Effects of miR-155 on High Glucose Induced-Cardiac Developmental Defects of Zebrafish Embryos

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Abstract

Background: Gestational diabetes mellitus (GDM) is known to have a teratogenic effect on heart development. However, the underlying mechanisms are still unclear. Former studies determined that miR-155 is elevated in GDM patients. Besides, miR-155 is a key molecule for development. In the present study, we explored the potential role of miR-155 in heart development and the effect of miR-155 on high glucose-induced cardiac developmental defects.

Methods: Zebrafish embryos were exposed to 2% D-Glucose in a fluctuating manner. Activators or inhibitors of miR-155, Ets1, and Igf1 were injected into one-cell stage embryos. The expression levels of miR-155, Ets1 and cardiac specific genes were evaluated by real-time PCR. The regulation of Igf1 by Ets1 was examined using luciferase assays. The levels of reactive oxygen species (ROS) were analyzed by DCFH-DA. Proteins involved in Igf1 pathway were detected by western blot analysis. Maternal serum miR-155 was determined using ELISA. Fetal cardiac structural and functional characteristics in diabetic or healthy pregnancies were performed by echocardiography.

Results: miR-155 levels are increased in serum from GDM patients and are correlated with fetal cardiac structural changes. High glucose exposure in zebrafish embryos altered the morphology of the heart, impaired the heart function, and increased the expression of miR-155 as well as cardiac specific genes. Upregulation of miR-155 activated Igf1-Akt-Gsk3 β pathway by targeting Ets1 and increased the production of ROS and may thereby exert teratogenic effect on cardiac development. In addition, knockdown of miR-155 blocked Igf1 survival pathway and induced apoptosis and may thus induced zebrafish cardiac developmental defects.

Conclusion: miR-155 is a key molecule for heart development and is involved in high glucose-induced cardiac malformation, and it might be a novel biomarker as well as a potential drug target of high glucose-induced cardiac defects.

Background

Diabetes mellitus (DM) is characterized by high levels of blood glucose. During pregnancy, DM is correlated with an elevated risk for congenital heart disease (CHDs), e.g., atrial or ventricular septal defects, Tetralogy of Fallot and transposition of the great arteries, in the offspring of humans and animals [1–3]. The potential teratogenic effect of high glucose on heart morphogenesis has been documented in previous research [4]; however, the mechanism remains unclear.

miR-155 is a well-known multifunctional microRNA that participates in diverse physiology and pathology processes including inflammation, metabolism, and tumor genesis [5, 6]. Recently, several studies reported that miR-155 also functions as a key molecular in development. For example, Burocchi et al [7] determined that miR-155 directly targets Ets proto-oncogene 1 (Ets1) to modulate human T cells development. Interestingly, previous studies identified that both miR-155 and Ets1 are also expressed in cardiomyocytes [8, 9]. Besides, it has been demonstrated that Ets1 plays a vital role in myocardial

development in chicken embryos [10]. Therefore, it would be interesting to explore whether miR-155 could regulate heart development via targeting Ets1. Besides, former study reported that miR-155 is upregulated in the heart of diabetic mice[8]. Moreover, circulating miR-155 is elevated in gestational DM (GDM) patients [11]. Considering the effect of high glucose on cardiovascular system and miR-155, and the potential role of miR-155 in heart development, we consequently hypothesized that miR-155 may be involved in high glucose-induced cardiac developmental defects.

In mammalian models, such as mice and rats, the observation of heart development can only be made through successive sections and immunohistochemical staining. These methods can not reflect the dynamic real-time development changes of heart [12]. Zebrafish is an outstanding model organism for studying development.[13]. Its rapid lifecycle, high productivity and transparent developing embryos make it easier to study dynamic changes in vivo which are not readily achievable in other animal models [14]. Moreover, the resting blood glucose levels of zebrafish embryos as well as adult zebrafish are very close to humans [15, 16]. Here, we investigated the effects of miR-155 on high glucose-induced cardiac developmental defects in zebrafish embryos. In addition, we detected the serum miR-155 of healthy and diabetic pregnancies and analyzed its correlation with cardiac structural and functional characteristics of their offspring.

Methods

Zebrafish usage and maintenance

Zebrafish maintenance and procedures were conducted in accordance with the medical ethics committee of Nanjing Medical University. All experiments were approved by the medical ethics committee of Nanjing Medical University. Wild-type Zebrafish and Cmlc2:GFP transgenic zebrafish were obtained from the Institute Of Hydrobiology, Chinese Academy Of Sciences (Nanjing, China). Normally developing embryos were selected and raised in embryonic medium , also referred as E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄), under standard conditions at 28.5 °C with a 14 h light/10 h dark cycle according to standard procedures.

Human subjects

We enrolled 43 patients that were diagnosed with GDM (according to guidelines of the American Diabetes Association in 2013) and treated from January 2017 to December 2018 in the Department of Obstetricians and Gynecologists, Jiangsu province Hospital. 20 of them showed poorly controlled blood glucose (according to the treatment targets recommended in the guidelines of American Diabetes Association, fasting blood glucose > 5.5 mmol/L and 2 h postprandial blood glucose > 6.7 mmol/L were regarded as uncontrolled). Patients with one or more exclusion criteria according to the previous study were excluded [17] . In addition, we recruited 25 gestational ages-matched healthy pregnant women as controls. Fetal echocardiography was performed in these female participants at 35 weeks using

VOLUSON 730 ultrasound systems (GE Medical Systems Kretztechnik GmbH & Co. OHG). All participants were informed about the study and a written informed consent was provided by each participant.

Treatment of zebrafish embryos with high glucose

To simulate blood sugar in a fluctuating manner, the environment of zebrafish embryos was alternated every 24 h between 2% D-glucose and E3 media until 72 hour post-fertilization (hpf) using a methodology modified previous study [18]. Control embryos were only maintained in E3 media.

Treatment of zebrafish embryos with N-acetylcysteine (NAC)

NAC was dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 10 mM. After treatment with high glucose or injection with miR-155 mimics at 3 hpf, zebrafish embryos were treated with 100 μ M NAC until 72hpf. Control embryos were treated with the equivalent amount of DMSO solution. Final concentration of DMSO is less than 0.1% and had no obvious side-effect on zebrafish embryonic development. Each treatment group was replicated three times using embryos obtained from three independent spawns.

Western blot analysis

For western blot analyses, embryos at 36 hpf were collected and then were lysed in RIPA lysis buffer and proteinase inhibitor mixture. The concentration of the protein was analyzed by bicinchoninic acid (BCA) method. Protein samples were separated via 10% SDS-PAGE and transferred to the PVDF (polyvinylidene difluoride) membranes for 100 min at the 100 V. The membranes were blocked with 5% non fat dry milk and then incubated with the appropriate primary antibodies at 4°C overnight, followed by incubation with the corresponding secondary antibodies. Antibodies against phospho (p)-Akt (Ser473) (#4060), Akt (#4685) were purchased from Cell Signaling Technology. Ets1 (#4685) was purchased from Santa Cruz biotechnology. Igf1 (#DF 6096), Bax(#AF0120), Bcl2 (#AF6139), p-GSK3 β (Ser 9) (#AF2016), GSK3 β (#AF5016) were purchased from Affinity Biosciences.

Total RNA extraction and RT-PCR

Total RNA from whole embryos at 72 hpf was extracted using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Shanghai, China). cDNA was synthesized by the PrimeScript™ reverse transcript reagent Kit (Takara, Tokyo, Japan). The qPCR reactions were prepared with SYBR Green Master (Roche), and then run 35 cycles of 32°C for 15 min, 42°C for 15 min, 72°C for 5 min, and 4°C for 5 min by real-time PCR detect system (Bio-Rad, Hercules, USA). Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with 18S used as the reference gene and normalized. PCR primer sequences were synthesized by Sangon Biotech Co., Ltd (Shanghai, China) and listed in Supplementary Table. S1.

Reverse transcription and qRT-PCR for miR-155

Stem-loop RT-PCR was performed as previously described [19]. Briefly, miR-155 and U6 specific cDNAs were synthesized with TaqMan MicroRNA Reverse Transcription Kit, according to the manufacturer's protocol. The Bulge-loopTM miRNA qRT-PCR Primer specific for dre-miR-155 and U6 is designed by RiboBio (Guangzhou, China). The qPCR reactions were mentioned above. The PCR primers were listed as follows: for U6, forward primer 5'- TTGGTCTGATCTGGCACATATAC -3' and reverse primer 5'- AAAAATATGGAGCGCTTCACG -3'; for miR-155, forward primer 5'- CGCCGTTAATGCTAATCGTGATAG -3' and reverse primer 5'- GCAGGGTCCGAGGTATTCCG -3'. Relative miR-155 expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with U6 used as the reference gene and normalized.

Detection of intracellular ROS

ROS activities were measured at 24 hpf using an ROS assay kit (Nanjing Jiancheng Bioengineering Institute, China). Briefly, 20 μ M of DCFH-DA (2,7-dichlorofluorescein diacetate) was added to the culture medium containing the embryos to be tested. After incubation at a constant temperature (28.5 °C) for 1.5 h, the medium was discarded and PBS was used to wash the embryos for three times. After washing with PBS for three times, embryos were lysed in RIPA lysis buffer and centrifuged at 12,000 g for 1 min at 4 °C. Finally, the supernatants were collected for detecting the activities of ROS at the excitation wavelength of 500 ± 15 nm and emission wavelength of 530 ± 20 nm. The fluorescence values were normalized to protein content for each group.

Measurement of miR-155 levels in maternal serum

Blood samples were collected from all participants upon their admission. Serum was immediately prepared by centrifugation at 4°C, 3000g, 10 min, and then stored at -80°C for subsequent use. Serum miR-155 levels were detected using a commercial double-sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China). The dynamic range of miR-155 detection varies from 38 to 1400 pg/mL. miR-155 levels were determined using standard curves, reading the optical density at 450 nm on an automatic plate reader (Biotek, Winooski, USA). All samples were run in duplicate, and concentration values were averaged.

Plasmids construction

The DNA fragment containing the zebrafish *Ets1* or *Igf1* promoter was amplified by PCR from zebrafish genomic DNA using the Pyrobest PCR kit (Takara, Tokyo, Japan). The PCR products were then subcloned into the *C1aI* and *XbaI* sites of the pCS2⁺ vector (Shanghai Qin cheng Biotechnology Co., Ltd, Shanghai, China) to construct the pCS2⁺-*Ets1* plasmid and pCS2⁺-*Igf1* plasmid or into the *KpnI* and *SacI* sites of pGL3-promoter vector (Promega, USA) to construct the pGL3-basic-zebrafish-*Igf1* promoter plasmid. Besides, pGL3-mutant-zebrafish-*Igf1* promoter plasmid was also constructed, which contained *Ets1*-binding site mutant in *Igf1* promoter. The integrity of these plasmids was confirmed by sequencing. The primers are listed in Supplementary Table 2.

Luciferase reporter assay

Dual-luciferase reporter assay to the analysis of promoter activity in zebrafish embryos was performed as previously described [20]. Briefly, 80 pg of corresponding Igf1 luciferase reporter DNA and 10 pg of Renilla DNA were injected either alone or together with 50 pg of Ets1 mRNA into one-cell stage embryos. Injected embryos were incubated for 12 h at 28.5°C in egg water and then were dissolved in passive lysis buffer for luciferase assays. Luciferase activities were measured using a dual-luciferase reporter assay kit (Promega, USA). The firefly luciferase activity was normalized using the Renilla luciferase activity levels.

Microinjection

Microinjection was performed using back-filled fine borosilicate glass capillary needles. Plasmids, mRNA and morpholinos (MOs) were injected into the cytoplasm of the embryos. The detail of microinjection has been described previously [21]. MOs against Ets1 or Ets2 were obtained from Gene Tools (Corvallis, OR). The morpholinos sequences are as follows: Control MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3'; Ets1 MO, 5'-GATGACTTACGTCTACTTTTTTCGGA-3'; Igf1 MO, 5'-GAAGAAATGACCGCTAGACATCCCT-3'. Activator and inhibitor of dre-miR-155 were purchased from RiboBio (Guangzhou, China). The dre-miR-155 mimic and inhibitor sequences are as follows: dre-miR-155 mimic, forward 5'-UUA AUGCUAAUCGUGAUAGGGG-3', reverse 5'-CCCCUAUCACGAUUAGCAUUA-3'; dre-miR-155 inhibitor, 5'-CCCCUAUCACGAUUAGCAUUA-3'. Besides, for mRNA injections, plasmids of pCS2⁺-*Ets1* and pCS2⁺-*Igf1* were linearized with C1aI. Capped mRNA for microinjection was synthesized with SP6 mMessage mMachine™ kit (Ambion, Thebarton, SA, Australia).

Statistical analysis

Experiments were conducted at least three times independently. SPSS software (Version 21, SPSS Inc., Chicago, IL, USA) was utilized for the statistical analyses. Dates were expressed as mean ± SD. Significance of differences among groups was evaluated by Student's t-test. Spearman correlation was used to analyze relation of maternal serum miR-155 levels to fetal cardiac ventricular walls thicknesses. Values of $P < 0.05$ were considered to be statistically significant.

Results

Serum miR-155 levels were increased in GDM patients and correlated with foetal ventricular walls thicknesses

To begin with, we detected the levels of miR-155 in maternal serum and analyzed its correlation with offspring. The general clinical data gathered for each group is presented in Table 1. Compared to healthy group, the BMI, FBG and 2 h OGTT in GDM groups were higher ($P < 0.01$). Besides, the foetal interventricular septal thickness (IVST), right ventricular wall thickness (RVWT), left ventricular wall thickness (LVWT) and birth weight were higher in the diabetic groups than those in non-diabetics ($P < 0.05$). Moreover, as shown in Fig. 1a, serum levels of miR-155 were significantly increased in GDM

groups ($P<0.01$). The concentration of miR-155 in pregnant women showed positive correlation with fetal IVST ($r=0.295$, $P<0.05$), LVWT ($r=0.297$, $P<0.05$) and RVWT ($r=0.248$, $P<0.05$) (Fig. 1b-d). These observations suggested that serum miR-155 levels were increased in GDM patients and associated with foetal ventricular walls thicknesses.

Table 1
Clinical characteristics of study participants

Characteristics	Health subjects(n = 25)	Controlled GDM(n = 23)	Uncontrolled GDM(n = 20)
Maternal information			
Age (years)	26.51 ± 4.17	28.72 ± 4.55	28.80 ± 3.86
Delivery weeks	39.13 ± 1.59	38.45 ± 1.92	38.42 ± 1.85
BMI (kg/m ²)	26.36 ± 2.87	29.33 ± 3.61 ^{**}	32.32 ± 3.29 ^{**,##}
FBG (mM)	4.49 ± 0.27	5.07 ± 0.33 ^{**}	5.69 ± 0.22 ^{**,##}
2 h OGTT (mM)	4.89 ± 0.50	6.06 ± 0.41 ^{**}	8.24 ± 0.99 ^{**,##}
Newborn information			
Birth weight (g)	3 205.35 ± 392.73	3516.97 ± 485.30 [*]	3 637.54 ± 570.97 ^{**}
IVSD thickness (mm)	2.97 ± 0.65	3.34 ± 0.44 [*]	3.74 ± 0.54 ^{**,#}
RVWD thickness (mm)	2.62 ± 0.40	2.94 ± 0.37 ^{**}	3.10 ± 0.42 ^{**}
LVWD thickness (mm)	2.74 ± 0.29	3.07 ± 0.62 [*]	3.30 ± 0.53 ^{**}
LVEF (%)	64.48 ± 3.60	63.13 ± 3.15	63.40 ± 3.21
Heart rate (bpm)	134.18 ± 7.63	132.42 ± 11.07	129.65 ± 15.22
HS: health subjects, GDM: gestational diabetes mellitus, BMI: body mass index, FBG: fasting blood glucose, OGTT: oral glucose tolerance test, IVSD: interventricular septal thickness at end diastole, RVWD: right ventricular wall thickness at end diastole, LVWD: left ventricular wall thickness at end diastole, LVEF: left ventricular ejection fraction			
Values were presented as mean ± SD. * $P<0.05$, ** $P<0.05$ vs corresponding health subjects. # $P<0.05$, ## $P<0.05$ vs corresponding controlled GDM.			

Knockdown of miR-155 can rescue high glucose-induced zebrafish cardiac developmental defects

Blood sugar levels are normally maintained within a narrow range; however, this precise physiological mechanism is disordered in women with pre-existing or gestational diabetes [15]. To simulate blood

sugar in a fluctuating manner, the environment of zebrafish embryos was alternated every 24 h between normal E3 medium and 2% D-glucose. High glucose exposures in zebrafish embryos altered embryonic heart morphology, caused pericardial edema, impaired heart rate and increased the expression of cardiac related genes (Fig. 1f-h). In addition, the levels of miR-155 were significantly increased in high glucose-treated embryos (Fig. 1e). Interestingly, the deformed phenotypes, decreased heart rate and abnormally expressed cardiac related genes were, at least partially, rescued when miR-155 inhibitors were treated (Fig. 1f-h). These results indicated that miR-155 may be involved in high glucose-mediated heart developmental defects in zebrafish embryos.

Overexpression of miR-155 causes zebrafish heart developmental defects

Considering miR-155 was up-regulated in high glucose-treated zebrafish embryos and was involved in high glucose-induced zebrafish cardiac developmental defects, we therefore supposed that overexpression of miR-155 may also impair zebrafish heart development. To confirm this, 3 μ M of miR-155 mimics were injected into zebrafish embryos. As shown in Fig 2a,c-d, overexpression of miR-155 resulted in cardiac morphology and functional defects, and increased the expression of cardiac related genes. Besides, we used the Cmlc2:GFP zebrafish to investigate the heart from fertilized eggs to the completely developed process. As shown in Fig 2b, overexpression of miR-155 caused chamber abnormalities, increased ventricular wall thickness and disorganized layers. Interestingly, the impaired heart morphology and function, and abnormal expression of cardiac specific markers were almost completely rescued when miR-155 mimics were injected together with miR-155 inhibitors (Fig 2). These results confirmed that miR-155 is essential for heart development, and up-regulation of miR-155 could cause zebrafish cardiac developmental defects.

miR-155 regulates zebrafish cardiac development by targeting Ets1

Previous studies demonstrated that the region of the 3' UTR of mouse and human Ets1 containing the conserved miR-155 binding site[22]. Using bioinformatics analysis, we found that zebrafish Ets1 gene also has at least one potential binding site at its 3'-UTR for miR-155 (Supplementary Fig S1). Given Ets1 plays a vital role in cardiac development, we were interested in exploring whether or not miR-155 mediates high glucose-induced zebrafish cardiac developmental defects by targeting Ets1. As shown in Fig 3b, high glucose declined the expression of Ets1, while knockdown of miR-155 could attenuate such abnormal decrease. Besides, overexpression of Ets1 was able to rescue high glucose-mediated zebrafish heart developmental defects and reduced the abnormal increase of cardiac gene expression (data not shown). In addition, overexpression of Ets1 could reverse cardiac developmental defects in miR-155 mimics-injected embryos (Fig 2). Moreover, knockdown of Ets1 resulted in the same abnormal phenotypes as overexpression of miR-155 did in zebrafish embryos, while downregulation of miR-155 could rescue Ets1 knockdown-caused cardiac developmental defects (Fig 3a, c-d). Taken together, these results determined that Ets1 is the downstream target gene of miR-155 in zebrafish embryos, and miR-155 could mediate high glucose-induced zebrafish cardiac developmental defects by targeting Ets1.

miR-155 regulates zebrafish cardiac development through Igf1 signaling

The above results showed that miR-155 was able to regulate zebrafish heart development by targeting Ets1; however, the potential downstream signal transduction pathways remain unclear. Using bioinformatics analysis, we found that Igf1 is probably a downstream of the transcription factor Ets1. To confirm this, luciferase assay was conducted. As shown in Fig 4b, overexpression of Ets1 markedly attenuated luciferase levels in pGL3-basic-zebrafish-*Igf1*-promoter plasmid-injected embryos, while had no effect on pGL3-mutant-zebrafish-*Igf1* promoter plasmid-injected embryos (pGL3-mutant-zebrafish-*Igf1* promoter plasmid containing Ets1-binding site mutant in Igf1 promoter). Besides, knockdown of Ets1 dramatically increased the expression of Igf1 (Fig. 3c). These results confirmed that Igf1 is the downstream target of Ets1 and Ets1 exerts a negative effect on the expression of Igf1 in zebrafish embryos. Moreover, overexpression of Igf1 resulted in the same phenotype as upregulation of miR-155 or downregulation of Ets1 did in zebrafish embryos (Fig 4a,c-d). Knockdown of miR-155 or overexpression of Ets1 could rescue cardiac defects in Igf1 mRNA-injected embryos (Fig 4a,c-d). Above all, these results suggested that miR-155 regulates zebrafish heart development through Igf1 signaling.

Upregulation of miR-155 activated Igf1-Akt-Gsk3 β pathway in zebrafish embryos

Previous studies determined that Igf1-Akt-Gsk3 β pathway plays a vital role in the process of myocardial hypertrophy [23, 24]. In the current study, we found that overexpression of miR-155 increased the levels of proteins (p-Akt, p-Gsk3 β) involved in Igf1 pathway and knockdown of miR-155 and Igf1, or overexpression of Ets1 could reverse these abnormal increases (Fig 4e,g). In addition, the protein levels of p-Akt and p-Gsk3 β were also markedly increased in high glucose-treated zebrafish embryos, and it is conceivable that knockdown of miR-155 or Igf1, or overexpression of Ets1 could reduce such abnormal increase (Fig 4f,h). These results indicated that under high glucose condition, upregulation of miR-155 could activate Igf1 pathway and may thereby cause zebrafish heart developmental defects.

Upregulation of miR-155 elevated the production of ROS in zebrafish embryos.

Previous study reported that reactive oxygen species (ROS) accumulation were partially reversed in miR-155 knockout mesenchymal stem cells [25]. Besides, plenty of researches determined that abnormal increase of ROS leads to developmental defects [26, 27]. In the current study, DCFH-DA was used to explore the levels of ROS in high glucose-treated zebrafish embryos. As shown in Fig 5b, high glucose increased the production of ROS, while such abnormal increase were partially prevented when miR-155 inhibitor was treated. Besides, NAC is a well-known ROS inhibitor. It could reduce the abnormal increase of ROS caused by high glucose (Fig 5b). In addition, NAC had no influence on the expression of miR-155 (Fig 5e). Although NAC could not reverse abnormal increase of cardiac gene expression (data not shown), it alleviated pericardial edema, improved heart rate in high glucose-treated zebrafish embryos (Fig 5a,c). Similarly, overexpression of miR-155 also elevated the production of ROS (Fig 5f). Overexpression of Ets1 or knockdown of Igf1 or treatment of NAC could reduce the abnormal increase of ROS (Fig 5f) and rescue the deformed phenotypes (Fig 5d,g). These results suggested that under high glucose condition, upregulation of miR-155 increased the production of ROS in zebrafish embryos and elevated ROS could exert teratogenic effect on zebrafish heart development.

Knockdown of miR-155 can also induce cardiac developmental defects in zebrafish embryos

According to the above results, upregulation of miR-155 could impair cardiac development and function. In the next study, we were interested in identifying whether downregulation of miR-155 affects the development of heart. We injected 5 μ M of miR-155 inhibitor into wild type as well as Cmlc2:GFP transgenic zebrafish embryos and found that downregulation of miR-155 interfered with normal cardiogenesis, caused cardiac looping defects, declined overall heart size, decreased the expression of cardiac related genes, and impaired embryonic heart function (Fig 6a-d). As expected, overexpression of Ets1 or knockdown of Igf1 resulted in the same phenotype as downregulation of miR-155 did (Supplementary Fig S2, Supplementary Fig S3). Besides, previous study determined that Bcl2 is a downstream of Igf1 and inhibits apoptosis in many cell types [28, 29]. In the current study, we found that knockdown of miR-155 significantly decreased the proteins levels of Bcl2 and markedly increased the Bax protein level, while these changes were attenuated by Ets1 MO or Igf1 mRNA treatment (Fig 6e). Above all, these results suggested that downregulation of miR-155 blocked Igf1 survival pathway, inducing apoptosis and may thereby cause cardiac developmental defects in zebrafish embryos.

Discussion

In the present study, we have made three major findings. First, miR-155 levels are increased in serum from GDM patients and are correlated with fetal cardiac structural changes. Second, high glucose elevated the expression of miR-155 in zebrafish embryos. Upregulation of miR-155 activated Igf1-Akt-Gsk3 β pathway as well as increased the production of ROS and may thereby exert teratogenic effect on cardiac development (Supplementary Fig S4). Third, knockdown of miR-155 inhibited Igf1 survival pathway and induced apoptosis and may thus hinder zebrafish embryonic and cardiac development (Supplementary Fig S4). These findings not only revealed the role of miR-155 in heart development, but also illustrated the effects of miR-155 on high glucose-induced cardiac developmental defects and highlighted the clinical potential of miR-155 as biomarkers for diagnosis and drug target of GDM-induced CHDs.

Congenital cardiovascular malformations form the most prevalent group of birth defects and about one in every 100–200 babies is born with a heart defect [30]. These defects can occur for many different reasons including genetic factors, exposure to environmental teratogens and birth injury. Diabetes in pregnant women is known to have a teratogenic effect on the development of fetal cardiovascular system [31]. In the present study, we found that foetal ventricular walls thicknesses were thicker in diabetic groups. Although the potential mechanisms for the correlation between GDM and fetal heart defects remain unclear, some evidences have proved that high glucose plays a vital role in the development of fetal cardiac anomalies [32]. In the current study, D-Glucose exposure in zebrafish embryos elevated the overall heart size and increased the expression of cardiac related genes, while L-glucose did not have such effect on zebrafish embryos development, suggesting that these abnormal phenotypes are not due to the osmotic effect. Interestingly, knockdown of miR-155 can rescue high glucose-induced zebrafish cardiac developmental defects. Moreover, overexpression of miR-155 caused the same abnormal phenotypes as high glucose exposure did in zebrafish embryos. Some possible

explanations may illustrate these results. Firstly, in the current study, we identified that miR-155 is involved in zebrafish heart development. Under high glucose condition, the expression of miR-155 was elevated and mediated high glucose-induced heart developmental defects. Secondly, we determined that miR-155 could regulate cardiac development through Igf1 pathway. It has been reported that Igf1 is crucial for cardiomyocyte proliferation during zebrafish heart development. Besides, Igf1 could cause cardiomyocyte hypertrophy via activation of Gsk3 β phosphorylation [23, 24]. In the current study, we found that upregulation of miR-155 activated Igf1-Akt-Gsk3 β pathway and may thus cause cardiac developmental defects.

Recently, several studies reported that high glucose could increase the production of ROS in cardiomyocytes [33, 34]. Given a number of researches reported that abnormal increase of ROS could cause developmental defects, it would be interesting to explore whether high glucose could increase the production of ROS and may thereby exert teratogenic effect on cardiac development [26, 27]. In the current study, we found that high glucose could increase ROS production in zebrafish embryos and cause heart developmental defects, while these deformed phenotypes and abnormal increase of ROS could be reduced in the presence of NAC or miR-155 inhibitor. These results could be explained by the following statement: To begin with, it has been demonstrated that upregulation of miR-155 increases ROS production via suppressing antioxidation-related genes expression in mesenchymal stem cells [25]. In the present study, we found that high glucose elevated the expression of miR-155 and may thus enhance the production of ROS. Knockdown of miR-155 could reverse such abnormal increase. Furthermore, NAC is a well-known ROS scavenger. It could scavenge excess ROS and may thereby rescue the deformed phenotypes. In addition, we also found that overexpression of Ets1 or knockdown of Igf1 could reduce the production of ROS in miR-155 mimics-injected zebrafish embryos. Reasons below may illustrate these discoveries: Initially, it has been demonstrated that Ets1 can regulate the expression and activity of intracellular glutathione peroxidase (GPX) expression that acts as a free radical scavenger [35]. Downregulation of Ets1 may decrease the glutathione levels and thus increase intracellular ROS. Additionally, former study suggested that Igf1 could increase midgut mitochondrial ROS synthesis [36]. Similarly, exogenous Igf1 is able to induce ROS production in mouse cortical collecting duct cells [37]. Above all, it may be possible that high glucose increased the expression of miR-155 and upregulation of miR-155 declined the expression of Ets1 and elevated the levels of Igf1 resulting in the abnormal increase in the production of ROS and may thereby cause zebrafish embryonic and cardiac defects.

In the current study, we also explored whether downregulation of miR-155 affects the development of heart and found that knockdown of miR-155 caused cardiac looping defects, decreased the expression of cardiac related genes and overall size of heart, and impaired embryonic heart function. We listed three possible reasons: Firstly, knockdown of miR-155 declined the levels of Igf1 and may thereby inhibit cardiomyocyte proliferation during zebrafish heart development [38]. Secondly, Bcl2 is a downstream of Igf1 and could inhibit apoptosis in many cell types [28, 29]. In the current study, we found that knockdown of miR-155 reduced the expression of Bcl2 but markedly increased the levels of Bax. Thirdly, previous study has proved that a decrease in Gsk3 β phosphorylation on Ser9 occurred concomitantly with cardiomyocyte apoptosis [39]. Moreover, we identified that p-Gsk3 β was also dramatically declined in

miR-155 inhibitors-injected embryos. Collectively, we suggested that knockdown of miR-155 blocked Igf1 pathway and induced apoptosis and may thereby cause cardiac developmental defects in zebrafish embryos.

Serum miRNAs are found to hold promise to serve as novel biomarkers since they are easy to detect and are also stable during sample handling. Recently, several studies detected miRNAs in maternal serum samples which may offer a noninvasive test for monitoring pregnancy-associated diseases. For example, Gu et al [40] revealed six miRNAs that are upregulated in the serum of pregnant women and could be used as biomarkers for the diagnosis of fetal neural tube defects. Mouillet et al [41] analyzed the serum levels of miRNAs of pregnant women with fetuses exhibiting fetal growth restriction (FGR) and determined that FGR is associated with increased miR-517a, -518b, -518e and - 524 levels in maternal serum. In the current study, we detected the serum levels of miR-155 and found that the levels of miR-155 were increased in serum from GDM patients, especially in these GDM patients whose blood glucose was poorly controlled. Besides, maternal serum concentration of miR-155 was positively correlated with the neonatal ventricular walls thicknesses, indicating that serum miR-155 might be a potential biomarker for GDM-induced CHDs.

Conclusions:

Collectively, the present study demonstrated for the first time that miR-155 is essential for cardiac development. Under high glucose condition, upregulation of miR-155 activated Igf1-Akt-Gsk3 β pathway and enhanced the production of ROS and may thereby exert teratogenic effect on cardiac development. Besides, knockdown of miR-155 could block Igf1 pathway and induce apoptosis and may thus cause cardiac developmental defects in zebrafish embryos. Moreover, miR-155 is increased in serum from GDM patients and correlated with fetal ventricular thickness, and it might be a novel diagnostic biomarker as well as a potential drug target of GDM induced-CHDs.

Abbreviations

GDM: gestational diabetes mellitus; ROS: reactive oxygen species; CHDs: congenital heart defects; RVWT: right ventricular wall thickness; LVWT: left ventricular wall thickness; IVST: interventricular septum thickness; ETS1: Ets proto-oncogene 1; NAC: N-acetylcysteine; DMSO: dimethyl sulphoxide; HPF: hours post-fertilization; BCA: bicinchoninic acid; IGF1: insulin-like growth factor 1; GSK3: glycogen synthase kinase-3 β ; MOs: morpholinos; DCFH-DA: 2',7'-Dichlorodihydrofluorescein diacetate; GPX: glutathione peroxidase; BCL2: B cell lymphoma/leukemia 2 gene; AKT: alpha serine/threonine-protein kinase; BAX: baxdelta2omega ; PVDF: polyvinylidene difluoride.

Declarations

Acknowledgments

None.

Authors' contributions

LY, ZXT conceived and designed the experiments; KZ, YJZ, XJY, BJ performed the qPCR, western blot, ROS detection and examined the serum miR-155; MYH, LYW, DLL constructed the plasmids and conducted micro-injection; YJZ, XJY, YZ, HJL monitored zebrafish embryos development and took the photo. KZ, LTW, XJW analyzed and interpreted the data; KZ, BJ, LY, ZXT wrote the paper; all authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available upon request

Ethics approval and consent to participate

All experiments were approved by the medical ethics committee of Nanjing Medical University and the ethics committee of the Jiangsu province Hospital.

Consent for publication

All authors have declared their consent for this publication.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Upregulation of miR-155 is correlated with high glucose-induced cardiac developmental defects. a Maternal serum levels of miR-155 in healthy subjects (HS) (n=25), controlled groups (n=23) and uncontrolled groups were detected by ELISA (n=20). b-d Maternal serum levels of miR-155 are positively associated with fetal IVST ($r=0.295$, $P=0.015$), LVWT ($r=0.297$, $P=0.014$) and RVWT ($r=0.248$, $P=0.041$). e The expression levels of miR-155 in high glucose-treated zebrafish embryos at 24 hpf were analyzed by qRT-PCR. f Zebrafish embryos were exposed to high glucose. In rescue experiments, zebrafish embryos were

injected with 3 μ M of miR-155 inhibitors. Bars, 250 μ m. g Cardiac specific markers mRNA levels at 72 hpf were analyzed by qRT-PCR. h Heart rate of zebrafish embryos were determined at 72 hpf. Data were presented as the mean $\pm$ SD of three individual experiments. *P<0.05, **P<0.01. §P<0.05, §§P<0.01 vs corresponding control groups. #P<0.05 vs corresponding high glucose-treated groups.

Figure 2

Overexpression of miR-155 causes zebrafish cardiac developmental defects. a Zebrafish embryos were injected with 3 μ M of miR-155 mimics. In rescue experiments, 3 μ M of miR-155 mimics were injected together with 3 μ M of miR-155 inhibitors or 30 pg of Ets1 mRNA. Bars, 250 μ m. b Cmlc2:GFP zebrafish embryos were injected with 3 μ M of miR-155mimics. In rescue experiments, 3 μ M of miR-155 mimics injected together with 30 pg of Ets1 mRNA. Bars, 100 μ m. c Heart rate of zebrafish embryos was determined at 72 hpf. d Cardiac specific markers mRNA levels at 72 hpf were analyzed by qRT-PCR. Data were presented as the mean $\pm$ SD of three individual experiments. *P<0.05, **P<0.01. §P<0.05, §§P<0.01 vs corresponding NC groups. #P<0.05, ##P<0.01 vs corresponding miR-155 mimics-injected groups.

Figure 3

miR-155 regulates zebrafish cardiac development by targeting Ets1. a Zebrafish embryos were injected with 5 ng of Ets1 MO. In rescue experiments, 5 ng of Ets1 MO were injected together with 3 μ M of miR-155 inhibitors or 30 pg of Ets1 mRNA. Bars, 250 μ m. b Zebrafish embryos were treated with high glucose. In rescue experiments, zebrafish embryos were injected with 30 pg of Ets1 mRNA or 3 μ M of miR-155 inhibitors. Protein levels of Ets1 at 36 hpf were analyzed by western blot analysis. c Cardiac specific markers mRNA levels at 72 hpf were analyzed by qRT-PCR. d Heart rate of zebrafish embryos were determined at 72 hpf. Data were presented as the mean $\pm$ SD of three individual experiments. *P<0.05, **P<0.01. §P<0.05, §§P<0.01 vs corresponding control groups. #P<0.05, ##P<0.01 vs corresponding Ets1 MO-injected groups.

Figure 4

Upregulation of miR-155 activated Igf1 pathway in zebrafish embryos. **a** Zebrafish embryos were injected with 100 pg of Igf1 mRNA. In rescue experiments, 100 pg of Igf1 mRNA were injected together with 3 ng of Igf1 MO or 3 μM of miR-155 inhibitors or 30 pg of Ets1 mRNA. Bars, 250 μm. **b** Luciferase activity was analyzed following co-transfection of zebrafish embryos with pGL3-basic-zebrafish-Igf1-promoter, pGL3-mutant-zebrafish-Igf1-promoter and Ets1 mRNA. **c** Heart rates of zebrafish embryos were determined at 72 hpf. **d** Cardiac specific markers mRNA levels at 72 hpf were analyzed by qRT-PCR. §P<0.05, §§P<0.01 vs corresponding control groups. #P<0.05, ##P<0.01 vs corresponding Igf1 mRNA-injected groups. **e,g** Zebrafish embryos were injected with 3 μM of miR-155 mimics, or 3 μM of miR-155 mimics were injected

together with 3 μ M of miR-155 inhibitors or 30 pg of Ets1 mRNA or 3 ng of Igf MO. Protein levels of Akt, p-Akt, Gsk3 β , p-Gsk3 β at 36 hpf were analyzed by western blot analysis. §P<0.05, §§P<0.01 vs corresponding control groups. #P<0.05, ##P<0.01 vs corresponding miR-155 mimics-injected groups. f,h Zebrafish embryos were exposed to 2% D-glucose. In rescue experiments, zebrafish embryos were injected with 3 μ M of miR-155 inhibitors or 30 pg of Ets1 mRNA or 3 ng of Igf MO. Protein levels of Akt, p-Akt, Gsk3 β , p-Gsk3 β at 36 hpf were analyzed by western blot analysis. §P<0.05, §§P<0.01 vs corresponding control groups. #P<0.05, ##P<0.01 vs corresponding high glucose-treated groups. Data were presented as the mean $\pm$ SD of three individual experiments.

Figure 5

Upregulation of miR-155 elevated the production of ROS in zebrafish embryos. a Zebrafish embryos were treated with high glucose. In rescue experiments, 100 μ M of NAC were treated. Bars, 250 μ m. b Reactive

oxygen species production was monitored in the zebrafish embryos treated with high glucose and 3 μ M of miR-155 inhibitor or 100 μ M of NAC. c,g Heart rates of zebrafish embryos were determined at 72 hpf. d Zebrafish embryos were treated with 3 μ M of miR-155 mimics. In rescue experiments, 100 μ M of NAC were treated. Bars, 250 μ m. e The expression of miR-155 in NAC-treated zebrafish embryos were analyzed by qRT-PCR. f Reactive oxygen species production was monitored in the zebrafish embryos treated with 3 μ M of miR-155 mimics and 100 μ M of NAC or 30 pg of Ets1 mRNA or 3 ng of Igf1 MO. Data were presented as the mean $\pm$ SD of three individual experiments. * P <0.05, ** P <0.01.

Figure 6

Knockdown of miR-155 can also induce cardiac developmental defects in zebrafish embryos. a Zebrafish embryos were injected with 5 μ M of miR-155 inhibitors. In rescue experiments, 5 μ M of miR-155 inhibitors were injected together with 5 μ M of miR-155 mimics or 7.5 ng of Ets1 MO or 150 pg of Igf1 mRNA. Bars,

250 μ m. b Cmlc2:GFP zebrafish embryos were injected with 5 μ M of miR-155 inhibitors or 5 μ M of miR-155 inhibitors injected together with 7.5 ng of Ets1 MO or 150 pg of Igf1 mRNA. Bars, 100 μ m. c Heart rate of zebrafish embryos were determined at 72 hpf. d Cardiac specific markers mRNA levels at 72 hpf were analyzed by qRT-PCR. e Zebrafish embryos were injected with 5 μ M of miR-155 inhibitors or 5 μ M of miR-155 inhibitors were injected together with 5 μ M of miR-155 mimics or 7.5 ng of Ets1 MO or 150 pg of Igf1 mRNA. Protein levels of Bcl2 and Bax at 36 hpf were analyzed by western blot analysis. Data were presented as the mean $\pm$ SD of three individual experiments. *P<0.05, **P<0.01. §P<0.05, §§P<0.01 vs corresponding con groups. #P<0.05, ##P<0.01 vs corresponding miR-155 inhibitor-injected groups.

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