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*Original Article*

# Reduced miR-423 is related to Hirayama disease via modulating the expression of MSTN

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**Abstract:** MiRNA is a group of endogenous short non-coding RNAs which may modulate the expression of more than 60% protein coding genes. MiRNAs have been confirmed to play an important role during the development of muscle and disturbance of miRNA profile has been found in the tissue or serum sample of patients with amyotrophic lateral sclerosis (ALS) and Duchenne muscular dystrophy (DMD). Herein, we detected 14 candidate miRNAs in the serum samples from Hirayama disease (HD) patients. We found the expression of miR-423 was significantly reduced and miR-206 was up-regulated in HD patients. Predicted by bioinformatics tools, confirmed by dual luciferase assay and immunoblotting, we identified that MSTN is a direct target of miR-423 and the target region is conserved between human and mouse. Functional study in mouse myoblasts C2C12 indicated that reduced miR-423 contributes to decreased cell proliferation. To our knowledge, we constructed the relationship between miRNA and HD pathogenesis for the first time and provide a potential new biomarker for HD clinical diagnosis.

**Keywords:** Hirayama disease, miR-423, miR-206, MSTN, cell proliferation

## Introduction

Hirayama disease (HD) is a rare type of cervical myelopathy commonly manifesting as brachial monomelic amyotrophy especially in adolescent boys and young men. It must be differentiated from motor neuron diseases because its natural history is different and because HD tends to stabilise in less than 5 years. Diagnosis is based on clinical findings and dynamic flexion MRI showing segmental spinal muscular atrophy, detachment of the posterior dura mater and venous congestion in the epidural space [1].

MiRNA is a group of endogenous, short non-coding RNAs, which regulates genes expression through targeting the 3'UTR of mRNA. MiRNAs have been found in various organisms, and many of them are evolutionary conserved. Meanwhile, it is estimated that more than a half of all human protein-coding genes are potentially regulated by miRNAs [2]. MiRNAs play important roles in modulating the development of skeletal muscle and motor neuron. Additionally, the dysregulation of several muscle specific miRNAs has been found to contribute to the pathogenesis of serious diseases such as amyotrophic lateral sclerosis (ALS) and Duchenne muscular dystrophy (DMD) [3-6]. And miR-206 has highlighted its potential biomarker for ALS and DMD [7, 8].

In this study, we screened 16 candidate miRNAs in the serum HD patients to unveil the relationship between miRNA dysregulation and the pathogenesis of HD. The target gene of disturbed miRNAs was predicted by bioinformatics tool and examined by dual luciferase assay and immunoblotting.

## Materials and methods

### *Participants*

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Human Ethics Committee of the Huashan Hospital in Shanghai and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Eighteen HD patients and eighteen controls were included in this study. Informed con-

sent was obtained from all individual participants included in the study. All participants underwent electromyography of the thenar and hypothenar muscles as conventional diagnosis method.

Criteria for diagnosis of HD were: (1) onset before 25 years of age; (2) unilateral or bilateral weakness of the distal upper limbs, accompanied by distal amyotrophy of the hand and forearm; (3) unaffected lower limbs; (4) unilateral or asymmetric; and (5) stabilization of the disease after an initial slow progression over several years [9, 10]. In addition, lower cervical compression resulting from anterior shifting of the posterior dura, and crescent abnormality posterior to the dura was evaluated by MRI under neck flexion.

*Serum collection*

Five milliliters of venous blood were collected from each participant at their first admission to the hospitals. To harvest cell free serum, the blood was drawn into a sterile tube without anticoagulant. After leaving the tube in standing position for 20 min, samples were centrifuges at 20°C 1500 g for 10 min, and the supernatant serum was quickly removed and stored immediately at -80°C.

*RNA isolation*

Total RNA was extracted from serum by using TRIzol LS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, about 250 μl serum sample was added into 750 μl TRIzol reagent, and were incubated at room temperature for 5 min. Chloroform was added to the samples, vigorously mixed, and incubated at room temperature for 5 min. Following incubation, the samples were centrifuged at 12,000 g for 15 min at 4°C. RNA was precipitated from the aqueous phase by addition of isopropyl alcohol to a fresh tube containing the supernatant aqueous phase. The integrity of the RNA was tested by spectroscopic analysis.

*MiRNAs quantification*

Quantitive RT-PCR analysis was used to determine the relative expression level of candidate miRNAs. The expression level of miRNAs was detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) together with miRNA-specific TaqMan MGB probes(Applied Biosystems, Foster City, CA, USA). The miR-16 miRNA was used for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times for the detection of miRNAs.

*Cell culture*

HeLa cells and mouse C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% $CO_2$.

*Dual luciferase assay*

A segment of 335 bp MSTN 3'UTR containing the predicted miR-423 target site was cloned into downstream of firefly luciferase coding region in pmirGLO vector (Promega, Madison, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, HeLa cells were seeded in 48-well plates. MiR-423 mimic or inhibitor and luciferase reporter vectors were co-transfected by using lipofectamine 2000 (Invitrogen, Carlsbad, CA USA). 48 hours post transfection, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

*Western blotting*

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 30 μg samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and then blotted onto PVDF membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-MSTN polyclonal antibody (Abcam, Cambridge, MA, USA), mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 2 h at 37°C. The specific protein-antibody complex was detected by using horseradish peroxidase conjugated goat

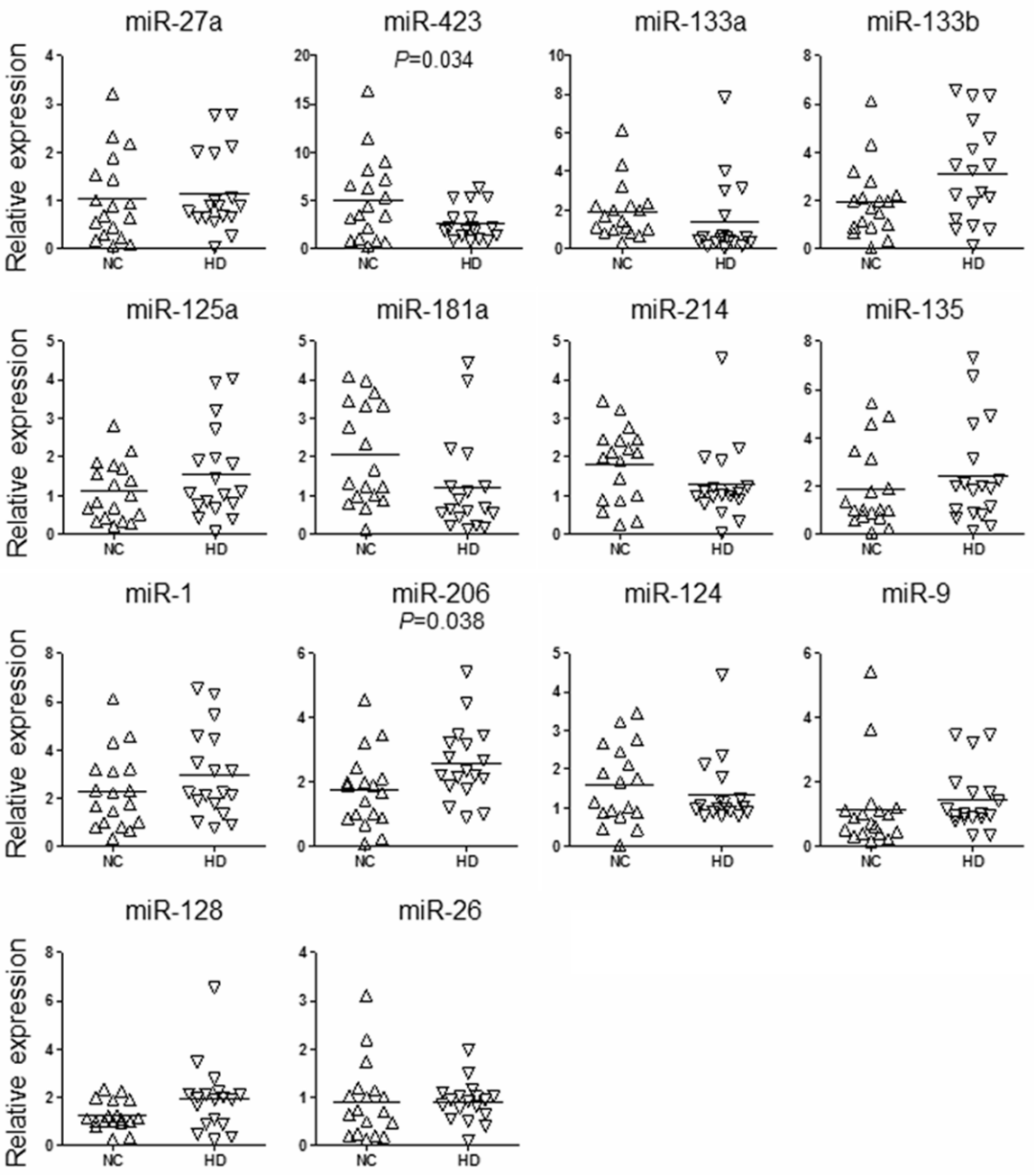


**Figure 1.** Detect the expression of 14 candidate miRNAs in serum samples from HD patients. Total RNA was extracted from 200 μl serum samples, using Trizol LS following the manufacturer's instructions. The expression level of candidate miRNAs was detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit and then amplified by using TaqMan Universal PCR Master Mix together with miRNA-specific TaqMan MGB probes. The miR-16 was used for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times for the detection of miRNAs. Student t-test was used to analyze the results and $P<0.05$ was considered statistically significant. *$P<0.05$, **$P<0.01$.

anti-rabbit or rabbit anti-mouse IgG. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The GAPDH signal was used as a loading control.

*Cell proliferation assay*

EdU (5-ethynyl-2'-deoxyuridine) proliferation assay. 8 hours after transfection with miR-423 mimic or inhibitor, cells were cultured with fresh

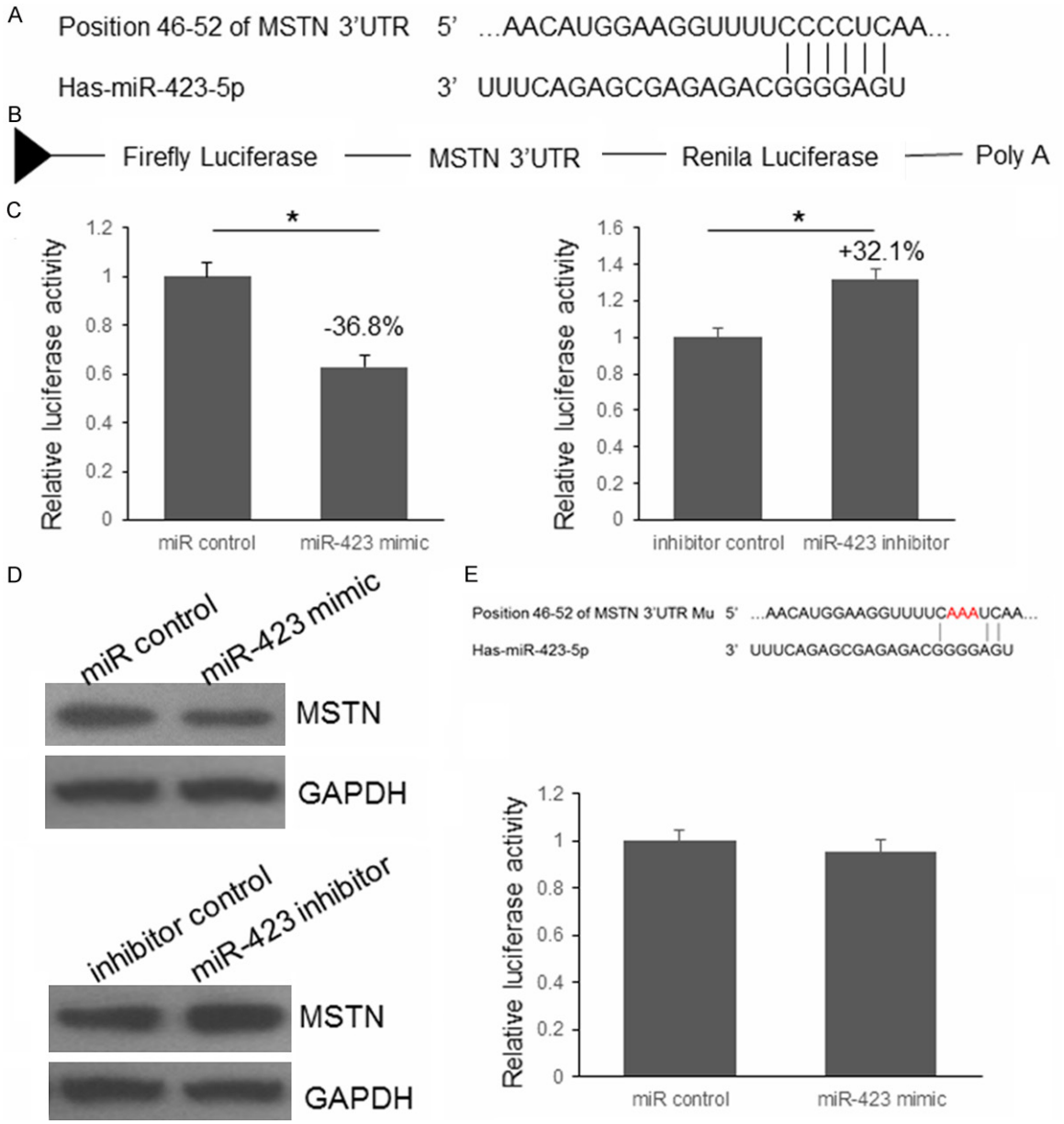


**Figure 2.** MSTN is a direct target of miR-423. A. The predicted interaction between miR-423 and the 3'UTR of MSTN MRNA. B. A schematic diagram of reporter vector construction. A segment of 335 bp MSTN 3'UTR containing the predicted miR-423 target site was cloned into downstream of firefly luciferase coding region in pmirGLO vector. C. Dual luciferase assay. HeLa cells were seeded in 48-well plates. MiR-423 mimic or inhibitor and luciferase reporter vectors were co-transfected by using lipofectamine 2000. 48 hours post transfection, cells were harvested and assayed with the Dual-Luciferase Assay kit. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC). D. Immunoblotting. The effect of miR-423 on MSTN expression was detected by immunoblotting and the signal of GAPDH was used as loading control. E. Mutant reporter vector with 3 nucleotides mutation was constructed for dual luciferase assay to confirm the target sites of miR-423. Student t-test was used to analyze the results and $P<0.05$ was considered statistically significant. *$P<0.05$.

growth medium containing EdU (10 μM) for 24 hours before fixation, permeabilization and EdU staining (Click-It EdU imaging Kit, Life technologies). EdU is a nucleotide analog of thymidine that is incorporated into DNA during active DNA synthesis only by proliferating cells. Cell nuclei were stained with DAPI at a concentration of 5 μg/ml for 30 min.

*Cell cycle flow cytometry*

48 hours after transfection with miR-423 mimic or inhibitor, trypsinized cells were fixed by 70% ethanol at 4°C. Samples were run on a BD FACSCalibur flow cytometry.

*Statistical analysis*

Data were analyzed by using SPSS Statistical Package version 16. Independent two group's analyses are used un-paired t-test. $P<0.05$ was considered statistically significant.

## Results

To investigate miRNA disturbance in the serum samples from 18 HD patients, we detected the expression of 14 candidate miRNAs which are muscle specific or associated with muscle cell differentiation or muscle atrophy by qRT-PCR. As shown in **Figure 1**, the serum miR-423 level was reduced significantly ($P=0.034$) in the HD patients and serum miR-206 level was up-regulated ($P=0.038$). Since miR-206 is one of the most studied and best characterized miRNA to date, which specifically expressed in skeletal muscle [11], our research mainly focused on exploring the roles of reduced miR-423 during the pathogenesis of HD.

Since the biological function of a miRNA mainly depends on the post transcriptional repression of its target genes, we first predicted the potential direct targets of miR-423 by online bioinformatics tool: TargetScan (http://www.targetscan.org/). **Figure 2A** shows the predictive direct interaction between miR-423 and MSTN 3'UTR, and there are 6 complementary sites between MSTN mRNA and the seed sequence of miR-423. To understand whether the expression of MSTN was modulated by miR-423, we cloned a 335 bp segment of MSTN 3'UTR containing the target site of miR-423 into pmirGLO vector to construct a reporter vector pmirGLO-MSTN (**Figure 2B**). MiR-423 mimic or inhibitor co-transfected with pmirGLO-MSTN into HeLa cells. 48 hours post transfection, the cells were lysed and luciferase activities were detected. As shown in **Figure 2C**, the relative luciferase activity was reduced by 36.8% in the miR-423 overexpressed cells and up-reduced by 32.1% in the cells co-transfected with miR-423 inhibitor. These results indicated that miR-423 target the 3'UTR of MSTN and repressed firefly luciferase expression.

To further explore whether endogenous MSTN was repressed by miR-423, HeLa cells were transfected with miR-423 mimic or inhibitor. 48 hours post transfection, protein was extracted and the expression of MSTN was detected by immunoblotting. As shown in **Figure 2D**, the expression of MSTN was repressed by miR-423 mimic and up-regulated by miR-423 inhibitor. To confirm the target site of miR-423, 3 nucleotides in the predicted target region of miR-423 were mutated. As shown in **Figure 2E**, the luciferase activity was not repressed by miR-423 when 3 nucleotides were mutated, indicating that miR-423 repress MSTN expression by targeting this predicted target site. After aligning with murine MSTN sequence, we found that miR-423 target sequence was conserved between human and mouse (**Figure 3A**). And in the same time, miR-423 can repress luciferase activities by targeting the 3'UTR of mouse MSTN (**Figure 3B**, **3C**). To understand whether endogenous murine MSTN was repressed by miR-423, C2C12 cells were transfected with miR-423 or miR-423 inhibitor. Proteins were extracted 48 hours after transfection and were then subjected to immunoblotting. As shown in **Figure 3D**, the expression of MSTN was repressed by miR-423 mimic and up-regulated by miR-423 inhibitor.

To study the effects of inhibition of miR-423 on proliferation of C2C12 cells, miR-423 mimic or inhibitor was transfected into C2C12 cells. The proliferation of C2C12 cells was measured by EdU proliferation assay and propdium iodine flow cytometry. The results showed that miR-423 accelerated proliferation of C2C12 cells; contrarily, miR-423 inhibitor repressed proliferation of C2C12 cells (**Figure 4A**). Quantitative analysis also demonstrated that these changes were statistically significant. The results of cell cycle analysis showed that the percentage of C2C12 cells in the G1 and G2 phases reduced by miR-423 mimic and increased by miR-423 inhibitor (**Figure 4B**). The percentage of C2C12 cells in S phage increased by miR-423 mimic and reduced by miR-423 inhibitor (**Figure 4B**). These results indicated that reduced miR-423 expression is related to decreased proliferation of C2C12 cells.

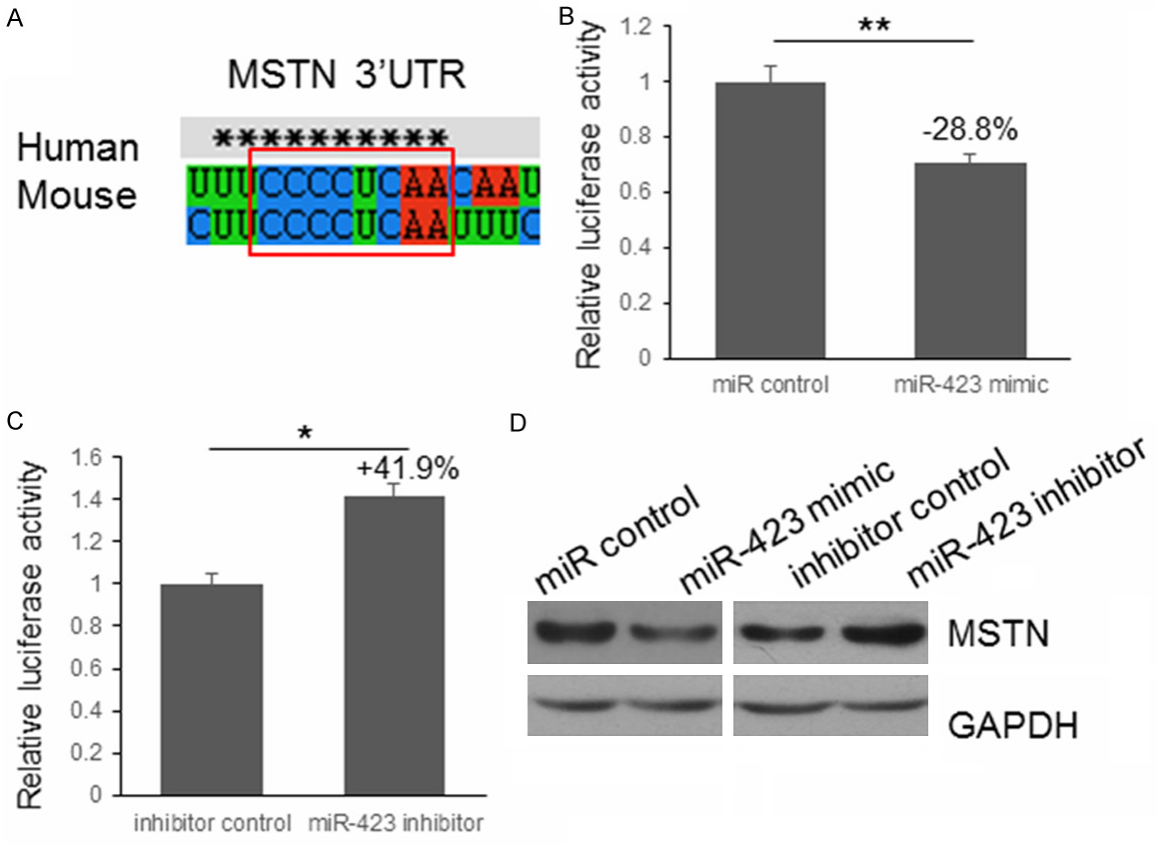


**Figure 3.** Mouse MSTN is repressed by miR-423. A. The sequence of 3'UTR of MSTN between human and mouse were aligned and red box indicates the target site of miR-423. B, C. Dual luciferase assay. A segment of 356bp mouse MSTN 3'UTR were cloned into downstream of firefly luciferase coding region in pmirGLO vector. HeLa cells were seeded in 48-well plates. MiR-423 mimic or inhibitor and luciferase reporter vectors were co-transfected by using lipofectamine 2000. 48 hours post transfection, cells were harvested and assayed with the Dual-Luciferase Assay kit. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC). D. Immunoblotting. C2C12 cells were transfected with miR-423 mimic or inhibitor. 48 hours post transfection, protein was extracted and the expression of MSTN was detected by immunoblotting. The signal of GAPDH was used as loading control.

## Discussion

In this study, we detected HD patients' serum level of 14 candidate miRNAs which are muscle specific (miR-206, -1, -133ab) or muscle development related (miR-27a, -423, -125a, -181a, -214, 135, -128, -26) or neuro specific (miR-9, -124). We found the expression of miR-423 was significantly reduced and miR-206 was up-regulated in HD patients. Predicted by bioinformatics tools, confirmed by dual luciferase assay and immunoblotting, we identified that MSTN is a direct target of miR-423 and the target region is conserved between human and mouse. Functional study in mouse myoblasts C2C12 indicated that reduced miR-423 contributes to decreased cell proliferation. To our knowledge, we constructed the relationship between miRNA and HD pathogenesis for the first time and provide a potential new biomarker for HD clinical diagnosis.

MSTN is a member of the transforming growth factor-β super family, functions as a negative regulator in muscle growth and plays important roles in activation, proliferation and self-renewal of skeletal muscle cells [12]. A previous study indicates that miR-27a targets the 3'UTR of MSTN and represses MSTN expression, which promotes C2C12 myoblast proliferation [13]. However, another group reported that miR-128 represses MSTN expression which inhibits proliferation but stimulates differentiation of C2C12 cells [14]. These various results about MSTN may be caused by multiple target genes of one miRNA. Since one miRNA may target

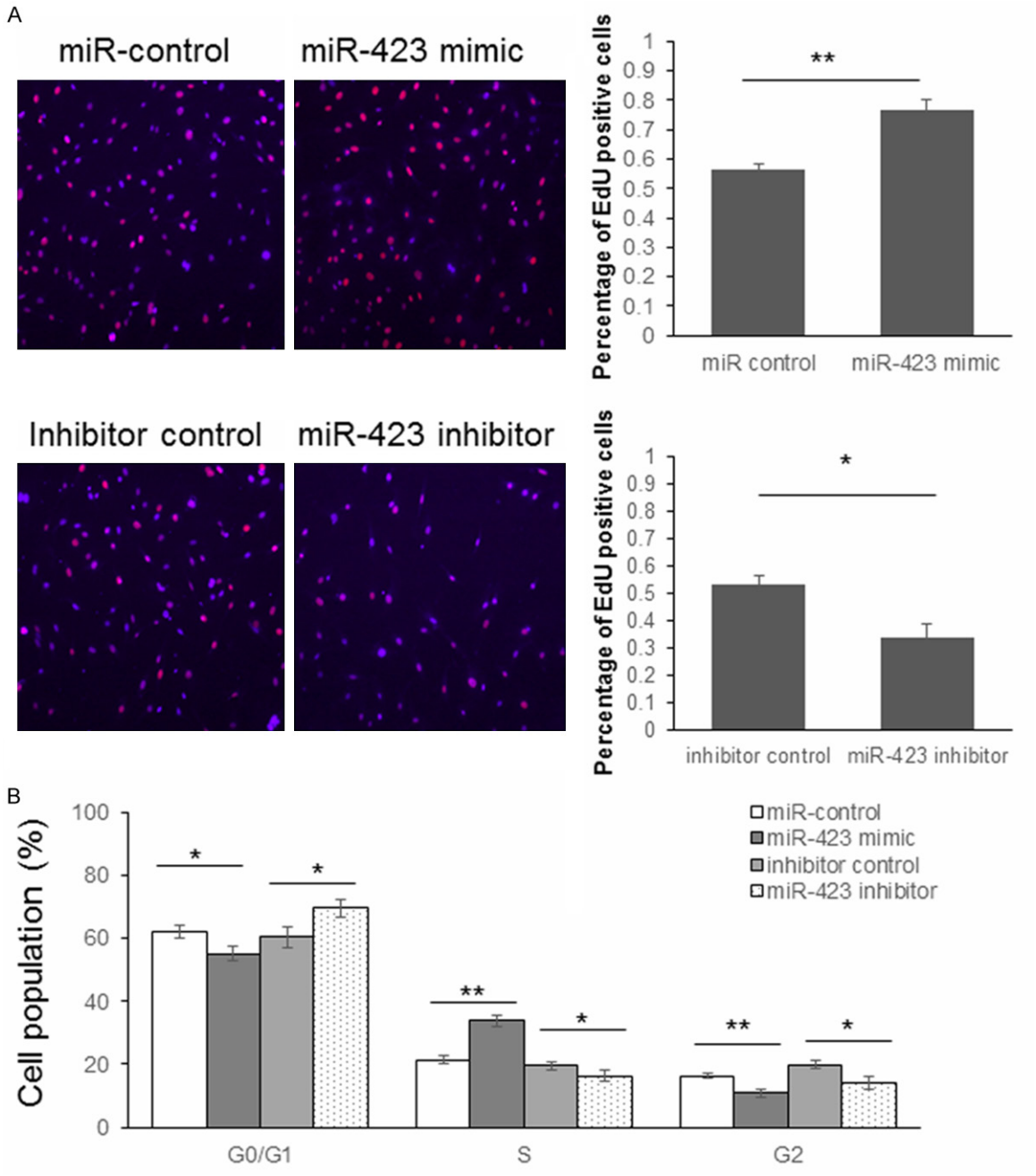


**Figure 4.** MiR-423 promotes cell proliferation. A. EdU proliferation assay. 8 hours after transfection with miR-423 mimic or inhibitor, cells were cultured with fresh growth medium containing EdU (10 μM) for 24 hours before fixation, permeabilization and EdU staining Cell nuclei were stained with DAPI at a concentration of 5 μg/ml for 30 min. B. Cell cycle flow cytometry. 48 hours after transfection with miR-423 mimic or inhibitor, trypsinized cells were fixed by 70% ethanol at 4°C. Samples were run on a BD FACSCalibur flow cytometry. Student t-test was used to analyze the results and $P<0.05$ was considered statistically significant. *$P<0.05$, **$P<0.01$.

tens or hundreds genes directly, to unveil the function of a miRNA, such as miR-423, needs a great deal of research.

MiR-206 specifically expressed in skeletal muscle, and up-regulated circulating miR-206 level has been found in ALS patients. In this study, serum miR-206 level of HD patients was significant higher than control, may representing the muscle atrophy conditions.

In conclusion, herein, we screened the expression of 14 miRNAs expression in the serum samples of HD patients and found reduced

miR-423 and increased miR-206 are related to HD. Subsequently, we confirmed that miR-423 targets MSTN 3'UTR and promote myoblasts proliferation. This is the first report that indicates a relationship between miRNA and HD, may provide a potential new biomarker for HD clinical diagnosis.

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## Disclosure of conflict of interest

None.

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