miR-92a represses the viability and migration of nerve cells in Hirschsprung’s disease by regulating the KLF4/PI3K/AKT pathway

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Hirschsprung's disease (HSCR) is an intestinal disease caused by defects in neural crest cell migration, proliferation, differentiation, and survival. Many reports have proposed that miRNA dysregulation is related to the occurrence of HSCR. However, the roles and mechanisms of miRNAs have not been thoroughly studied. The levels of miR-92a and KLF4 were examined using qRT-PCR and immunohistochemistry, respectively. Cell viability, migration and apoptosis were evaluated by MTT, Transwell and flow cytometry assays, respectively. A dual-luciferase reporter assay was employed to verify the binding relationship between miR-92a and KLF4. Levels of PI3K/AKT signals were further determined by western blot assay. Herein, elevated expression of miR-92a and reduced expression of KLF4 were found in HSCR tissues, and their expression patterns were negatively correlated. Overexpression of miR-92a inhibited cell viability and migration but enhanced cell apoptosis. However, overexpression of KLF4 had the opposite effects. Mechanistically, KLF4 was a target of miR-92a and it negatively affected biological functions by activating PI3K/AKT signaling. These results proved that miR-92a inhibited the proliferation and metastasis of nerve cells by regulating the KLF4/PI3K/AKT axis.

Key words: Hirschsprung's disease, miR-92a, KLF4, PI3K/AKT signaling

INTRODUCTION

Hirschsprung's disease (HSCR) is a type of intestinal disease caused by a congenital enteric nervous system (ENS) malformation (Butler Tjaden et al., 2013). It usually manifests as an inability to pass the meconium, with bloating and discomfort (Kenny et al., 2010). Generally, HSCR appears quickly after birth and affects one in every 5,000 live births worldwide (Kenny et al., 2010). Many reports have suggested that the onset of HSCR has hereditary characteristics. For example, the incidence in boys is higher than in girls (4:1) (Moore, 2017; Tilghman et al., 2019). Surgical removal of the aganglionic bowel is the main treatment strategy for HSCR (Butler Tjaden et al., 2013). However, some patients may have severe perianal erosion and malnutrition after surgery; in particular, the incidence of enterocolitis is significantly increased, leading to many controversies about the treatment of HSCR. Therefore, an in-depth study of the pathogenesis of HSCR can provide a theoretical basis for the prevention and treatment of HSCR.

microRNA (miRNA) is an endogenous noncoding RNA of 19 to 25 nucleotides in size (Bartel, 2004; Di Leva et al., 2006). miRNAs regulate gene expression mainly by targeting complementary mRNAs, thereby participating in regulating a series of biological processes, such as cell proliferation, apoptosis and autophagy (Ambros, 2004). Current researches have indicated that abnormal levels of miRNA may contribute to the development of HSCR (Sharan et al., 2015; Chen et al., 2017). For instance, miR-431-5p was upregulated in HSCR, and knockdown of miR-431-5p enhanced proliferation of enteric nerve cells (ENCC), and eventually inhibiting HSCR incidence (Hu et al., 2019). Zhi et al. (2018) also

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discovered that miR-483-3 derived from IGF2 promoted the proliferation and migration of 293T and SH-SY5Y cells, alleviating the progression of HSCR. Importantly, a previous study described that miR-92a was significantly upregulated in the serum of HSCR patients, and its expression was closely related to the occurrence of HSCR (Tang et al., 2014). However, the biological functions of miR-92a in HSCR remain unclear.

Krüppel-like factor 4 (KLF4) is a transcription factor that participates in the regulation of various cell processes, such as cell differentiation and proliferation (Morales-Martinez et al., 2019). For example, the conditioned loss of KLF4 in the gastrointestinal epithelium led to proliferation and differentiation changes in gastric epithelial cells (Ou et al., 2019). Ectopic expression of KLF4 accelerated proliferation and differentiation while reducing apoptosis in neural stem cells (Miao et al., 2017). Studies have shown that KLF4 was significantly downregulated in the colon tissue of HSCR patients, suggesting that it may be related to the pathogenesis of HSCR (Nakamura et al., 2018). However, its roles and mechanism are not fully clear.

miRNAs are capable of regulating the expression of target genes at the posttranscriptional level by directly binding to the 3’-UTR of targets. By prediction analysis, we found that miR-92a had a potential binding site for KLF4 3’-UTR. Therefore, we examined the biological roles and molecular mechanism of miR-92a and KLF4, which might provide new insights for understanding the pathogenesis of HSCR.

**METHODS**

**Clinical sample collection**

After diagnosis by pathological detection, colon tissue samples were collected from 25 HSCR patients and 25 patients who were diagnosed without HSCR or other intestinal nerve malformations (no ischemic or necrotic sites). All samples were obtained from Hunan Children’s Hospital, and their clinical information is shown in Table 1. After surgical removal, all samples were stored at -80°C until further detection. All participants’ guardians gave written informed consent, and all procedures in the present study were conducted in accordance with the Helsinki Declaration and government policies. In addition, the experiment was approved by the Ethics Committee of Hunan Children’s Hospital (Changsha, China, No.HCHLL-2019-96).

**Cell culture**

Due to the limitations of the HSCR enteric nervous system (ENS) cell model, we selected two cell lines suitable for this study after referring to several papers (Sharan et al., 2015), namely, human HEK-293T (ATCC® CRL-3216™, VA, USA) and SH-SY5Y (ATCC® CRL-2266™). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sangon, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Sangon), 100 U/mL penicillin (Invitrogen, CA, USA) and 100 μg/mL streptomycin (Invitrogen). The cells were cultured in a cell incubator at 37°C and 5% CO₂. qRT-PCR assays were performed to determine whether there was mycoplasma contamination.

**Plasmid constructs and transfection**

The whole cDNA sequence of KLF4 was inserted into the pcDNA3.1 vector to construct KLF4-overexpressing vectors (pcDNA-KLF4). miR-92a mimics and its negative control (mimics NC) were obtained from Sigma-Aldrich (MO, USA). Before transfection, 1 x 10⁵ cells were cultured in 24-well plates with 500 μL complete medium for 24 h until they reached 90% confluence. For *in vitro* transfection, 0.4 μg pcDNA-KLF4/pcDNA-NC or 80 nM miR-92a mimics/mimics NC and 0.4 μL Lipofectamine 3000 (Invitrogen) were mixed with 50 μL Opti-MEM medium (Ther
mo Fisher Scientific, MA, USA). Then, the diluted RNAs and diluted Lipofectamine 3000 were mixed together and kept at room temperature for 20 min. Subsequently, this mixture was added to cells whose density was approximately 90%. After transfection for 48 h, qRT-PCR was applied to determine the transfection efficiency.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific). A NanoDrop One/OneC trace nucleic acid protein concentration analyser (Thermo Fisher Scientific) was employed to detect the RNA concentration and purity (A260/A280=2.0, and the concentration was greater than 5 μg/μL). CDNA was synthesized with reverse transcriptase kits (Toyobo, Japan) in accordance with the manufacturer’s instructions. Then, CDNA was used for qRT-PCR assay conducted on an Eppendorf MasterCycler RealPlex4 (Eppendorf, Wesseling-Berzdorf, Germany) using a SYBR kit (Toyobo). The relative expression levels of the genes and miRNAs were calculated by the 2^−ΔΔCT method. GAPDH and U6 were used to normalize the expression of the genes and miRNAs, respectively. The specific primer sequences were listed below (5’-3’):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (F)</td>
<td>CTGACTTCAACACGGCACC</td>
<td>GAPDH (R): GTGGTCCAGGGGTCTTACTC</td>
</tr>
<tr>
<td>GAPDH (R):</td>
<td>GTGGTCCAGGGGTCTTACTC</td>
<td>U6 (F):</td>
</tr>
<tr>
<td>U6 (F):</td>
<td></td>
<td>U6 (R):</td>
</tr>
<tr>
<td>miR-92a (F):</td>
<td>CTGTCCTGTTATTGAGCACTGGTCTATGG</td>
<td>miR-92a (R):</td>
</tr>
<tr>
<td>KLF4 (F):</td>
<td>CGCACTAGTATCCCACGTAGTGGATG</td>
<td>KLF4 (R):</td>
</tr>
</tbody>
</table>

MTT assay

After transfection for 48 h, the cells in each group were harvested and suspended in complete DMEM mixed with 5 mg/mL MTT solution for 4 h at 37°C. After incubation, the medium was removed, and dimethyl sulfoxide (DMSO) was added and oscillated for 10 min. The absorbance at 490 nm was detected by a microplate reader (Biateke, Beijing, China). Each data point was obtained from at least three independent experiments.

Transwell assay

Transwell assay was conducted to assess cell migration by using a 24-well Transwell plate (Corning, NY, USA). After transfection for 48 h, the cells were starved in serum-free medium for 12 h before the experiment. Then, the cells (1 × 10^5) were resuspended in 100 μL of serum-free medium and added to the upper chamber. Next, 500 μL of medium mixed with 10% FBS was added to the lower chamber. After incubation for 48 h, a cotton swab was used to gently remove the cells from the upper surface. Then, the cells in the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China). After washing with PBS, the migrating cells were observed by a microscope (Nikon, Tokyo, Japan).

Flow cytometry

Cell apoptosis was determined by an Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, Shanghai, China) in accordance with the manufacturer’s instructions. After treatment, the cells were harvested and washed twice with prechilled PBS. Then, the cells were resuspended in 100 μL of 1× binding buffer, followed by incubation with 5 μL of Annexin V-FITC and 10 μL of PI staining solution protected from light at room temperature for 30 min. Then, cell apoptosis was detected by a FACSARia Flow Cytometer (BD Biosciences, NJ, USA), and the data were analyzed in FlowJo V7 software (TreeStar, OR, USA).

Immunohistochemistry

The tissue samples were fixed in formalin and then embedded in wax blocks to prepare 4 μm thick sections. After deparaffinization and rehydration, the sections were incubated with 10% goat serum in PBS mixed with 0.2% Triton X-100 at room temperature for 2 h. Next, the sections were incubated with the primary antibody, anti-KLF4 (1:200) (Abcam, Cambridge, UK) at 4°C overnight. After washing with PBS, the sections were incubated with the corresponding secondary antibody (1:500) (Abcam). After staining with a DAB kit, the sections were observed and photographed under a microscope (Nikon).

Dual luciferase reporter assay

The binding site of miR-92a on the 3’-UTR of KLF4 was predicted by a StarBase online target prediction tool (http://starbase.sysu.edu.cn/). The 3’-UTR sequence of KLF4 containing/without the binding site of miR-92a (KLF4-wt and KLF4-mut) was synthesized by GenePharm (Shanghai, China) and then cloned into the pmirGLO vector (Promega, WI, USA). KLF4-wt or KLF4-mut plasmids (0.4 μg) were cotransfected with
mimics NC or miR-92a mimics (80 nM) into 293T and SH-SY5Y cells by Lipofectamine™ 3000 (Invitrogen). After 48 h, the relative luciferase activity (firefly luciferase activity/renal luciferase activity) was measured using a dual-luciferase reporter assay system (Promega) following the manufacturer’s instructions.

**Protein extraction and western blot**

The proteins were isolated from the cells by using RIPA mixed with 1% protease inhibitor and phosphorylase inhibitor, and the protein concentrations were determined by a BCA protein assay kit (Beyotime). The proteins were separated by 6% and 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Then, the proteins were transferred to a PVDF membrane (Millipore, MA, USA). According to the size of the target protein, membranes at the corresponding positions were cut apart and the pieces were blocked with blocking solution (Beyotime) for 1 h at room temperature. Then, the membranes were incubated with primary antibodies against KLF4 (1:1000, Abcam, Cambridge, UK), p-PI3K (1:1000, Abcam), PI3K (1:1000, Abcam), p-AKT (1:1000, Abcam) and AKT (1:1000, Abcam). Anti-GAPDH antibody (Sigma–Aldrich) served as a loading control. After washing with PBST, the membranes were incubated with the corresponding secondary antibody labelled with HRP at room temperature for 1 h. The blots were visualized by ECL WB detection reagents (Beyotime), and the images were obtained by a GEL imaging system (Bio-Rad, CA, USA). The quantification of the proteins was analyzed by ImageJ software.

**Data analysis**

The results were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using StarView. The differences between two groups were analyzed by Student’s t-tests. One-way analysis of variance (ANOVA) was employed to evaluate the differences among multiple groups. P values less than 0.05 were considered to be significant.

**RESULTS**

**Increased expression of miR-92a and decreased expression of KLF4 in HSCR**

Tissue samples from HSCR patients (n=25) and matched controls (n=25) were enrolled, and the expressions of miR-92a and KLF4 were examined using qRT-PCR. The results showed that the level of miR-92a was markedly upregulated in HSCR patients (Fig. 1A), while the mRNA level of KLF4 was reduced (Fig. 1B) compared with the matched controls. Next, the immunohistochemistry result showed that the KLF4 level was markedly reduced in HSCR bowel tissues compared to controls (Fig. 1C). Pearson analysis also revealed a negative correlation between the expression of miR-92a and KLF4 in HSCR (Fig. 1D).

**The biological roles of miR-92a in cell viability, migration and apoptosis**

Based on the above data, we sought to determine the biological functions of miR-92a. Therefore, miR-92a-overexpressing 293T and SH-SY5Y cells were established by transfecting miR-92a mimics. The level of miR-92a was significantly elevated in the miR-92a mimics group compared to the control group, indicating that miR-92a was successfully overexpressed (Fig. 2A). MTT and Transwell assays results revealed that miR-92a overexpression markedly inhibited cell viability (Fig. 2B) and migration (Fig. 2C). Moreover, flow cytometry assay revealed that apoptosis was markedly promoted by miR-92a overexpression (Fig. 2D). Moreover, the protein levels of p-PI3K and p-AKT were obviously reduced following miR-92a overexpression (Fig. 2E). Taken together, miR-92a might exert anti-viability, anti-migration and pro-apoptosis roles in 293T and SH-SY5Y cells.

**The biological effects of KLF4 on cell viability, migration, and apoptosis**

Likewise, to investigate the biological functions of KLF4, pcDNA-KLF4 plasmids were transfected into 293T and SH-SY5Y cells, and then the expression of KLF4 was detected. qRT-PCR assay showed that pcDNA-KLF4 plasmids markedly enhanced KLF4 expression (Fig. 3A). Next, functional experiments were performed, and the data suggested that ectopic expression of KLF4 accelerated cell viability (Fig. 3B) and cell migration (Fig. 3C) but reduced the proportion of apoptotic cells (Fig. 3D). Western blot assay showed that the phosphorylation of PI3K and AKT was markedly increased by KLF4 overexpression (Fig. 3E). These data showed that KLF4 accelerated nerve cell viability, migration and cell survival.

**KLF4 acted as a target of miR-92a**

Previous reports have proven that KLF4 was a target of miR-92a affecting cell proliferation and metas-
Fig. 1. Upregulation of miR-92a and downregulation of KLF4 in HSCR. Twenty-five case clinical samples from HSCR patients and matched controls were obtained. (A) and (B) The expression levels of miR-92a and KLF4 were determined by qRT-PCR. (C) The level of KLF4 was tested by immunohistochemistry assay. (D) The expression levels of miR-92a and KLF4 were analyzed by Pearson correlation analysis. The data were expressed as the mean ± SD. The experiments were repeated in triple. *P<0.05, **P<0.01.
The biological roles of miR-92a in cell viability, migration, and apoptosis. miR-92a mimics or mimics NC were transfected into 293T and SH-SY5Y cells. (A) The expression of miR-92a was detected by qRT-PCR. (B) cell viability was determined using MTT assay, C, cell migration was tested by Transwell assay. (D) Cell apoptosis was detected using flow cytometry. E, The protein levels of PI3K, p-PI3K, AKT and p-AKT were examined using western blot assay. The data were expressed as the mean ± SD. All data were obtained from at least three replicate experiments. *P<0.05, **P<0.01, ***P<0.001.
Fig. 3. The biological effects of KLF4 on cell viability, migration, and apoptosis. pcDNA-KLF4 or pcDNA-NC was transfected into 293T and SH-SY5Y cells. (A) The mRNA level of KLF4 was detected using qRT-PCR assay. (B) Cell viability was determined using MTT assay. (C) Cell migration was verified by Transwell assay. (D) Cell apoptosis was measured using flow cytometry. E, The protein levels of PI3K, p-PI3K, AKT and p-AKT were detected using western blot assay. The data were expressed as the mean ± SD. All data were obtained from at least three replicate experiments. *P<0.05, **P<0.01, ***P<0.001.
The function and mechanism of miR-92a in HSCR (Zhang et al., 2017; Chen et al., 2018; Liu et al., 2019). However, it remains not well-studied in HSCR. The potential binding site between miR-92a and KLF4 was analyzed using bioinformatics software (http://starbase.sysu.edu.cn/) (Fig. 4A). A dual-luciferase reporter assay showed that KLF4-wt and miR-92a mimics combined treatment markedly reduced the luciferase activity of 293T and SH-SYSY cells, while the luciferase activity of the cells was not significantly different after cotransfection with KLF4-mut and miR-92a mimics (Fig. 4B). Moreover, qRT-PCR assay showed that the mRNA level of KLF4 was significantly downregulated by miR-92a overexpression (Fig. 4C). Additionally, Fig. 1D suggested an inverse association between the expression of miR-92a and KLF4 in HSCR, further supporting that miR-92a negatively regulated the expression of KLF4.

KLF4 negatively affected the biological roles of miR-92a

To further examine the functional connection between KLF4 and miR-92a, KLF4 and miR-92a were co-overexpressed in 293T and SH-SYSY cells. The results showed that the mRNA level of KLF4 was markedly reduced by overexpression of miR-92a, while pcDNA-KLF4 transfection dramatically reversed this effect (Fig. 5A). Next, western blot assay showed that restoration of KLF4 reversed the inhibitory effects of miR-92a on the phosphorylation of AKT and PI3K (Fig. 5B). Similarly, the inhibitory impacts on cell viability (Fig. 5C) and migration (Fig. 5D) resulting from miR-92a overexpression were obviously weakened by KLF4 overexpression. Likewise, overexpression of KLF4 notably impeded the promoting effect of miR-92a upregulation on cell apoptosis (Fig. 5E). These evidences corroborated that miR-92a inhibited cell viability and migration and induced cell apoptosis by targeting KLF4/PI3K/AKT signaling.

DISCUSSION

Increasing amounts of evidences have shown that the lack of the enteric nervous system in the distal bowel due to dysfunctional ENCC migration and proliferation are the cause of HSCR (Luzón-Toro et al., 2015; Nishikawa et al., 2015). HSCR is a multifactorial disease, and heredity and gene polymorphisms play a role in the pathogenesis of HSCR (Li et al., 2017). It has been widely reported that miRNAs exhibit dysfunction in HSCR and play key roles in regulating HSCR progression. For instance, miR-140-5p was significantly downregulated
Fig. 5. KLF4 negatively affected the biological functions of miR-92a. 293T and SH-SY5Y cells were cultured and then transfected with miR-92a mimics or cotransfected with miR-92a mimics and pcDNA-KLF4. (A) The mRNA level of KLF4 in 293T and SH-SY5Y cells was examined by qRT-PCR assay. (B) The protein levels of PI3K, p-PI3K, AKT and p-AKT were quantified using western blot assay. (C) Cell viability was determined using MTT assay. D, Cell migration was measured using Transwell assay. E, Cell apoptosis was detected using flow cytometry. The data were expressed as the mean ± SD. All data were obtained from at least three replicate experiments. *P<0.05, **P<0.01, ***P<0.001.
in HSCR tissue, and knockdown of miR-140-5p inhibited cell migration and proliferation and promoted apoptosis in SH-SYSY5 cells (Du et al., 2020). miR-181a was validated to be involved in the onset and progression of HSCR by augmenting RAP1B (Chen et al., 2017). By referring to previous studies (Chen et al., 2017), Z93T and SH-SYSY5 cells were selected in this work to examine the roles and regulatory network of miR-92a and KLF4.

The miR-92a family, including miR-25, miR-92a-1/2 and miR-36, is a class of highly conserved miRNAs that originate from three distinct homologous clusters of miR-17-92, miR-106a-363, and miR-106b-25 and share the same seed region (Li et al., 2014). miR-92a has been proven to play vital regulatory functions in multiple diseases by influencing biological processes (Li et al., 2014). For instance, miR-92a was reported to inhibit cell proliferation and metastasis and promote cell apoptosis in Wilms’ tumor by targeting FRS2 (Li et al., 2020). Similar effects were observed in Zhu et al.’s study (2018). In fibroblast-like synoviocytes, miR-92a suppressed proliferation and migration abilities by directly targeting AKT2 (Yu et al., 2018). Furthermore, miR-92a was also confirmed to play an oncogenic role, accelerating tumor cell proliferation and metastasis (Zhou et al., 2015). These data implied that the physiological function of miR-92a was related to the heterogeneity of the disease. In this study, our results proved that the expression of miR-92a was elevated in HSCR, and overexpression of miR-92a obviously inhibited cell viability and migration and induced cell apoptosis in HSCR-associated nerve cells, suggesting that increased expression of miR-92a might be involved in HSCR.

Recent studies have shown that miRNAs can directly bind the 3’-UTR of the target gene and then influence its expression (Valastyan et al., 2009). KLF4 is a member of the Krupp-like factor (KLF) family, which is a group of transcription factors that regulate cell biological processes (Black et al., 2001; Kaczynski et al., 2003). In our study, we found that KLF4 was significantly down-regulated in HSCR tissues, which was consistent with previous studies (Nakamura et al., 2018). KLF4 has been widely reported to act as a tumor suppressor to inhibit proliferation, metastasis and tumor growth in most cancers (Ma et al., 2017; Xue et al., 2019). Likewise, KLF4 has been confirmed to promote the proliferation and migration of endothelial progenitor cells. Similar data were observed in human retinal microvascular endothelial cells, and inducible expression of KLF4 was able to facilitate cell proliferation, migration and tube formation by transcriptionally upregulating VEGF (Wang et al., 2015). Moreover, our data demonstrated that KLF4 upregulation enhanced cell viability and migration but reduced apoptosis of HSCR-related nerve cells. By using a dual-luciferase assay, KLF4 was verified as a downstream target of miR-92a, and restoration of KLF4 obviously impeded miR-92a upregulation-mediated effects, indicating that KLF4 might be an effector of miR-92a.

PI3K/AKT signaling has been revealed to be involved in a variety of biological processes, having an essential role in many diseases, including cell proliferation, differentiation, apoptosis, and tumorigenesis. In glioblastoma, the activation of PI3K/AKT signaling promoted glioma progression (Turner et al., 2015); while knockdown of AKT3 suppressed the invasiveness of T98G glioblastoma multiforme cells (Paul-Samojedny et al., 2015). Recently, PI3K/AKT signaling was shown to be abnormal in HSCR (Ohgami et al., 2010; Li et al., 2016), but the mechanism remains unclear. Tang et al. found that KLF4 had a binding site in the promoter of PIK3CA and activated its transcription, thus enhancing the activation of PI3K/AKT signaling (Tang et al., 2018), indicating a potential association between PI3K/AKT and KLF4. Interestingly, our results illustrated that PI3K/AKT signaling was suppressed by miR-92a overexpression, while the effects were abolished by KLF4 co-overexpression. These data illustrated that miR-92a could inactivate PI3K/AKT signaling by targeting KLF4.

Finally, our experimental data revealed that miR-92a inhibited cell viability and migration and induced apoptosis in nerve cells by regulating KLF4/PI3K/AKT signaling, indicating that miR-92a upregulation might be involved in HSCR. Additionally, these data further enriched our understanding of miR-92a and provided a basis for exploring more mechanisms of miR-92a during HSCR development in the future.

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REFERENCES


