MiR-125a Rs12976445 Polymorphism is Associated with the Apoptosis Status of Nucleus Pulposus Cells and the Risk of Intervertebral Disc Degeneration

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Key Words
Rs12976445 • Apoptosis • Nucleus pulposus cells • Intervertebral disc degeneration

Abstract

Background: Spinal degenerative diseases are a major health problem and social burden worldwide. Intervertebral disc degeneration (IDD) is the pathological basis of spinal degenerative diseases and is characterized by loss of nucleus pulposus cells due to excessive apoptosis caused by various factors. MicroRNAs (miRNAs) have been reported to be functionally involved in the control of apoptosis. Methods: computational analysis and luciferase assay were used to identify the target of miR-125a, and cell culture, transfection were used to confirm such relationship. Sequencing was used to determine the genotype of each participant. Results: We confirmed the previous report that the presence of the minor allele (T) of rs12976445 polymorphism significantly downregulated the expression level of miR-125a in nucleus pulposus cells, leading to less efficient inhibition of its target gene. We also validated TP53INP1 as a target of miR-125a in nucleus pulposus cells using a dual luciferase reporter system, and the transfection of miR-125a significantly reduced the expression of TP53INP1. The expression level of TP53INP1 was significantly lower in nucleus pulposus cells genotyped as CT or TT than in those genotyped as CC, and the apoptosis rate was consistently lower in the CC group than in the nucleus pulposus cells collected from individuals carrying at least one minor allele of rs12976445 polymorphism. To study the association between rs12976445 polymorphism and the risk of IDD, we enrolled 242 patients diagnosed with IDD and 278 normal controls, and significant differences were noted regarding the genotype distribution of rs12976445 between the IDD and the control groups (OR = 2.69, 95% C.I. = 1.88–3.83, p < 0.0001). In summary, rs12976445 polymorphism is significantly associated with the risk of IDD in the Chinese population. Conclusion: The present study indicated that miR-125a is a promising potential target for patients with IDD in clinical practice.

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Introduction

Spinal degenerative diseases are a major health problem and social burden worldwide, and it is generally accepted that intervertebral disc degeneration (IDD) is the pathological basis of spinal degenerative diseases [1, 2]. Although significant progress has been achieved in recent decades, the exact molecular mechanism remains largely unknown. Some risk factors, including age, genetics, mechanical shear and toxins, have been identified [3, 4], and these risk factors may make nucleus pulposus cells susceptible to apoptosis. The intervertebral disc (IVD) is composed of annulus fibrosus, an external fibrous layer and internal gelatinous core loaded with proteoglycans, the nucleus pulposus. The extracellular matrix (ECM), such as aggrecan and type II collagen, secreted by nucleus pulposus cells plays an essential role in maintaining the integrity of the structure of the spine, and IDD is characterized by ECM depletion caused by the loss of nucleus pulposus cells [5], which can be attributed to excessive apoptosis caused by various factors [6].

Recent studies showed that microRNAs (miRNAs), a class of 21–23 nucleotides, non-coding RNA, are functionally involved in the regulation of a wide range of biological processes, such as apoptosis, differentiation, oncogenesis and proliferation [7], and are responsible for the development of various medical disorders, such as cancer, neurodegeneration and cardiovascular disease [8]. MiRNAs negatively regulate the expression of the target genes via binding to cis-regulatory elements located within the 3' untranslated region (3'UTR) of the genes, resulting in mRNA degradation or translational repression [9, 10]. Up to one third of human genes are under the control of miRNAs [11], but the study of the relationship between miRNAs and the development of IDD is limited [12, 13]. A growing body of evidence suggests that single nucleotide polymorphisms (SNPs) in pre-miRNA genes may interfere with the mature processing or the degradation of miRNA by compromising the secondary structure and causing a reduction of the amount of mature miRNA, which attenuates or abolishes the inhibition of the target genes [14-16]. Furthermore, functional SNPs located in pre-miRNA may be associated with altered susceptibility to numerous diseases, including cancer, reproductive disorders and degenerative diseases [17, 18]. MiR-125a is significantly down regulated in the nucleus pulposus cells of the patients diagnosed with IDD [19], but the downstream mediators and signaling pathways have not been identified. The role of miR-125a in the pathogenesis of the development of IDD remains largely elusive. Hu et al. reported that rs12976445 polymorphism in the pre-miR-125a caused a significant reduction in the amount of mature miR-125 by compromising the mature processing of the miRNA, leading to less efficient inhibition of target genes, LIFR, and increased risk of recurrent pregnancy loss in a Chinese population [20].

Based on the above evidence, we hypothesize that rs12976445 polymorphism may cause down regulation of miR-125a and therefore excessive apoptosis in nucleus pulposus cells, which may be the underlying molecular mechanism of the increased risk of IDD. To test the hypothesis, we determined and compared the expression of miR-125a in human tissue samples of different genotypes and evaluated the association between rs12976445 polymorphism and risk of IDD in a Chinese population.

Materials and Methods

Patients and samples

A total of 520 subjects, including 242 with diagnosed IDD according to the modified criteria of the International Society for the Study of the Lumbar Spine, and 278 normal controls, were recruited in this study. The demographic data and MRI scores [21] of the participants are presented in Table 1. In addition, human lumbar nucleus pulposus specimens were collected from 54 patients (MRI score, IV: 32 and V: 22) who received surgical treatment in our hospital. Tissues specimens were washed three times with phosphate-buffered saline (PBS), and the nucleus pulposus was isolated from the annulus fibrosus with a stereotaxic microscope and was stored at -80°C for future use. The study protocol was approved by the
Research Ethics Committee of our hospital, and written consent was obtained from each participant prior to donation of 4 ml peripheral blood.

Isolation and primary culture of human nucleus pulposus cells
The isolation of nucleus pulposus cells was conducted following the previously described protocol [22, 23]. Tissue specimens were obtained from 54 patients who received surgical treatment in our hospital. NP was separated from NF (observed under a stereotaxic microscope) and was sectioned (2–3 mm). NP tissues were incubated with 0.25 mg/ml type II collagenase to release nucleus pulposus cells.

Rs12976445 polymorphism genotyping
Genomic DNA was isolated from the peripheral blood using a DNA extraction kit (Sangong, Shanghai, China). The quality and quantity of the isolated genomic DNA were determined by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA), and the DNA was then stored at -80°C until use. Chromosome segments containing rs12976445 polymorphism were PCR-amplified (primer set for PCR: 5'-TCT TTC ACA GTG GAT CCT CTG AC-3' and 5'-CCA TCG TGT GGG TCT CAA G-3') and were subsequently sequenced to determine the genotype of the Rs12976445 polymorphism.

Real-time RT-PCR
Total RNA was isolated from nucleus pulposus cells using Trizol (Invitrogen, Carlsbad, CA). The integrity of the isolated RNA was examined by agarose electrophoresis, and the concentration of the RNA was evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). To determine and compare the expression of miR-125a and TPS3INP1 among the genotype groups, the TaqMan assay was used to detect the expression of the miRNA and mRNA. The TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesize single-stranded cDNA, which was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The signals were detected by an ABI 7900 (Applied Biosystems, Foster City, CA). Each sample in each group was measured in triplicate, and the experiment was repeated at least three times. U6 was used for normalization.

Apoptosis evaluation by flow cytometry analysis
The nucleus pulposus was stripped using 0.25% trypsin/EDTA (Invitrogen, Carlsbad, CA), and $1 \times 10^6$ cells were washed twice with PBS and resuspended in binding buffer (2.5 mM CaCl$_2$; 140 mM NaCl; 10 mM
HEPES, pH 7.4). Subsequently, the cells were stained with FITC Annexin V/PI (BD Biosciences, San Diego, CA) and cultured for 10 min at room temperature before loading for flow cytometry analysis.

**Dual luciferase assays**

3′UTR of TP53INP1 was amplified using the following primers: 5′-TAG TTT CAA GTT TTG TTG GTG GTT-3′ and 5′-AAA GAT TTT GCC TGA AGT TAA AAC-3′. The PCR product was inserted into a pGL-3 vector. The accuracy of the construct was verified by direct sequencing. Then, the seed sequence was mutated by introducing the complementary sequence using the following primers: 5′-CAA GCA AAA ATT TAT TGA GTC CCT CAG CTG TAC ACTTT-3′ and 5′-AAA GTG TAC AGC TGA GGG ACT CAA TAA ATT TTT GCTTG-3′. For the luciferase assay, 1 × 10^5 cells were harvested 24 h after transfection and were then analyzed using the Dual Luciferase Assay (Promega, WI, USA), according to the manufacturer’s instructions. Firefly luciferase results were converted to the Renilla signal. All assays were repeated at least three times.

**Oligonucleotides and transfections**

MiR-125a mimics (5′-UCC CUG AGA AGA UUU AAC CUG UGA-3′) and anti-TP53INP1 siRNA (5′-GAT AGT GGT TAA TCC ACCTG-3′) were synthesized and purchased from GenePharma (Shanghai, China). All transfections were performed using DharmaFECT1 reagent (Dharmacon, Austin, TX, USA) according to the instructions provided by the manufacturer. Duplicate or triplicate experiments were conducted for all cell transfections.

**Western blotting**

RIPA lysis buffer was used to lyse the cells, and the concentration of the lysate was determined using a BCA Kit (Pierce, Rockford, IL). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to separate the proteins. After separation, the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% defatted milk and were cultured overnight at 4°C with primary antibodies. After washing with Tris-buffered saline with 0.1% Tween (TBST), the membranes were cultured with horseradish peroxidase-linked secondary antibody for 1 h. The signals on the blots were detected using an enhanced chemiluminescence kit (Applygen, Beijing, China). The primary antibodies, including anti-TP53INP1 antibody and anti-β-actin antibody, and secondary antibody were obtained from Santa Cruz Biotech (Santa Cruz, CA).

**Statistical analysis**

Statistical analysis was performed with SPSS 21 software (IBM Inc. Chicago, IL). All data were generated from a minimum of three independent experiments and are expressed as the mean ± standard deviation (SD). The comparisons between two different groups were performed using Student’s t-test (for two groups) or one-way ANOVA (for more than two groups). Logistic regression analysis was used to adjust the potential confounding factors. Chi square test was used to test the Hardy-Weinberg equilibrium. P < 0.05 was considered statistically significant.

**Results**

As shown in Fig. 1A, rs12976445 polymorphism is located 54 bp upstream of pre-miR-125a. Although the in silico analysis demonstrated that rs12976445 polymorphism could alter neither the predicted secondary structure nor the predicted ΔG, the northern blot results demonstrated that the presence of a minor allele of rs12976445 polymorphism significantly changed the ratio of pre-miR-125a and mature miR-125a, suggesting that the polymorphism significantly interfered with the mature processing of the miRNA [20].

To identify the downstream mediators and signaling pathway of miR-125a in nucleus pulposus cells, we searched the miRNA database (www.mirdb.org) and identified TP53INP1 as a possible target of miR-125a in nucleus pulposus cells. To verify TP53INP1 as a target gene of miR-125a, a luciferase assay reporter system was set up by amplifying and inserting the 3′UTR of TP53INP1 into pGL3 (wild-type pGL3-TP53INP1) that contained downstream firefly luciferase. The predicted seed sequence was replaced by its complementary sequence...
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Fig. 1. Schematic depiction of the location of rs12976445 polymorphism, and sequence comparison of miR-125a and TP53INP1. (A) rs12976445 polymorphism is located 54 bp upstream of pre-miR-125a; (B) Sequence comparison of miR-125a and wild-type/mutated TP53INP1.

Fig. 2. The luciferase activity was significantly decreased in cells transfected with miR-125a mimics and wild-type pGL3-TP53INP, whereas the luciferase activity was comparable between the cells transfected with mutated pGL3-TP53INP/miR-125a mimics and scramble control.

(mutated pGL3-TP53INP1), as shown in Fig. 1B. The constructs were then transfected with miR-125a mimics and scramble controls, and the luciferase activity was significantly decreased in the cells transfected with miR-125a mimics and wild-type pGL3-TP53INP, whereas the luciferase activity was comparable between the cells transfected with mutated pGL3-TP53INP/miR-125a mimics and scramble control (Fig. 2).

To further validate TP53INP1 as a target of miR-125 and to evaluate the effect of overexpression of miR-125a on the endogenous expression of TP53INP1, we transfected nucleus pulposus cells with miR-125a mimics and anti-TP53INP1 siRNA and found that TP53INP1 mRNA and the protein expression level were similarly but significantly downregulated by miR-125a mimics and anti-TP53INP1 siRNA compared with the scramble control (Fig. 3).

To study the effect of rs12976445 polymorphism on the expression of miR-125a and TP53INP1, we collected 54 tissue samples genotyped as CC (n=32), CT (n=18), and TT (n=4) and performed real-time PCR and western blot to determine the expression levels of the miRNA and the gene. As shown in Fig. 4, the expression levels of miR-125a were similar between TT and CT, both of which were significantly lower than that of CC, indicating a dominant model of the minor allele of rs12976445 polymorphism. Consistent with the
hypothesis that miR-125a negatively regulates the expression of TP53INP1, we found that the mRNA and protein expression levels were comparable between CT and TT, whereas the expression of TP53INP1 was much lower in CC than in the CT and TT groups (Fig. 5). Considering the role of TP53INP1 in the control of apoptosis, we further isolated the nucleus pulposus cells of each genotype and used flow cytometry to determine the apoptosis status of the cells. As shown in Fig. 6, the fraction of live cells was significantly higher in the CC group than in the CT and TT groups.

To study the association between rs12976445 polymorphism and the risk of IDD, we enrolled 242 patients diagnosed with IDD and 278 normal controls. The distributions of
rs12976445 polymorphism were in Hardy-Weinberg equilibrium among the case group and the controls. The expression levels of miR-125a were similar between TT and CT, both of which were significantly lower than that of CC, indicating a dominant model of the minor allele of rs12976445 polymorphism. Meanwhile, because the frequency of TT is relatively

Fig. 5. The expression of TP53INP1 grouped by rs12976445 genotype. (A) The mRNA expression levels were comparable between CT and TT, whereas the expression of TP53INP1 was much lower in CC than in the CT and TT groups; (B) The protein expression levels were comparable between CT and TT, whereas the expression of TP53INP1 was much lower in the CC than the CT and TT groups.

Fig. 6. The fraction of live cells was significantly higher in the CC group than in the CT and TT groups.
low in the population we studied, we combined CT and TT. Significant differences were noted regarding the genotype distribution of rs12976445 between the IDD and the control (OR = 2.69, 95% C.I. = 1.88–3.83, p < 0.0001), as described in Table 1.

**Discussion**

MiR-125a is mapped to chromosome 19q13.41 and plays a crucial role in organ development of adult tissues [24, 25]. Accumulating evidence has demonstrated that miR-125a is functionally involved in the tumorigenesis of various types of cancers, including gastric cancer, breast cancer, and lung cancer, by regulating its target genes, which might be oncogenes or tumor suppressive genes [26-32]. The exact role of miR-125 in the development of malignancies may vary in different cancer types and largely depends on the function of its target gene. For example, in some types of cancer, such as lung cancer [29, 30], leukemia [27, 32], gastric cancer [28, 31], and breast cancer [26], the target genes of miR-125a are tumor suppressor genes and the expression of miR-125a is substantially up regulated in those tumor tissues, indicating a oncogenic role of miR-125a in the development of miR-125a. In contrast, in other cancer types, such as multiple myeloma, miR-125a functions as a tumor suppressive gene that is p53-dependent [25]. MiR-125a is also involved in the control of apoptosis of various cells. Kim et al. found a significant upregulation of miR-125a in diffuse large B-cell lymphoma, resulting in an over-activated NF-kB pathway via stimulating signals of TNFAIP3, inhibiting apoptosis, and thereby facilitating the onset of a malignant and anti-apoptotic phenotype in B cells [33]. In this study, we confirmed the previously reported downregulation of miR-125a in nucleus pulposus cells collected from patients with diagnosed IDD, and rs12976445 polymorphism located in pre-miR-125a caused a significant reduction of the mature form of the miRNA. To identify the downstream mediators and signaling pathway of miR-125a in nucleus pulposus cells, we searched the miRNA database (www.mirdb.org) and identified TP53INP1 as a possible target of miR-125a in nucleus pulposus cells. The luciferase reporter system was used to validate TP53INP1 as a target gene of miR-125a, which was confirmed by the following in vitro analysis, showing that TP53INP1 mRNA and protein expression levels were similarly but significantly down regulated by miR-125a mimics and anti-TP53INP1 siRNA compared with the scramble control.

The role of TP53INP1 as a tumor suppressive agent has been described in previous studies [34, 35], and TP53INP1 can induce apoptosis via targeting p53 and facilitate cell growth arrest by regulating transcriptional activity of p53 [34, 35]. In addition, TP53INP1, as well as the apoptosis status of the host cells [36], has been reported to be under the control of the miRNAs [37]. Hu et al. reported that rs12976445 polymorphism in the pri-miR-125a causes a significant reduction in the amount of mature miR-125 by compromising the mature processing of the miRNA, leading to less efficient inhibition of target genes, LIFR, and an increased risk of recurrent pregnancy loss in a Chinese population [20]. As shown in Fig. 1A, rs12976445 polymorphism is located 54 bp upstream of pre-miR-125a. Although the in silico analysis indicated that rs12976445 polymorphism could alter neither the predicted secondary structure nor the predicted ΔG, the northern blot results demonstrated that the presence of the minor allele of rs12976445 polymorphism significantly changed the ratio of pre-miR-125a and mature miR-125a, suggesting that the polymorphism significantly interfered with the mature processing of the miRNA [20]. In this study, we collected 54 tissue samples genotyped as CC (n=32), CT (n=18), and TT (n=4) and performed real-time PCR and western blot analysis to determine the expression levels of the miRNA and the gene. As shown in Fig. 4, the expression levels of miR-125a were similar between the TT and CT groups, both of which were significantly lower than that of the CC group, indicating a dominant model of the minor allele of rs12976445 polymorphism.

Considering the essential role of nucleus pulposus cells in maintaining the integrity of the structure of IVD, the observation that excessive apoptosis of nucleus pulposus cells was significantly associated with an increased risk of IDD, and the fact that rs12976445
polymorphism in the pri-miR-125a causes a significant reduction in the amount of mature miR-125, we hypothesize that rs12976445 polymorphism influences the risk IDD by affecting the TP53INP1 signaling pathway. In this study, we examined the expression of TP53INP1 in human tissue samples and found that mRNA and protein expression levels were comparable between CT and TT; however, the expression of TP53INP1 was much lower in the CC than in the CT and TT groups, and the fraction of live cells was significantly higher in the CC group than the CT and TT groups. Furthermore, to study the association between rs12976445 polymorphism and risk of IDD, we enrolled 242 patients diagnosed with IDD and 278 normal controls and identified significant differences regarding genotype distribution of rs12976445 between the IDD and the control groups (OR = 2.69, 95% C.I. = 1.88–3.83, p < 0.0001).

In summary, the findings of this study confirmed the down regulation of miR-125a in nucleus pulposus cells from patients diagnosed with IDD and verified that rs12976445 polymorphism located in pre-miR-125a caused a significant reduction of the mature form of the miRNA. In addition, rs12976445 polymorphism is significantly associated with the risk of IDD in the Chinese population. The present study indicated miR-125a is a promising potential target for patients with IDD in clinical practice.

Disclosure Statement
No potential conflicts of interest were disclosed.

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