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Original Article

Expression and effects of miR-137 in patients with erectile dysfunction

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Abstract: Objective: We analyzed the expression of microRNA (miR)-137 in elderly patients with erectile dysfunction (ED) and young healthy volunteers. Methods: The morphology and blood flow of the penile corpus cavernosum of all patients were analyzed via angiography. The miR-137 expression was detected by real-time fluorescent RT-PCR in stem cell samples taken from peripheral blood. Western blot was used to detect the expression of CDK16, the target protein of miR-137. Results: miR-137 expression was significantly higher in elderly ED patients than in young healthy volunteers, and were negatively correlated with IIEF-5 scores. CDK16 protein expression was lower in prostate secretion samples of ED patients than in healthy controls. The average rate of penile blood flow was 98.83 ± 1.32 mL/min in healthy volunteers and 31.28 ± 1.75 mL/min in elderly ED patients. There were significant differences in clinical signs between these groups. Conclusion: There was a close correlation between the degree of ED and miR-137 expression, which may inhibit the physiological expression of CDK16.

Keywords: Erectile dysfunction, miR-137, CDK16, expression

Introduction

During mens' sexual activity, if they are without the ability to develop or maintain an erection of their penis, it is defined as erectile dysfunction (ED) disease, or sexual dysfunction [1]. The main causes of ED are cardiovascular disease or diabetes, neurological problems (e.g., trauma from prostatectomy), hormonal insufficiencies (e.g., hypogonadism), or drug side effects [1-4]. As for the vascular diseases cause, an epidemiological survey showed that smoking, aging, hyperlipidemia, diabetes, and hypertension were major high risk factors of ED [5, 6]. Other causes of ED include cavernous nerves injury, endocrine disorders, fibrosis of the penile vasculature and corporal smooth muscle [7-9]. The most direct results of vasculogenic and neurogenic ED are loss of normal cellular function or cell death, from information in clinical and etiological implications field.

When vascular diseases or with cavernous nerve injury, there might be some changes with genes expression for cellular dysfunction or cell proliferation and differentiation. microRNAs (miRNAs) are non-coding RNAs typically ~20 nucleotides in length and transcribed by RNA polymerase II from individual miRNA genes or coding gene introns. The large number and variable functions of miRNA identified to date indicate that miRNAs are involved in a vast array of cellular processes, including development, growth, and tumorigenesis [10, 11]. microRNA (miR)-137 is a short non-coding RNA molecule that regulates the expression of other genes through a variety of mechanisms. Dysfunctional miRNA could cause an abnormally high transcription level of the target mRNA and related clinical consequences, which is located on human chromosome 1p22 [7, 8], and in mouse embryonic stem cells has been shown to regulate neural stem cell proliferation and differentiation. When analyzed its effect on the biological functions of vascular endothelial cells, its over-expressed trophoblast cells can regulate the biological functions of vascular endothelial

cells, e.g. reduce proliferation and migration activity [12, 13].

On the other side, the roles of miRNAs in the nervous system have been studied extensively, from physiology to pathology [14, 15]. Refering to health conditions related to genetic changes, as mutations happen miR-137 has a relationship with schizophrenia disease [16].

Regardless of the process, initiated by sexual arousal, signals are transmitted from the brain to nerves in the penis. A penile erection is the result of the hydraulic effect of blood entering and being retained in sponge-like bodies within the penis. So, if miR-137 has a function through brain, nerves or serum system, this needs to analyzed.

Furthermore, considering miR-137 is related to downstream signaling pathways, it can also directly inhibit cyclin-dependent kinase 6 (CDK6) expression and decrease expression of the downstream target protein of CD16, the significance of which is deep-seated. miR-137 has also been shown to induce differentiation, and inhibit the proliferation, of adult mouse neural stem cells, oligodendroma-derived stem cells, and human glioblastoma multiforme-derived stem cells [7, 9]. Researchers [17] have identified 14 target sites predicted by target scans, including testis-specific serine kinase 6 (TSSK6).

However, no miRNAs have been reported to directly regulate ED so far through over-expression, without research on its downstream signaling pathways.

In this study, we studied the expression of miR-137 in ED patients compared to normal healthy controls, by nucleic acid and protein detection, which might be helpful in analyzing the molecular biological mechanisms of ED.

In the meantime, recently, stem cell transplantation via corpus cavernosum angiography surgery was shown to have a curative effect in an animal model of ED [18]. Thus, how to screen for useful donors has become another important issue need to be addressed. In this study, we investigated the relationship between miR-137 and ED and its effect on ED pathogenesis, which can provide the basis data for future transplantation research.

Materials and methods

Patients

This prospective study recruited 11 ED patients in January 2016. The patients were diagnosed by the Department of Andrology, Inner Mongolia Autonomous Region Comprehensive Center for Disease Control and Prevention. We employed a questionnaire to confirm a clear diagnosis by two trained attending physicians. Patients ranged in age from 53 to 76 years old, with an average age of 55.6 years. Their course of ED ranged from 5 to 22 years, with an average duration of 17 years according to epidemiological follow-up. The entire course of treatment and follow-up continued for 1 year from 2016 to 2017.

The International Index of Erectile Function 5 (IIEF-5) was used to evaluate the degree of ED. Two patients were diagnosed with a moderate degree of ED with scores ranging from 12 to 21, three patients had scores ranging from 8 to 11, and six patients had scores ranging from 5 to 7.

Including criteria: first admitted to a participating Inner Mongolia Autonomous Region CDC, aged 50 years or older, clear diagnosis of ED, and IIEF-5 score $\leq$ 21.

Excluding criteria: disease caused by psychological factors; lack of compliance with treatment regimens; suffering from hypertension, diabetes, high cholesterol, heart disease; and/or presence of blood-borne infectious diseases including human immunodeficiency virus (HIV), treponema pallidum (TP), and hepatitis B and C viruses.

Volunteer recruitment

Ten senior students from the College of Life Sciences of Inner Mongolia University were recruited as volunteers. The volunteers, which were used as healthy controls, were 27 years of age and had no genetic history of ED. The volunteers were assessed using the IIEF-5 tool to exclude ED. All volunteers and patients were assessed in the same manner and sampled at the same time.

All study participants provided written informed consent. The study was conducted according to

the declaration of Helsinki, implemented by the guidelines of the World Health Organization (WHO). The research was conducted under the supervision of the ethics committee of Inner Mongolia University (No. IMUU20150301421).

Separation of vascular endothelial progenitor cells (vEPCs)

Monoclonal cells (10 mL) from each group were collected using the COBE 6.1 Spectra device (Genbro Company, Somerset, NJ, USA) on the eighth morning following abstinence from ejaculation for 7 days. Then, $CD133^{+}VEGFR\text{-}2^{+}$ peripheral blood stem cells were separated by cell sorting reagent (Miltenyi Biotec, Germany). Employing the flow cytometry counting method, the concentration of 2.2×10^{6} stem cell was used for research, which are referred to as vEPCs.

Laboratory testing

Total RNA extraction: Total RNA including mRNA and miRNA from fresh CD133 stem cells were extracted using the RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity were measured as the absorbance (A) at 280 nm and 260 nm and samples with A_{260}/A_{280} ratios ranging between 1.8 and 2.1 that were used for subsequent experiments.

Micro-array hybridization: All isolated RNA samples were used to synthesize complementary DNA (cDNA), and double-stranded cDNA was labeled and hybridized to the micro-array (Arraystar, Rockville, MD, USA). Following hybridization and washing, processed slides were scanned with the Axon GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA).

Reverse transcription polymerase chain reaction: cDNA synthesis was performed using the RT2 First Strand Kit (Takara, Japan) and real-time PCR was conducted using SYBR Green PCR Master Mix (Promega, Madison, WI, USA) with the Bio-Rad IQ5.0 system (Bio-Rad Laboratories, Hercules, CA, USA). The primers sequences were as follows: F, 5'-*AAAGCAAUGAGACUGA*-3' and R, 5'-*UCUCAUUGCUUUAUA*-3'.

Prostate press

A prostate press was used to stimulate the male prostate gland for subsequent tests. Due to its proximity to the anterior rectal wall, the prostate can be stimulated from the anterior wall of the rectum or externally via the perineum. Digital rectal examination (DRE) was performed to obtain an expressed prostate secretion specimen (EPS) for microscopy and Western blotting analyses.

SDS-PAGE and Western blot

EPSs were collected following DRE. The specimen (1 mL) was mixed with 1 mL lysis buffer (Beyotime, China) and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), 25 mM NaF, and 1 mM Na_3VO_4 were added.

The mixture was frozen at -80°C, thawed at room temperature four times, and centrifuged at 10,000 × *g* for 30 min at 4°C (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was collected and analyzed using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Hayward, CA, USA).

Membranes were blocked with 5% bovine serum albumin-Tris-HCl buffered saline with 0.05% Tween-20 for 2 hours at 25°C followed by incubating with primary mouse anti-CDK16 monoclonal antibody and secondary rabbit anti-mouse monoclonal antibody. GADPH was used as an internal standard and protein bands were visualized using an enhanced chemiluminescence system (Bestbio, China). Samples from the healthy volunteers were used as a control. The relative expression level of CDK16 was calculated by measuring the gray value of the target protein in each group. Relative expression amount = target gene gray value/GADPH gray value.

Cavernosography

Cavernosography was used to evaluate venous occlusion function and arterial inflow to the corpus cavernosum. All operations were performed according to the standard operating procedures of corpus cavernosum assessment. Briefly, intracavernous injection of vasoactive drugs was performed, with re-dosing if needed. Then, two punctures were made in the corpora cavernosa, and a peristaltic pump was used to maintain the intracavernosal pressure (ICP) at 90 mmHg. Non-ionic contrast (50 to 100 mL) was added and X-ray images were taken.

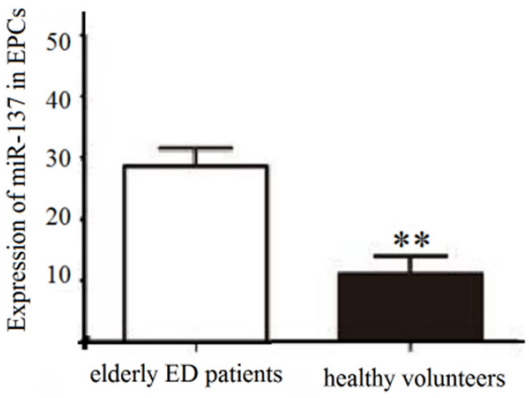

Figure 1. Expression of miR-137 in endothelial progenitor cells (EPCs).

Two intracavernosal angio-catheters were placed following local anesthesia with 1% plain xylocaine, using 21 gauge butterfly needles. One catheter was attached to a pressure transducer to record changes in ICP; the second catheter delivered heparinized saline through a controlled pump at the required rate. Patients were injected with vasoactive drug (prostaglandin E1 or a combination of prostaglandin E1, papaverine, and phentolamine) through one of the intracavernosal catheters.

Statistical analyses

Statistical analyses were performed using SPSS software version 19.0 (SPSS, Inc., Chicago, IL, USA). Differences between two groups were evaluated using a one-way analysis of variance. The Mann-Whitney U test was used to calculate the significance of detection rates between two groups. *P* values below 0.05 were considered significant.

Results

miR-137 expression higher in vEPCs

To determine the expression of miR-137 in peripheral blood stem cells and analyze its effects, we performed micro-array analyses of 96 human miRNAs. In the miRNA array, miR-137 expression was significantly higher in elderly ED patients group. To validate this result, we repeated the measurements using quantitative real-time RT-PCR, with β-actin as an the internal standard ($P < 0.01$, **Figure 1**). Expression levels were negatively correlated with IIEF-5 scores (**Table 1**). There was no significance in the age distribution of the patient group as 8 of the 11 patients ranged in age from 53 to 63 years.

CDK16 decreasing expression in ED patients by western blot

The western blot results were consistent with the real-time RT-PCR results. Several miR-137 target genes play important roles in human diseases. miR-137 has also been shown to inhibit CDK6 expression in elderly ED patients (their gray value were 0.232 ± 0.056 vs. 0.936 ± 0.218). We found that CDK16 protein expression was 75% lower in ED patients (**Figure 2**) compared to healthy volunteers. By T-testing, the t value was 13.452 and $P < 0.001$, between these groups. So, CDK16 expression was significantly reduced in elderly patients with ED.

Penile blood flow slowly by corpus cavernosometry

Cavernosography was used to measure the vascular pressure in the corpus cavernosum. Saline is infused under pressure into the corpus cavernosum with a butterfly needle, and the flow rate needed to maintain an erection indicates the degree of venous leakage. Digital subtraction angiography (DSA) was performed and the X-ray images were acquired digitally (**Figure 3**). Venous leakage could not be identified in any subjects. Bilateral penile corpus cavernosum with symmetrical distribution; contrast agent with evenly distribution and consistent density; penis with smooth edges and clear intervals.

Penile blood flow was also assessed in all subjects, and was almost three times higher in healthy volunteers than in ED patients ($P < 0.05$; 98.83 ± 1.32 mL/min vs. 31.28 ± 1.75, respectively).

Discussion

To the extent of our knowledge, this is the first study to correlate the expression of miR-137 with ED, particularly in elderly patients. The erectile reflex interruption induced by cavernous nerve damage is a direct cause of ED. In addition, apotheosis of smooth muscle cells and endothelial cells in the corpus cavernosum can increase the incidence of ED [19-22].

Table 1. Relationship between miR-137 expression levels and IIEF-5 scores in patients with different severity of erectile dysfunction

Disease severity	IIEF-5 score range	n	Average Ct value of real-time fluorescence
Normal	22-25	10	40.88
Slight	12-21	2	31.57
Moderate	8-11	3	27.93
Severe	5-7	6	25.44

Figure 2. Expression of CDK16 in the two groups.

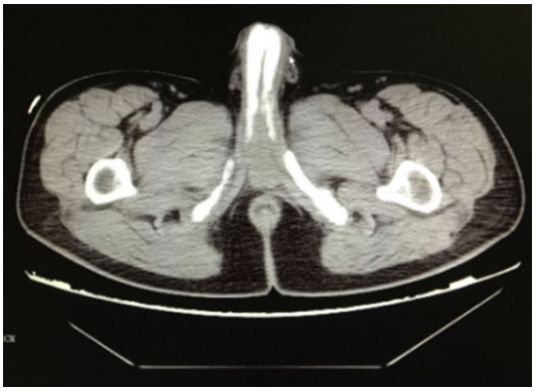

Figure 3. Corpus cavernosometry, using digital subtraction angiography, confirmed that elderly ED patients' penis had normal vascular structure.

All these aspects might result in changes of gene expression, e.g. micro-RNA.

A previous study investigated the expressions of miR-93, miR-320, and miR-16 and evaluated their role in the diagnostic value of ED [7]. It identified there was an obvious negative correlation between the incidence of ED and serum levels of total testosterone, which had a positive correlation with HbA1c levels regulated by these three types of miRNAs. According to this study, the expression levels of the three types of miRNAs, including miR-137, were higher in ED patients than in controls. Therefore, the miR-137 expression changes could be useful for the early diagnosis of ED.

Other scientists found that aging-related erectile dysfunction (AED) patients had series complicated pathophysiological mechanisms about vascular and refractory disorder [17], AED patients had less smooth muscle and endothelium than controls. miRNAs have impacts on the function of the endothelium, which might be involved in the pathophysiological processes of this dysfunction.

Not only gene expression changes, but also target proteins are involved in the ED disease. For example, SIRT1 inhibition regulated by miR-200a could attenuate ED in AED [8]. Following *in vitro* transfection, miR-200a up-regulated SIRT1 and levels of eNOS, then down-regulated cGMP. This study verified that there was up-regulation of not only miR-200a but also miR-137 in ED patients. But it's down-regulation effect needs more data, especially in the eNOS/NO/PKG pathway. Actually, in the present study, the expression of miR-137 was significantly higher in elderly ED patients than in healthy volunteers. Importantly, it was also negatively correlated with IIEF-5 scores. Protein levels of CDK16 were lower in ED patients, as was penile blow flow rate (31.28 ± 1.75 mL/min vs. 98.83 ± 1.32 mL/min). Thus, there was a close correlation between ED and miR-137 expression, which could have inhibited the expression of CDK16. However, further studies are needed on miR-137 and other miRNAs involved in ED.

With aging, the expression of certain genes (e.g., miR-137) is altered to some degree. However, it is not feasible to collect specimens from the same person at different ages over the course of several decades. Thus, we compared 50 year old's and 20 year old's in different populations. We also analyzed the expression and effects of miR-137 expression in patients with ED in an effort to find more suitable gene therapy options or stem cell transplantation treatments.

When we performed corpus cavernosometry using DSA, we wanted to illustrate that there was not only a change in gene and protein expression, but also morphological changes in tissues and organs, pathological changes, and degenerative changes in ED patients. The re-

sults suggest that the miR-137 gene plays an important role in ED.

There were some limitations to this study. Due to Chinese culture, patients with ED are often ashamed and will not seek help. When they do finally see a doctor, there is often a long history of illness. Moreover, in China, ED has not been included in the management of chronic diseases. Therefore, only 11 patients were included in this study. Future studies should include a larger number of patients.

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Disclosure of conflict of interest

None.

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