invasion and metastasis are all promoted[22,23]. Recent reports[24,25] have confirmed that MACC1 is overexpressed in lung cancer and liver cancer. MACC1 expression is related to malignant progression of tumors. In this study, we found that MACC1 is overexpressed in cervical cancer tissues than that of paracancerous tissues. MACC1 participated in the development of cervical cancer by promoting cell proliferation via HCP5 regulation.

Conclusions

We showed that overexpressed HCP5 promoted the development of cervical cancer through increasing MACC1 expression by microRNA-15a adsorption.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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