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MiR-199b-5p promotes tumor growth and metastasis in cervical cancer by down-regulating KLK10

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ABSTRACT

MiR-199 b-5p and kallikrein-related peptidase 10 (KLK10) are related to various disease processes and pathogenesis. However, little is known about the molecular mechanisms of miR-199 b-5p and KLK10 in human cervical cancer. In the present study, we found that miR-199 b-5p was highly expressed in cervical cancer tissues and cell lines, and was positively correlated with overall survival (OS) and progression-free survival (PFS), higher incidences of larger tumor sizes, late International Federation of Gynecology and Obstetrics (FIGO) stages and preoperative metastasis. Further, we found that transfecting miR-199 b-5p mimics into cervical cancer cells promoted tumor progression through enhancing the cell viability, migration, and suppressing apoptosis by using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), wound healing and flow cytometry analysis. Luciferase reporter assays indicated that miR-199 b-5p targeted the 3′-untranslated region (3′-UTR) of KLK10. Overexpressing KLK10 reversed the role of miR-199 b-5p in accelerating cervical cancer progression. Suppressing miR-199 b-5p expressions improved apoptosis and reduced the cell viability, while the process was reversed in KLK10-knockdown cervical cancer cells. In vivo analysis verified the effects of miR-199 b-5p on promoting cervical cancer progression, accompanied with reduced KLK10 expressions. In summary, we identified that miR-199 b-5p played as a tumor promoter in cervical cancer cell growth by targeting KLK10, and miR-199 b-5p might function as a novel biomarker for diagnosis or therapeutic targets of human cervical cancer.

1. Introduction

Cervical cancer is a common gynecological malignancy, a leading cause of cancer-associated mortality among women worldwide [1,2]. Radiotherapy, chemotherapy, and surgery are known as standard therapeutic treatments for cervical cancer, but the 5-year survival rate for advanced patients still remains very low [3–5]. As reported, metastasis to the lymph node and distant organs is a main cause of treatment failure [6,7]. Presently, elucidation of the molecular mechanisms revealing cervical cancer tumorigenesis and progression is important for individualized treatments.

MicroRNA (miRNA)-regulated modulation of post-transcriptional gene expression has been identified in the progression and metastasis of various cancer types [8]. MiRNAs are endogenous noncoding short RNAs, which inhibit gene expression through binding to target mRNAs at their 3′-UTR [9]. They have been involved as oncogene or anti-oncogene of cancer [10]. MiR-199 b-5p has been indicated to be aberrantly expressed in different human tumors with varied roles in the development of cancers [11–13]. For instance, miR-199 b-5p expression is lost in metastatic patients with medulloblastoma [14]. In addition, miR-199 b-5p has been reported to be increased in human osteosarcoma tissues and participate in the Notch signaling in osteosarcoma cells [15]. MiR-199 b-5p could regulate human hepatocellular carcinoma invasion by targeting discoidin domain receptor-1 (DDR1) [16]. However, its clinical significance and roles in cervical cancer remain unknown. KLK10 is a member of the human tissue kallikrein family of secreted serine proteases, encoded by a family of 15 genes clustered in a tandem on chromosome 19q13.3–4 [17]. It has been reported that KLK10 may act as a tumor suppressor gene, and its over expression

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in nude mice was shown to suppress tumor progression [18]. KLK10 gene suppresses proliferation, induces apoptosis and decreases glucose metabolism of multiple cancer cells [19–21]. However, the involvement of KLK10in cervical cancer has not been elucidated.

In the present study, miR-199 b-5p was shown to be frequently up-regulated in human cervical cancer tissue samples and might function as an oncogene. MiR-199 b-5p significantly promoted cervical cancer cell growth through targeting KLK10.

2. Materials and methods

2.1. Patients

A total of 70 human cervical cancer samples and matched normal adjacent (non-tumorous) cervical tissues were obtained from the First Affiliated Hospital, Zhengzhou University (Zhengzhou, China) with the informed consent of patients and approval for experimentation from the First Affiliated Hospital, Zhengzhou University. Diagnoses were according to pathological evidence. No patients underwent chemotherapy, immunotherapy, hormone therapy, or radiotherapy before samples collection. The clinical stage and histological grades were based on FIGO. Tissue samples were divided into two portions with one snap-frozen immediately in liquid nitrogen and stored in −80 °C for RT-qPCR analysis.

2.2. Cells and transfection

Human cervical cancer cells, including Hela, CaSki, SiHa, ME-180, MS-751 and C-33 A, were purchased from American Type Culture Collection (ATCC, Manassas, USA). Normal cervix uteri cell lines, including Ect1/E6E7 and H8, were obtained from the Chinese Academy Medical Science (Beijing, China). Normal cervix uteri cell line of HcerEpic was purchased from ScienCell Research Laboratories (USA). Cells were cultured in complete growth medium as recommended by the manufacturer. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. miR-199 b-5p mimics, miR-199 b-5p inhibitor and corresponding negative controls (NC) were synthesized from RiBoBo (Guangzhou, China). The pcDNA-KLK10 plasmid were obtained from Genechem Co.,Ltd. (Shanghai, China). KLK10 knockdown was achieved by transfection of si-KLK10 (Santa Cruz, USA). For transfection, Hela, CaSki and SiHa cells were seeded in 24-well or 6-well plates and then transfected with the indicated plasmids or siRNA for 48 h using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's protocol.

2.3. Luciferase reporter assay

Cells were planted in 24-well plates. Cells were then co-transfected with luciferase reporters, either wild-type (WT) or mutant (MUT) klk10 3′-UTR, in combination with miR-199 b-5p mimics and a miR-NC, or miR-199 b-5p inhibitor and miR-inhibitor-NC using Lipofectamine 2000 (Invitrogen). 48 h later, cells were harvested and analyzed for luciferase activity by a luciferase assay kit (Promega, USA) following the manufacturer's instructions.

2.4. Quantitative real time-PCR (RT–PCR)

Total RNA was extracted from cells or tissues using a miRNeasy Mini kit (Qiagen, Germany). MiRNA was reverse transcribed to complementary DNA with miScript Reverse Transcription Kit (Qiagen). miRNA expression was determined using a miScript SYBR Green PCR Kit (Qiagen) on the ABI7500 Real-Time PCR System (Applied Biosystems, USA). Primers for miRNAs and the endogenous control (U6) gene were showed as followings:

miR-199 b-5p (forward), 5′-AGATGCGAGATGATCTCACATAGCT-3’;
miR-199 b-5p (reverse), 5′-CTCTTTGTCTTAGTAGCTTGCTCGT-3’;
U6 (forward), 5′-TCTAGTCGGTTCACTACAAACTATAA-3’;
U6 (reverse), 5′-GTCACTTCAGCCGATTTGCAGG-3’;
KLK10 (forward), CTTCCGGGCAAGCAACATAAGCC;
KLK10 (reverse), GATGTGAGTGAGGCCGGT;
GAPDH (forward), ATTAGTCCAGGGCGTCGCAC;
GAPDH (reverse), CTTCCATCTTCCTCGGCTTACG.

All primers used here were obtained from GenePharma (Shanghai, China). The relative fold expression of the target was analyzed via the comparative Ct method and was normalized to control.

2.5. Cell viability assay

Cell viability was measured using a MTT analysis (KeyGen BioTECH, Jiangsu, China) following the manufacturer's protocol.

2.6. Western blot analysis

Cells and Tissues were lysed in lysis buffer (KeyGen BioTECH) for protein extraction. Protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, USA). After blocking in 5% skim milk, the membranes were incubated with the specific primary antibodies at 4 °C overnight (Supplementary Table 1). The membranes were then incubated in HRP-conjugated secondary antibodies. Positive signals were developed by ECL (Thermo Scientific, USA) and analyzed by Image J (National Institutes of Health, USA). GAPDH was used as an internal control.

2.7. Apoptosis and flow cytometry analysis

The cells were labeled with PI and annexin V following the manufacturer's instructions (BD Biosciences, USA). A minimum of 10 000 events for each sample were collected and analyzed with a FACScalibur Flow Cytometer (BD Biosciences).

The activities of Caspase-3/7 were determined using the Caspase-Glo Assay Kit purchased from Promega (USA). Cancer cells were seeded in a 96-well plate at 4×10^3 per well and cultured overnight to ensure that the cells have enough time for proper adherence. Next, the culture media were replaced with serum deprivation medium for 24 h. Caspase-3/7 activity was evaluated following manufacturer's protocol and analyzed.

2.8. Wound healing analysis

Wound healing assays were performed as previously described [22]. The wounds pictures were photographed after 24 h and closures were recorded.

2.9. Cervical tumor xenograft model

Animal experiments were performed in accordance with the guideline for the Regulations for Animal Experiments and Related Activities at the First Affiliated Hospital, Zhengzhou University. The backs of 6-week-old female Balb/c nude mice were purchased from Experimental Animal Centre of Zhengzhou University. 2.0×10^7 Hela cells stably transfected with miR-199 b-5p mimics and a miR-NC, or miR-199 b-5p inhibitor and miR-inhibitor-NC were subcutaneously injected into the flank region of nude mice to establish cervical cancer xenograft model ($n = 6$ each group). Tumor size was monitored at 2-day intervals by

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