MiR-29a is a potential protective factor for fibrogenesis in gluteal muscle contracture

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Short title: the role of miR-29a in gluteal muscle contracture.

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Summary

Circulating miRNAs have been proposed as the effective diagnostic biomarkers for muscular fibrosis-associated diseases. However, circulating biomarkers for early diagnosis of contracture muscles are limited in gluteal muscle contracture (GMC) patients. Here we sought to explore the abnormally expressed miRNAs in plasma and contraction bands of GMC patients. The results showed miR-29a-3p expression in plasma and contraction bands tissue was significantly reduced in GMC patients compared with normal control. Cell viability and levels of proliferation-associated protein cyclin D1 and cyclin-dependent-kinase 2 (CDK2) were powerfully inhibited by miR-29a mimics and enhanced by miR-29a inhibitor compared with negative control. Furthermore, miR-29a mimics effectively impeded, while miR-29a inhibitor enhanced the expression of collagen I and collagen III, followed by the secretion of transforming growth factor β1 (TGF-β1), TGF-β3 and connective tissue growth factor (CTGF) in primary human contraction bands (CB) fibroblasts. The miR-29a-3p negatively regulated the expression of TGF-β1 through binding to the 3′ UTR region of *SERPINH1* [encoding heat shock protein (HSP) 47], but had no effect on Smad2 activity. The miR-29a-3p was inversely correlated with HSP47 in contraction bands tissue from GMC patients. Collectively, miR-29a was notably depressed and regulated cell viability and fibrosis by directly targeting HSP47 in GMC, which suggest that circulating miR-29a might be a potential biomarker for early diagnosis and provides a novel therapeutic target for GMC.

Key words: Gluteal muscle contracture; miR-29a; fibrosis; HSP47; TGF-β1

Introduction

Gluteal muscle contracture (GMC) is characterized by contracture of the gluteal muscles and related fascia. The patients clinically present as abduction and external rotation of the affected hip and unable to keep the knees together while squatting, and the patients also have difficulties in maintaining a normal gait (Aggarwal *et al.* 2005, Scully *et al.* 2015). The disease is much more frequent in China with an incidence rate of 1% to 2.5% (Liu *et al.* 2009). GMC not only causes a hip deformity with abnormal hip motion in different degrees but also reported to have femoral head osteonecrosis (Liu *et al.* 2011). Current methodologies for GMC diagnosis are confined in some physical examinations including Ober's sign, flattened or cone-shaped buttock and dimpling of skin in the buttock area. Magnetic resonance imaging (MRI) and computed tomography (CT) scan are also being used for probing GMC; unfortunately, all of them have a disadvantage of not being able to predict early (Rai *et al.* 2017). The surgical treatment is only the effective way to alleviate the symptom of GMC at present (Rai *et al.* 2018, Xu *et al.* 2014). However, intraoperative hemorrhage and postoperative complications such as infection, incision scar and incomplete remission or recurrence of symptoms are problems that cannot be ignored (Zhang *et al.* 2017). Therefore, it is urgent to search for a new biomarker for early diagnosis of GMC that might provide a reliable basis for the definitive treatment of GMC.

Multiple etiologies have been reported to cause the GMC that leads to chronic fibrosis of the gluteal muscles (Al Bayati *et al.* 2016, Zhao *et al.* 2009). There is proliferation of fibroblasts and immoderate expression of collagen in the extracellular matrix of GMC (Zhao *et al.* 2010). Some studies uncovered that fibrotic muscle of the GMC patients is associated with the accumulation of transforming growth factor β1 (TGF-β1), type I and type III collagen (Oleas-Santillan *et al.* 2019, Zhao *et al.* 2010). TGF-β1 can independently act as a potent fibrogenic cytokine for tissue fibrosis by stimulating fibroblasts growth (Zhang *et al.* 2015). TGF-β1/Smad2 signalling pathway plays an extremely important role during fibrotic processes of gluteal muscles. Once activated by TGF-β1, Smad2 can cause the increase of matrix proteins and decrease of matrix-degrading proteases, which are involving in the process of extracellular matrix

(ECM) generation and tissue fibrosis (Yang *et al.* 2011). Furthermore, Other fibrotic related protein such as HSP47, a collagen-specific molecular chaperone, can promote the effects of TGF-β1 on the abduction of expression of collagen type I, collagen type IV in human renal cortical cells (Xiao *et al.* 2012). In human primary tenon's fibroblasts, HSP47 removal can notably inhibit TGF-β1-induced differentiation from Tenon's fibroblasts to myofibroblasts (Hong *et al.* 2012). These data suggest that correlation of HSP47 and TGF-β1 participates in the fibrotic processes. However, the relationship of HSP47 and TGF-β1 are blurry in the progression of GMC.

Numerous evidence indicates that miRNAs is of utmost importance to the progress of muscular fibrosis (Hesse *et al.* 2019, Zanotti *et al.* 2018). For instance, the expression of miR-29 and miR-21 was much more abundant in Duchenne fibroblasts than in Duchenne muscle and myoblasts (Zanotti *et al.* 2015). The miRNAs expression pattern of Duchenne muscular dystrophy patients also shows the upregulation of miR-31, miR-34c, miR-206, miR-335, miR-449, and miR-494 in adductor muscles (Greco *et al.* 2009). Muscular dystrophy (MD) accompanied by fibrosis present an increased expression of miR-21 in the mouse of Laminin-deficient congenital muscular dystrophy (LAMA2-CMD) (Moreira Soares Oliveira *et al.* 2017). Some studies also demonstrated that the expression of miR-30c and miR-181a increased 7- and 6-fold, respectively, in the serum of muscular dystrophy patients (Llano-Diez *et al.* 2017). The profile of circulating miRNA of muscular dystrophy patients in serum shows that miR-1, miR-133a and miR-206 are up-regulated, which is coincident with abnormal expression in muscle-specific miRNAs (Mizuno *et al.* 2011). These above studies suggest that serum miRNAs and muscle miRNAs are potentially useful and reliable biomarkers for early diagnosis in muscle fibrosis-related diseases. Nevertheless, there are no evidence that circulating miRNA or other biomarkers for early diagnosis in GMC progression.

Herein, we demonstrated that the expression of miR-29a was lower in plasma and contraction bands in GMC patients than that in normal control. The miR-29a regulation affected the cells viability and fibrosis of fibroblasts by regulating HSP47 and TGF-β1 expression. The expression of miR-29a was negatively associated with the transcription and protein level of HSP47. These findings suggest that miR-29a is likely to be a useful

biomarker for early diagnosis and monitoring prognosis of GMC.

Materials and Methods

Subjects

This study was approved by the ethics committee of PEKING University Shenzhen Hospital, and all patients had signed informed consent. Blood samples were collected from 13 healthy people and 18 GMC patient ranging from March 2017 to June 2018 in PEKING University Shenzhen Hospital. Briefly, peripheral venous blood samples were collected from individual and stored in EDTA anticoagulant tube. Each sample (5 ml) was diluted with an equal volume red blood cell lysis buffer (R1010, Solarbio, China) for 15 min on ice. After centrifugation at 400 g for 10 min, the supernatant containing lysate of red blood cell was removed and the bottom sediment (leukocyte) was resuspended by PBS buffer. The mixture was centrifugated at 400g for 10 min again to collect bottom sediment followed by the extraction of total RNA by using Trizol regents. Besides, fresh contraction bands (CB) tissues were collected from GMC patients (n=8) with CB releasing surgery according to previous study. Then CB tissues were used for next experiment. The clinical features of the cohort were presented in **Table 1** and **Table 2**.

Immunohistochemical staining

Fresh contraction bands (CB) and adjacent normal muscle samples of GMC patients (n=8) were collected and fixed with 4% paraformaldehyde for 24h at 4 °C. After dehydrating with graded ethanol, tissues were vitrified by dimethyl benzene and embedded in paraffin. The 5 μm sections of paraffin-embedded tissue were dewaxed in xylene for 10 min x 3 times and rehydrated in an ethanol gradient. Antigen retrieval was performed with high-pressure method in fresh sodium citrate solution for 15 minutes, followed by blocking in 5% Bovine Serum Albumin (BSA) for 1 h at room temperature. Blocked slides were then incubated with the primary antibody against HSP47 (Ab109117, 1:200, Abcam, USA) diluted with 5% BSA overnight at 4°C. Next day, the slides were washed in 1x phosphate buffered solution-Tween 20 (PBST) for 5min x 3 times and incubated with specific-secondary antibody (PV9001, ZSGB-BIO,

China) according to the manufacture's instruction. Finally, HSP47 positive cells were visualized by using standard diaminobenzidine (DAB) chemiluminescence reagents (ZLI 9018, ZSGB-BIO, China). After mounting with glycerinum, HSP47-positive cells were observed under inverted biological microscope and counted using Image J software.

Cell isolation and culture

Fresh CB tissues from GMC patients were used for human CB fibroblasts isolation. Briefly, contraction bands were separated into blocks with 2×2 mm^2 and washed with sterile PBS buffer for 3 times. Then, the small blocks were placed into 10-cm tissue culture dishes and incubated with DMEM (11320-033, GIBCO, USA), 10% FBS (10091-148, GIBCO, USA), 10 μg/ml gentamicin (15750078 Invitrogen, USA), and 10 mg/ml amphotericin B (R01510, Invitrogen, USA) overnight. Cells were incubated in at 37 °C in a humidified incubator in 5% CO2. Culture medium was changed three times each week. The cells adopted in this study were confined in 3-5 generations. Human CB fibroblasts were harvested when cells at 80-90 % confluence using 0.1% trypsin and were seeded in 6-well plate. After 24h, miR-29a mimic and miR-29a inhibitor (RiboBio, China) were transfected in cells by lipo2000 (11668-027, Invitrogen, USA) for 48h and then performed further examinations.

Real-time quantitative PCR (qRT-PCR)

Total RNA from leukocyte and contraction band tissues were extracted by Trizol (15596026, Invitrogen, USA) under the manufacture's introduction. Then 1μg RNA was reverse-transcribed into cDNA by using cDNA reverse transcription kit (4368813, Applied Biosystems, USA) according to the specification. Real-time quantitative PCR (qRT-PCR) was performed to measure the relative mRNAs expression of target genes by using SYBR Green (AQ131-01, Transgen Biotech, China). All data were normalized to the control of GAPDH. PCR primer sequence was shown in **Table 3**.

For miRNA detection assay, total RNA from blood samples and tissues were extracted as described above. Then 10 ng RNA was reverse-transcribed into cDNA by TaqMan™ MicroRNA Reverse Transcription Kit (4366596, ThermoFisher Scientific, USA). TaqMan Universal PCR Master Mix was used to perform RT-PCR amplification

by Applied Biosystems ABI 7500 (ThermoFisher Scientific, USA). U6 was served as the internal control for qRT-PCR assay. All primer information of miRNAs was presented in **Table S1**. Stem–loop RT primer (custom RT primer) was used to perform reverse transcription. The universal stem-loop sequence was as follow: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC. After real-time PCR reaction, specific RT primers and probes were used for fluorescence quantification.

CCK8 assay

Primary human CB fibroblasts were cultured in 96-well plates at a density of $3x10^3$ cells/well. After 12h, miR-29a mimics and inhibitors were transfected in cells by lipo2000 (11668-027, Invitrogen, USA) for 8h followed by changing fresh culture medium. After 24, 48, 72 and 96 h, cells were added in 10μL CCK8 assay (C0037, Beyotime, China) reagents for 4h and the cells viability were measured by a Multiscan plate reader (MK3, Thermo Fisher Scientific, Waltham, MA, USA) at the wavelength of 450 nm.

ELISA assay

Levels of TGF-β1, TGF-β3 and CTGF in cell supernatant were measured by ELISA assay. In brief, when human CB fibroblasts cells were transfected with miR-29a mimics or miR-29a inhibitors for 48 h, culture mediums were collected and centrifugated at 2500 rpm for 10 min, the supernatants of mediums were used to detect the concentration of fibrogenic cytokines according to the manufacture's instruction of TGF-β1 kits (88-8350-22. Invitrogen, USA), TGF-β3 kits (KA4402, Abnova, USA) and CTGF kits (ABIN6730903, Abnova, USA). Absorbance readings were taken at the wavelength of 405 nm using a Multiscan plate reader.

Dual-luciferase assay

The human normal untranslated regions sequence (3′ UTR) of *SERPINH1* (HSP47) containing miR-29a binding site and its mutated 3′ UTR sequence were synthesized by PCR amplification and inserted into the pYr-MirTarget basic vector. 293T cells were seeded in 24-well plates with a density of 2×10^4 cells/well. After 12h, cells were transfected with 50 nM miR-29a mimic or negative controls by using lipo2000,

followed by co-transfection with 50 ng of the wild type (WT) or 3′ UTR-mutant ofHSP47, respectively. Luciferase activities were proceeded with Luciferase Reporter Detection kits (E1910, Promega, USA) at 48 h post-transfection. Each sample was duplicated at least 3 times.

Western Blotting

Total proteins were extracted by using RIPA protein lysate (89900, Invitrogen, USA). 50 μg total protein were used to determine the levels of proliferation- and fibrosis-related protein. All samples were performed at least 3 independent experiments. Primary antibodies information were as follows: cyclin D1 (55506S, Cell Signaling, USA), CDK2 (2546S, Cell Signaling, USA), P-SMAD2 (18338S, Cell Signaling, USA), SMAD2 (5339S, Cell Signaling, USA), TGF-β1(ab92486, Abcam, USA), collagen I (ab34710, Abcam, USA), Collagen III (ab7778, Abcam, USA), HSP47 (Ab109117, 1:200, Abcam, USA), GAPDH (AF0006, Beyotime, China)

Statistical analyses

All experiments were repeated at least 3 times. Correlation analysis was performed by Pearson's coefficient using SPSS software. Relative quantitative analysis of protein was proceeded using Quantity One software. All values were presented as means ± SD, and these data were statistically analyzed by a two-tailed Student’s t test and two-way ANOVA by using GraphPad Prism 5.0 software. Statistically significant differences were accepted at $P < 0.05$.

Results

MiR-29a is apparently reduced in GMC patients

MiRNAs, as direct post-transcriptional repression, play an essential role in muscular dysfunction (Amin *et al.* 2015, Friedman *et al.* 2009). However, its role in the progression of GMC remains unclear. In the present study, we first evaluated the expression pattern of muscular dysfunction- and fibrosis-related miRNAs such as miR-29a-3p, miR-26a-5p, miRNA-133a-3p, miRNA-206, miRNA-222-3p, miRNA-342a-3p and miRNA-378a-5p in GMC patients. The results showed that only miR-29a-3p among these miRNAs was drastically declined in the plasma of GMC patients

compared with normal individuals **(Fig. 1A)**.

Furthermore, the expression of these miRNAs were determined in contraction bands tissues of GMC patients and the paired adjacent normal muscle samples. As shown in Figure 1B, the levels of miR-29a-3p and miR-342a-3p was decreased, whereas the expression of miR-133a-3p and miR-222-3p presented a significant increase in GMC patients' tissues compared to that in adjacent normal muscle **(Fig. 2B)**. Therefore, these findings prove that the reduced miR-29a-3p in plasma is consistent with the expression pattern in contraction bands of GMC patients, which suggest that miR-29a-3p is possibly an effective diagnostic and prognostic circulating marker for GMC.

MiR-29a impairs cell proliferation of human contraction bands fibroblasts

It has been demonstrated that decreased cell viability of muscle fibroblasts contribute to the improvement of muscular dystrophy and muscle fibrosis (Morin *et al.* 1995). To explore the biological functions of miR-29a on human contraction bands (CB) fibroblasts, we firstly performed CCK8 assay to determine the role of miR-29a on cell ability of human CB fibroblasts. Once transfected with miR-29a mimics, cells showed a significant decline at Day 2 and Day 3 compared with control mimics and a more apparently reduced at Day 4 compared with human CB fibroblasts transfected with control mimics **(Fig. 2A)**. However, miR-29a inhibitors markedly enhanced cell viability of fibroblasts compared to negative control cells at Day 2 and the promotion effect became more significant at Day 3 and Day 4 **(Fig. 2A)**. Besides, we further determined the impact of miR-29a on cell cycle-related proteins of fibroblasts. After cells were treated with miR-29a mimics for 48h, the proteins of cyclin D1 and cyclin-dependent kinase 2 (CDK2) were powerfully crippled, which expression were significantly enhanced in fibroblasts incubated with miR-29a inhibitor compared with negative inhibitor group **(Fig. 2B)**. These results showed that miR-29a inhibition promotes cell viability of primary human contraction bands (CB) fibroblasts possibly by regulating cyclin D1 and CDK2.

MiR-29a hampers the fibrotic process in human CB fibroblasts

GMC is featured by gluteal muscle fibrosis, which is mediated by numerous fibrotic regulators including some collagens and transforming growth factor (TGF) members (Karsdal *et al.* 2017, Meng *et al.* 2016). As shown in Fig 3A, miR-29a mimics transfection obviously inhibited the protein expression of collagen I and collagen III, whereas miR-29a inhibitors enhanced their levels in human CB fibroblasts compared with negative control mimics groups **(Fig 3A)**. The relative protein quantitative analysis of collagen I and collagen III confirmed the regulatory role of miR-29a on the expression of collagens molecule **(Fig 3A)**. Furthermore, a weaker expression of *collagen I, collagen III* and alpha-SM-actin (α-SMA) mRNA were showed in fibroblasts transfected with miR-29a mimics than cells treated with negative control mimics, but fibroblasts with miR-29a inhibition significantly promoted the transcription level of *collagen I, collagen III* and α-SMA **(Fig. 3B)**. At the same time, we measured the concentration of fibrosis-associated factors including TGF-β1, TGF-β3 and CTGF in culture mediums. As expected, compared with negative control mimics groups, miR-29a mimics drastically inhibited the secretion of TGF-β1, TGF-β3 and connective tissue growth factor (CTGF), whereas miR-29a removal accelerated the release of these fibrosis-associated factors in human CB fibroblasts **(Fig. 3C)**. Thus, these results indicate that miR-29a involved in fibrotic process by obstructing the expression of collagens and the secretion of fibrogenic cytokine in primary human CB fibroblasts.

MiR-29a inhibits signal transduction of fibrosis by targeting HSP47

HSP47 and TGF-β1 have been widely reported in fibrotic related diseases, such as intestinal fibrosis and hepatic fibrosis and so on (Ali *et al.* 2014, Inoue *et al.* 2011). To elucidate the regulatory mechanism of miR-29a on fibrosis *in vitro*, we evaluated the changes of fibrosis-related signal transduction including HSP47 and Smad2/TGF-β1 in fibroblasts treated with miR-29a mimics or miR-29a inhibitors. Fibroblasts suffered from miR-29a mimics exhibited more depressed expression of HSP47 and TGF-β1 than cells exposed with negative control mimics, and no changes were observed in the expression of phosphorylated Smad2 and total Smad2. However, Fibroblasts exposed

with miR-29a inhibitors showed the enhancement of TGF-β1 expression, followed by the increase of HSP47, compared with cells transfected with negative control inhibitors **(Fig. 4A and B).** Further study demonstrated that there were the potential binding sites between miR-29a-3p and *SERPINH1* (HSP47) analyzed by using Starbase database (**Fig. S1**). Then luciferase reporter assay showed that miR-29a mimics significantly decreased the transcriptional activity of wild-type *SERPINH1,* but had no impact on its luciferase activity in cells transfected with *SERPINH1*-3'-UTR-Mut reporter **(Fig. 4C)**. These results suggest that miR-29a blunts the expression of HSP47 by directly targeting its' 3'-UTR, implying that miR-29a negatively regulates the expression of TGF-β1 possibly through affecting HSP47, but not Smad2 activity.

MiR-29a is negatively correlated with HSP47 in gluteal muscle contracture patients

Next, we measured the expression of *SERPINH1* in contracture band tissue and paired-adjacent tissues from 8 **gluteal muscle contracture** (GMC) patients. The results showed that *SERPINH1* mRNA was much higher in gluteal muscle contracture than adjacent tissues **(Fig. 5A)**. Combined with the results of miR-29a level in contracture band tissue, we discovered that there had a negative association between *SERPINH1* and miR-29a expression in contracture bands of GMC patients **(Fig. 5B).** We also assessed the protein level of HSP47 in contracture band tissue from 8 GMC patients by immunohistochemistry. As shown in Figure 5C, in comparison to adjacent muscle tissues, HSP47-positive cells were robustly enhanced in contracture bands tissue (**Fig. 5C**). And there was also a significant negative correlation of HSP47-positive cells ratio and miR-29a expression in GMC patients **(Fig. 5D)**.

Discussion

Circulating microRNAs has been widely used as a useful biomarker in numerous disease including cancer, diabetes, and cardiovascular (Lees *et al.* 2017, Yang *et al.* 2018, Zhao *et al.* 2017). In this study, we discovered that circulating miR-29a is downregulated in GMC patients and miR-29a participated in the fibrotic process in

human CB fibroblasts, implying that miR-29a might be a helpful biomarker for early diagnosis of GMC.

Many studies in human muscular disorders have reported MiRNAs dysregulation. One hundred and eighty-five miRNAs were enhanced or dwindled in muscle specimens of muscular disorders patients in humans including DMD and Becker muscular dystrophy (BMD), both of which arise from muscular fibrosis (Ardite *et al.* 2012, Eisenberg *et al.* 2007, Ohlendieck *et al.* 2017). Subsequently, miRNAs profiling in blood serum and plasma were abnormal in muscular fibrosis-related diseases (Cacchiarelli *et al.* 2011, Hu *et al.* 2014, Matsuzaka *et al.* 2014). However, numerous circulating miRNAs were not correspondence with the ones in serum and plasma, for instance that miR-1, miR-133a, and miR-206 were highly abundant in serum but downregulated or modestly upregulated in muscle (Roberts *et al.* 2012). Fortunately, we found an extraordinary reduced expression of miR-29a in plasma and contraction bands of GMC patients. These findings demonstrate that miR-29a inhibition potentially take part in specific pathological pathways of GMC disease and miR-29a in plasma is perhaps an efficient biomarker for early diagnosis of GMC.

The miRNAs is closely associated with the development of fibrosis in various tissues through controlling the expression of anti- and profibrotic genes, the secretory profibrotic cytokine and transforming growth factor (Bowen *et al.* 2013). The miR-21 level is elevated in DMD patient's muscle, and miR-21 inhibitor significantly hampers the transcription of fibrosis-related genes, such as *COL1A1* and *COLI6A1* by facilitating the expression of PTEN, resulting in violent reduction of collagen I and VI, and inhibition of fibrosis progression (Heller *et al.* 2017). Importantly, miR-29b overexpression prevents endometrial fibrosis via blockade of the Sp1-TGF-β1/Smad-CTGF pathway (Li *et al.* 2016). MiR-29c suppresses the expression of collagen type I (COL1), collagen type III alpha 1 chain (COL3A1), TGF-β3, CDK2 to curb the leiomyoma growth and fibrosis (Chuang *et al.* 2017). Administrated with miRNA-29c significantly alleviates the accumulation of fibrosis by directly depressing the transcription of *COL1A1*, followed by the reduction of *Col1A*, *Col3A*, *fibronectin* and *Tgfb1 expression* (Heller *et al.* 2017). In this study, we detected that miR-29a

powerfully inhibited the cell viability of fibroblasts. Besides, miR-29a activation notably activated the fibrotic process by promoting the expression of collagens and the secretory of fibrosis-related factors. Our data proves that miR-29a-mediated the inhibition of cell viability and fibrotic process possibly affects the development of GMC.

HSP47-positive myofibroblasts in the skin significantly induce collagen deposition and aggravate skin fibrosis in chronic graft-versus-host disease (Yamakawa *et al.* 2018). It is confirmed that miR-29b disrupts collagen structure and collagen maturation in hepatic stellate cells by targeting 3'-UTRs of *SERPINH1* (Zhang *et al.* 2014). MicroRNA-29b-3p also prevents Schistosoma japonicum-induced liver fibrosis by down-regulating the expression of TIMP-1, HSP47, α-SMA, COL1A1, and COL3A1 (Tao *et al.* 2018). Besides, the upregulation of miR-29a is able to alleviate idiopathic pulmonary fibrosis (IPF) by regulating HSP47 (Cushing *et al.* 2011, Kamikawaji *et al.* 2016). Here, we identified that miR-29a mimics attenuated fibrosis of CB fibroblasts by downregulating the expression of TGF-β1 via directly binding to HSP47. And, miR-29 modification has no impact on the activity of Smad2. These findings reveal that Smad2/TGF-β1 pathway is not involved in miR-29a-mediated the improvement of fibrotic processes and HSP47/TGF-β1 should be the response for its principal function. Our further study illustrated that there had a negative correlation between miR-29a and HSP47 in GMC patients, which confirmed that miR-29a-mediated the inhibition of fibrosis in fibroblasts should involve with the downregulation of HSP47 in GMC patients. These results firstly and validly suggest an indispensable role for miR-29a in the progression of GMC by interfering fibrosis through regulating HSP47. However, the study is limited by small sample size, and the larger sample size is needed to support the theory postulated in this study. Additionally, effective animal models should be established to further confirmed the new prognostic biomarker of miR-29a in GMC disease.

Conclusion

In summary, low miR-29a was observed in plasma and contraction bands in GMC

patients. Upregulation of miR-29a inhibited the cells viability and fibrosis of fibroblasts through regulating TGF-β1 by targeting HSP47. Therefore, circulating miR-29a might be served as a potential early diagnosis index for GMC. Possibly, miR-29a is likely to be a protective factor and novel therapeutic target for GMC by blocking fibrosis progress.

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Not Applicable.

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Figures and Figure legends

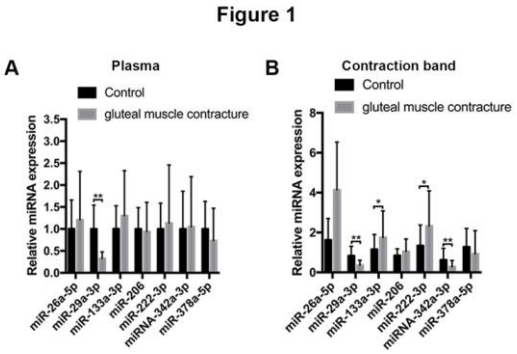

Figure 1. The miRNAs profiles of plasma and tissue in human normal individuals and Gluteal muscle contracture (GMC) patients. MiRNAs profiles including miR-26a-5p, miR-29a-3p, miR-133a-3p, miR-206, miR-222-3p, miRNA-342a-3p and miR-378a-5p of plasma (healthy samples: n=13; GMC patients: n=18) in human normal individuals and GMC patients (**A**) and gluteal musculature tissue (n=8) **(B)** in contraction bands and adjacent normal muscle samples as determined by qRT-PCR. * indicates normal individuals vs GMC patients or adjacent normal muscle vs contraction bands of GMC patients. Data represent the means ± SD of three experiments and analyzed by GraphPad Prism 5.0 software using two-way ANOVA. *, $P<0.05$. **, $P<0.01$.

Figure 2

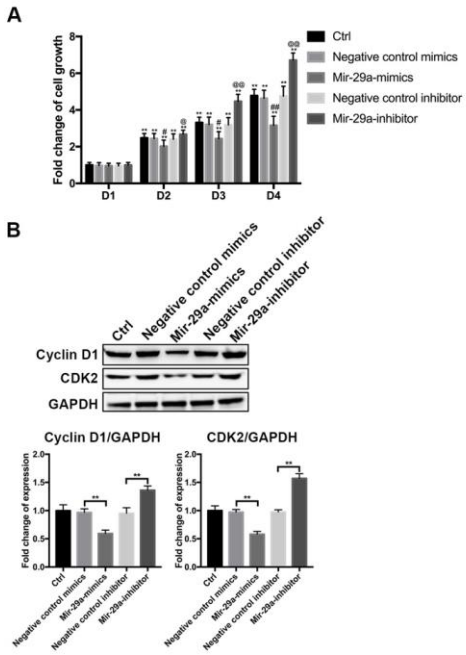

Figure 2. Effects of miR-29a on cell viability in primary human contraction bands fibroblasts. (**A**) Human contraction bands (CB) fibroblast cells were transiently transfected with miR-29a mimics (50 nM), miR-mimic NC, miR-29a inhibitors, or miR-inhibitor NC for 48 h. Then cell viability of different group analyzed by CCK8 experiment at Day 1 to Day 4. (**B**) Cell cycle related protein cyclin D1 and CDK2 were evaluated by western blotting in human CB fibroblast cells. Relative quantitative analysis of protein was proceeded using Quantity One software. Protein expression of collagen I and collagen III were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ctrl, control; NC, negative control. * indicates each group vs the former day of each group. $^{\#}$indicates miR-29a mimics groups vs negative control mimics groups a more significant difference with P <0.05; $^{\#}$ indicates miR-29a mimics groups vs negative control mimics groups. $^{@}$ indicates miR-29a inhibitors groups vs negative control inhibitor groups. Data represent the means ± SD of three experiments, each experiment performed in triplicate. Fold change of cell growth was analyzed by

GraphPad Prism 5.0 software using two-way ANOVA. # and @, P<0.05. **, ## and @@, P<0.01.

Figure 3

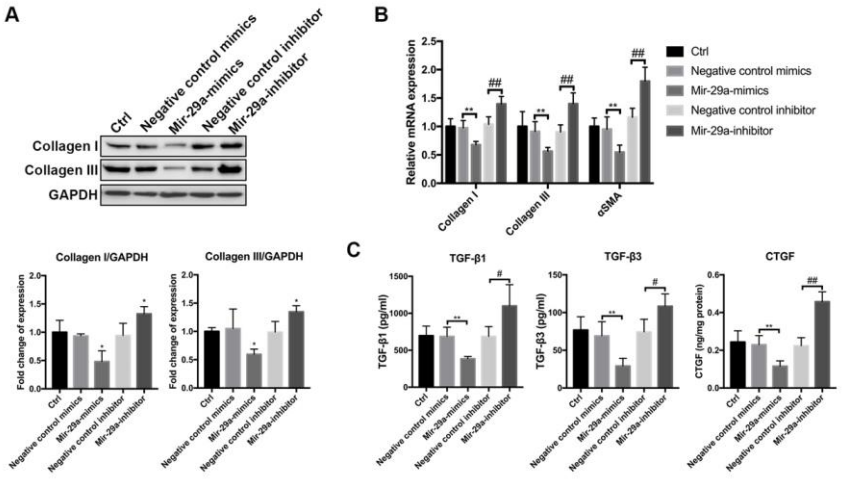

Figure 3. Effects of miR-29a on fibrosis and collagen deposition in primary human contraction bands fibroblasts. Primary human contraction bands (CB) fibroblasts were transiently transfected with miR-29a mimics (50 nM), miR-mimic NC, miR-29a inhibitors, or miR-inhibitor NC for 48 h. Then the protein expression of collagen I and collagen III (**A**) and mRNA expression of collagen I, collagen III and α-SMA (**B**) were measured by western blotting and qRT-PCR, respectively. Protein expression of collagen I and collagen III were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative quantitative analysis of protein was proceeded using Quantity One software. Ctrl, control; NC, negative control; α-SMA, alpha-SM-actin. **(C)** Profibrotic factor TGF-β1, TGF-β3 and connective tissue growth factor (CTGF) in cellular supernatant were measured by ELISA assay. * indicates miR-29a mimics groups vs negative control mimics groups. # indicates miR-29a inhibitors groups vs negative control inhibitor groups. All data were statistically analyzed by GraphPad Prism 5.0 software using a two-tailed Student's t test. * and #, P <0.05. ** and ##, P <0.01.

Figure 4

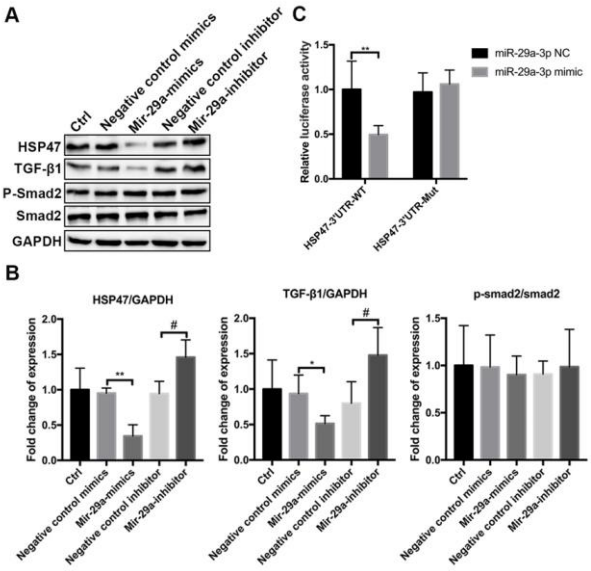

Figure 4. Effects of miR-29a on HSP47 and subsequent signaling pathway in primary human contraction bands fibroblasts. (**A**) Human CB fibroblasts were transiently transfected with miR-29a mimic (50 nM), miR-mimic NC, miR-29a inhibitor, or miR-inhibitor NC for 48 h. Then the protein expression of HSP47, TGF-β1, P-Smad2 and Smad2 were analyzed by western blotting. Ctrl, control; NC, negative control. (**B**) Grayscale quantitation of HSP47, TGF-β1 and P-Smad2 protein in primary human CB fibroblasts. Relative quantitative analysis of protein was proceeded using Quantity One software. (**C**) Effect of miR-29a on transcription activity of HSP47 as determined by luciferase report assay in fibroblasts. * indicates miR-29a mimics groups vs negative control mimics groups. # indicates miR-29a inhibitors groups vs negative control inhibitor groups. All values were presented as means ± SD, and these data were statistically analyzed by GraphPad Prism using two-way ANOVA. * and #, $P<0.05$. ** and ##, $P<0.01$.

Figure 5

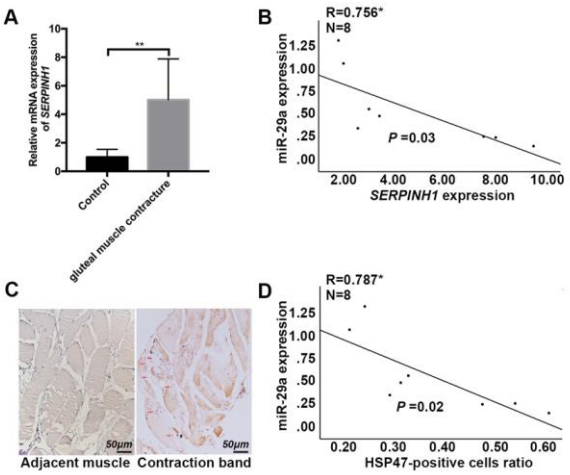

Figure 5. The correlation between miR-29a and HSP47 in Gluteal muscle contracture patients. (**A**) The expression of *SERPINHI* mRNA of control and GMC tissues were analyzed by qRT-PCR. **(B)** The correlation between miR-29a and *SERPINHI* mRNA in GMC patients by Pearson analysis. (**C**) HSP47-positive cells determined by immunohistochemistry staining (IHC) using HSP47 antibody. Scale bar: 50 μm. **(D)** The correlation between miR-29a and HSP47 positive cells in GMC patients by Pearson analysis. Data in panel A was statistically analyzed by GraphPad Prism using a two-tailed Student's t test. Correlation analysis was performed by Pearson's coefficient using SPSS software. *, P <0.05; **, P <0.01.

Table 1. Clinical features in the assay analyzed miRNAs profiles of plasma and tissue.

	Control	Gluteal muscle contracture (GMC)
Gender		
Male	7	5
Female	6	13
Age	38.15 ± 3.55	26.06 ± 1.243
Weight (kg)	62.38 ± 2.787	54.7 ± 2.316
Disease type		
Bilateral GMC	0	18
Unilateral GMC	0	0
Treatment	/	Arthroscopic release

Table 2. Clinical features of patients in the correlation analysis assay.

	Gluteal muscle contracture (GMC)
Gender	
Male	4
Female	4
Age	27.88 ± 1.597
Weight (kg)	58.21 ± 4.734
Disease type	
Bilateral GMC	8
Unilateral GMC	0
Treatment	Arthroscopic release

Table 3 Primer sequences of qRT-PCR.

Gene name	**Forward (5'-3')**	**Reverse (5'-3')**
Collagen I	GTCGAGGGCCAAGACGAAG	CAGATCACGTCATCGCACAAC
Collagen III	TGGTCCCCAAGGTGTCAAAG	GGGGGTCCTGGGTTACCATTA
αSMA	GTGTTATGTAGCTCTGGACTTTGAAAA	GGCAGCGGAAACGTTCATT
SERPINH1	CGCCATGTTCTTCAAGCCA	CATGAAGCCACGGTTGTCC
β-actin	CTCCATCCTGGCCTCGCTGT	GCTGTCACCTTCACCGTTCC

Supplementary figure legends

Fig. S1

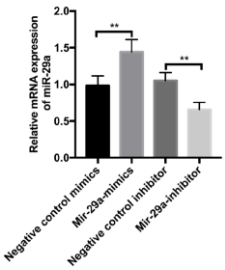

Figure S1. Validation of transfection efficiency in fibroblasts. Human CB fibroblasts were transiently transfected with miR-29a mimic (50 nM), miR-mimic NC, miR-29a inhibitor, or miR-inhibitor NC for 48 h. Then miR-29a expression in fibroblasts measured by qRT-PCR. All data were statistically analyzed by GraphPad Prism using a one-tailed Student's t test. **, P <0.01.

Fig. S2

3'-UTR (57-63)
SERPINH1 (HSP47): 5'—CUCCUGAGACACAUG**GGUGCUAU**—3'
Has-miR-29a-3p: 3'—AUUGGCUAAAGUCUA**CCACGAU**—5'
SERPINH1 mutant 3'-UTR: 5'—CUCCUGAGACACAUGGCCUUGAU—3'

Figure S2. Targeted binding sites between miR-29a and *SERPINHI*-3 'UTR and *SERPINHI*-3 'UTR mutant sequences. The gene sequences labeled by black bold are binding site between miR-29a and *SERPINHI*-3 'UTR, and the red ones are mutant *SERPINHI*-3 'UTR mutant sequences.

Table S1: Primer information of miRNAs

miRBase ID	TaqMan miRNA Assay ID (Life technologies, USA)	TaqMan Assay name (Life technologies, USA)
hsa-miR-26a-5p	000405	hsa-miR-26a
hsa-miR-29a-3p	002112	hsa-miR-29a
hsa-miR-133a-3p	002246	hsa-miR-133a
hsa-miR-206	000510	hsa-miR-206
hsa-miR-222-3p	002276	hsa-miR-222
hsa-miR-342-3p	002260	hsa-miR-342
hsa-miR-378a-5p	000567	hsa-miR-378a