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Original article

Effects of miRNA-197 overexpression on proliferation, apoptosis and migration in levonorgestrel treated uterine leiomyoma cells

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ABSTRACT

Background/aims: Uterine leiomyoma is the ahead benign tumor of the female genital tract, which resulted in menstrual abnormalities, recurrent pregnancy loss, and other serious gynecological disorders in women. Recently, as the process of exploring the brief molecular mechanisms of tumorgenesis, microRNAs (miRNAs) have attracted much more attention.
Methods: In this study, we first confirmed that microRNA-197 (miR-197) was down-regulated significantly in human uterus leiomyoma by quantity real-time polymerase chain reaction, compared to normal uterus myometrium. Then we observed the potential effects of miR-197 overexpression on human uterus leiomyoma cells by cell counting kit 8, wound healing assay, and flow cytometric assessment separately.
Results: The data showed that miR-197 could inhibit cell proliferation, induce cell apoptosis, and block cell migration in vitro. Coincidently, levonorgestrel (LNG), a well-known uterus leiomyoma therapy, could induce miR-197 expression in human uterus leiomyoma cells, and over-expression of miR-197 showed a synergy effect on human uterus leiomyoma cell proliferation and apoptosis with LNG.
Conclusion: In this study, the data showed that miR-197 could play an anti-oncogenic role in human uterus leiomyoma cells, and cooperate with LNG on the cell proliferation and apoptosis, which suggested that miR-197 might be a potential target and provided database for clinical treatment.

1. Introduction

Uterine leiomyoma is the most common benign uterine tumor in reproductive women, which occurs in 30–50% of reproductive-aged women [1], increasing to a 70–80% cumulative incidence by age 50 years [2]. Usually, uterine leiomyomas is the reason for hysterectomy and myomectomy in clinical, and has brought kinds of injuries to women, such as menstrual abnormalities, recurrent pregnancy loss, gynecological disorders, and pelvic pressure, since its invasive and metastatic properties are not like other malignancies [3]. Despite their prevalence, the etiology and pathogenesis remain poorly understood.

Uterine leiomyoma is considered to initiate from myometrial cells transformation, and identified to be estrogen/progesterone-dependent with a polygenic inheritance pattern [4,5]. Up to now, a series of studies, including familial, epidemiological, and cytogenetic studies, have indicated that genetic factors play important roles during the initiation and development of uterine leiomyomas [6–8]. Stewart et al. demonstrated that hundreds of genes dysregulated in leiomyoma versus normal myometrium [5]. Genomic instability of these identified genes, involving estrogen and progesterone receptors were suspected to be associated with an increased risk of leiomyoma development, however the evidence has been inconsistent in a large scale of cases [9–11]. Chegini et al. and Boryana et al. have afforded further insights into the molecular mechanisms of leiomyoma initiation and development by high-throughput sequencing technology [12,13], demonstrating that

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many miRNAs are deregulated in leiomyoma compared to normal tissue. However, the regulation and biological function of many of these miRNAs during the process of initiation and development of leiomyoma remain to be uncovered.

MicroRNAs (miRNAs) are a new class of small, noncoding and single-stranded RNAs of 18–22 nucleotides, derived from miRNA duplex complexes processed from larger pre-miRNAs by the RNase III enzyme Dicer [14]. General mechanism of miRNA action in animal and human cells is the inhibition of translation after forming a complex called the RNA-interference-induced silencing complex (RISC) or the induction of mRNA cleavage [15]. MiRNAs play important roles in cell growth, proliferation, differentiation and apoptosis through interaction with particular RNA species. It has been reported that there are many deregulation of miRNAs in leiomyoma [16,17], and miR-197 was found to be significantly down-regulated in leiomyoma [17]. MiR-197 is transcribed from the genomic region of chromosome 1p13.3 and many recent studies have indicated that miR-197 is up-regulated in some malignant tumors, including small-cell lung cancer cell lines (SCLC), non-small-cell lung cancer cell lines (NSCLC) [18], invasive ductal adenocarcinoma (IDA) [19] and hepatocellular carcinoma (HCC) [20]. But its function and mechanism in uterus leiomyoma is still unknown.

The aim of this study was to validate miR-197 expression module and initially identify its function in leiomyoma. In the present study, we confirmed that miR-197 was de-expressed in the leiomyomas compared to normal tissues. Interestingly, levonorgestrel, a well-established leiomyoma therapy [21], could induce miR-197 expression in uterus leiomyoma cells (UtLMCs). Subsequently, we found that miR-197 could inhibit cell proliferation, induce cell apoptosis, and enhance cell metastasis of UtLMCs. Furthermore, we confirmed that levonorgestrel appeared cooperation with miR-197 on the cell physiologies in a dose-dependent manner. These preliminary findings suggested that miR-197 might play an anti-oncogenic role in the molecular etiology of UtLMCs and may be a useful target for leiomyoma therapeutics.

2. Materials and methods

2.1. Tissue collection

Twenty-four female patients aged 36–48 years (mean age ± SD, 42.5 ± 0.9 years) were recruited. They did not receive any hormone treatment for at least 3 months before the hysterectomy. Their menstrual cycles were still regular and diagnoses of leiomyoma were clear. The surgery was performed 3–10 days after cessation of menstruation. The tissues were collected after surgery, and the histopathologic diagnoses were all confirmed as uterine leiomyoma and without degeneration. Informed consent about the use of these samples was obtained from all patients. Ethical approval was obtained from the hospital ethics committee.

2.2. Uterus leiomyoma muscle cell isolation and culture

The UtLMCs were isolated from small portions of leiomyoma, and cultured by the digestion and tissue adherence methods as previously described [5]. All cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and antibiotics at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Briefly, after being digested with trypsin and cultured for 24 h, the medium was replaced with fresh medium. Afterwards, the medium was replaced every 48 h and the cells were examined under a light microscope. When the cells reached confluence after culturing for 7–8 days, cell passage was carried out. The experiment only included the cells within the sixth generation of the leiomyoma cells. Prior to use, the cell cultures were characterized using antibodies to α-smooth muscle actin based on immunofluorescence microscopy. This cell culture was morphologically homogenous and at initial isolation and after first passage displayed 100% immuno-staining for α smooth muscle actin (α-SMA).

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA of tissue samples and cells were extracted using Trizol method according to the manufacturer's protocol. The integrity of the RNA was assessed by agarose gel-electrophoresis and measured by spectrophotometry at 260 nm by Nanodrop 2.0. To monitor the levels of miRNA, cDNA was synthesized from total RNA by using TaqMan miRNA Reverse Transcriptase Kit (ABI, Foster City, CA, USA) with specific probes, human U6 as an endogenous control. Quantitative real time-PCR (qRT-PCR) was performed by using an Applied Biosystems ViiA 7 Sequence Detection System (ABI ViiA 7 SDS, USA) following the manufacturer's guidelines. Briefly, samples were incubated at 95 °C for 10 min for an initial denaturation, followed by 40 PCR cycles of incubation at 95 °C for 15 s and then 60 °C for 1 min. MiR-197 expression was normalized to snRNA U6. Primer identification numbers are MI0000239 for hsa-miR-197 (*RT: GTCGTATCCAGTGCAGGGTCC; UP: GCGTTCACCACCTTCTC C*), and 001973 for snRU6 (ABI, Foster City, CA, USA). For detection of mRNAs levels, cDNA was synthesized from total RNA using a High-Capacity cDNA Reverse Transcriptase Kit (ABI, USA). All reactions were done in a 20 μl reaction volume in triplicate. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Lentiviral infection of leiomyoma cells

UtLMCs were incubated in 6-well plate (Greiner, Germany) at density of 1×10^5 cells per well, and then infected with the miR-197 expression lentiviral vector or unrelated sequence negative control (miR-NC) when the cell confluence reached about 60%. After 24 h post-infection, fresh culture medium with 10% FBS were injected instead of the existing. The infected efficiency of lentiviral vector was observed by fluorescence microscope at 48 h and total RNA was extracted at 72 h after infection. The transfected UtLMCs that had increased levels of hsa-miR-197 were identified by TaqMan-based, quantitative real-time PCR.

2.5. CCK-8 assay for cell viability

The CCK-8 assay was used to determine the relative cell growth. UtLMCs were plated at 5×10^3 cells per well in 96-well plates with six replicate wells at the indicated concentrations, then proliferation assays were performed. The absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay plate reader. Each data point represents the mean of a minimum of six wells. The viability of untreated cells was assumed to be 100%.

2.6. Wound migration assay

A density of 1×10^5 UtLMCs cells per well were seeded into 6-well plates. When they were approximately 80% confluent, UtLMCs cells were transfected with hsa-miR-197 or negative control vector for 24 h. The monolayer was scratched with a sterile 10 μl pipette tip, and rinsed with PBS to remove cellular debris. Subsequently, fresh medium was added and cells were incubated for 48 h to allow time for migration into the cell-free area. The images of the migratory cells were captured at times 0, 24 and 48 h post-wounding using an Olympus IX70 microscope equipped with digital camera (Olympus Inc., Melville, NY). Images were analyzed