Role of MiR-215 in Hirschsprung's Disease Pathogenesis by Targeting SIGLEC-8

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Key Words
Host Gene • MicroRNA • Gene regulation • Hirschsprung's disease • Enteric Neurons

Abstract
Background/Aims: Hirschsprung's disease (HSCR), known as aganglionosis, is an infrequent congenital gut motility disorder characterized by absence of enteric neurons. In this study, we focus on the role of the intronic miR-215 and its host gene isoleucyl-tRNA synthetase 2 (IARS2) in the pathogenesis of HSCR. Methods: Quantitative real time PCR and Western blot were used to detect the miRNA, mRNAs, and proteins levels. The dual-luciferase reporter gene assay confirmed the direct regulation of the specific mRNA and miRNAs in cell lines. Transwell assays, CCK8 assay, and flow cytometry were used to measure cell function of the human 293T and SH-SY5Y cells. Results: Expression levels of miR-215 in HSCR patient colon tissues were outstandingly lower than controls, which was positively correlated with expression of the host gene IARS2 and negatively correlated with predicted target gene: sialic acid binding Ig-like lectin 8 (SIGLEC-8). The loss of miR-215 inhibited cell migration and proliferation, which was consistent with the reduction of IARS2. The dual-luciferase reporter gene assay confirmed that miR-215 resulted in the inhibition of SIGLEC-8 by directly binding to the 3'-UTR of SIGLEC-8. Moreover, knocking-down SIGLEC-8 rescued the extent of suppressed cell migration and proliferation that resulted from the diminishment of miR-215. Conclusions: Our findings indicate that miR-215 acts in concert with the host gene IARS2 to affect neuron migration and proliferation through the target gene SIGLEC-8. We infer that the IARS2-miR-215-SIGLEC-8 pathway may play a crucial role in the pathogenesis of HSCR.

Introduction

Hirschsprung's disease (HSCR), also known as aganglionosis, is an infrequent congenital gut motility disorder characterized by absence of enteric neurons along a variable length of the distal gut. HSCR is caused by either failed migration of enteric neural crest-derived cells (ENCCs) into the intestinal tract or due to a failure in survival, proliferation or differentiation of ENCCs once they reach the gut [1]. HSCR, which occurs 1 in 5000 live births H. Lei and H. Li contributed equally to this work.

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with an overall 4:1 male predominance, often presents with delayed passing of meconium as well as abdominal distension and ileus [2]. Extensive research has identified that HSCR has a complex genetic etiology with a number of genes including those rearranged during transfection (RET) proto-oncogene, glial cell-derived neurotrophic factor (GDNF), GDNF family receptor alpha 1 (GFRα1), neurturin (NRTN), endothelin receptor type B (EDNRB), endothelin 3 (ET3), zinc finger homeobox 1B (ZFHX1B), paired-like homeobox 2b (PHOX2b), SYR-box 10 (SOX10) and sonic hedgehog (SHH). These genes encode for receptors, ligands, transcriptional factors or other cell elements that are usually involved in the neural crest cell development and migration during the ENS development [3]. Moreover, the RET proto-oncogene is considered the major disease causing gene in HSCR [4].

MiRNAs are small non-coding RNA molecules of about 22 nucleotides, which bind to the 3′-UTR of target mRNAs leading to either mRNA degradation or prevention of translation in numerous tumors and biological processes [6, 7]. But to date, few studies have looked at miRNAs in the context of HSCR. MiR-200a/141, the miR-200 family, plays an important role in the pathogenesis of HSCR, which has been reported in our previous study [8]. It is critical to pay attention to the effects of miRNAs during ENCCs development and migration in human HSCR generation in order to gain a better understanding of this disease.

Host gene is the concept that a miRNA is transcribed in parallel with its host transcript. The miRNA sequence is often located in an exon or intron of its host gene [9]. Many miRNAs have host genes and in turn these miRNAs have effects on disease progression [10, 11]. For instance, miR-218-2 and its host gene SLIT3 cooperate to promote invasion and progression of thyroid cancer [12]. Similarly miR-149, hosted by GPC1, fine-tunes the angiogenic response to fibroblast growth factor in human endothelial cells [13]. But, few miRNAs and their related host genes have been observed to be involved in embryo peripheral nervous system development, especially during ENS development.

In this study, we investigated the functional involvement of miR-215 and its host gene IARS2 in HSCR progression. We also identified the target of the miR-215, Sialic acid binding Ig-like lectin 8 (SIGLEC8), which is one member of the CD33-like subgroup of SIGLECs. In this study, we conducted the experiment to explore that the IARS2-miR-215-SIGLEC8 pathway may play a crucial role in the pathogenesis of HSCR.

Materials and Methods

Statement of ethics and sample collection
In our study, colon tissue samples from 70 HSCR patients were collected. These patients were undergoing surgical treatment in Nanjing Children’s Hospital Affiliated to Nanjing Medical University from October 2009 to June 2013. HSCR patients were diagnosed by barium enema and anorectal manometry before surgery. Pathological analysis was performed to confirm the absence of ganglion cells in stenosis colon tissues. The 62 matched controls were acquired from non-ischemic non-necrotic colon tissues of patients receiving surgical treatment for intussusception or incarcerated inguinal hernia. The tissues were immediately frozen and stored at −80°C. The study was approved by the institutional Review Board of Nanjing Medical University and all samples were collected after obtaining informed consent from participating patients.

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA, including miRNA, was extracted from colon tissues using TRIzol reagent as described by the manufacturer (Life Technologies, CA, USA). Quantitative real time polymerase chain reaction (qRT-PCR), performed using ABI Prism 7900HT (Applied Biosystems, CA, USA) according to the manufacturer’s instructions, was utilized to detect the expression levels of miR-215 and mRNAs of all related genes. TaqMan® MicroRNA Assays (Applied Biosystems, CA, USA) were used for detecting the expression level of hsa-miR-215 and human U6 RNA was amplified as an internal control. The miRNA or mRNA levels were calculated according to \(2^{-\Delta\Delta C_t}\). Forward (F) and reverse (R) primer sequences were as follows: IARS2 (F) 5'
ACT GCC CGA AGT TTG TGGG-3' and (R) 5'-CGG TAT CTG CCA CTA TTC GAGTT-3'; SIGLEC8(F) 5'-CAA TAT AGG CTG AGAAC-3' and (R) 5'-GGA TCT CGC TCC TGG AAG ATG-3'.

Protein analysis

RIPA buffer, which includes phenylmethylsulfonyl fluoride (PMSF), was applied to extract protein from colon tissues. A bicinchoninic acid (BCA) assay was used to quantify protein concentration. The protein samples (80 μg) were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Antibodies used to detect IARS2 (sc-137528) and SIGLEC-8 (sc-47418) were purchased from Santa Cruz Biotechnology (CA, USA). The anti-rabbit and anti-goat HRP-linked secondary antibodies were purchased from Beyotime (Nantong, China). Enhanced chemiluminescent (ECL) western blotting was used to detect protein expression levels. We also utilized this method to measure the protein level of GAPDH, which was used as the internal control. The ImageJ software (NIH) was used to analyze and measure protein band intensities.

Cell culture and reagents

Human 293T and SH-SY5Y cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas VA, USA). These cell lines were cultured in complete growth medium DMEM (HyClone, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C, 5% CO₂. The miR-215 mimics, miR-215 inhibitor, siRNAs of IARS2 and SIGLEC-8 and the negative controls (GenePharma, Shanghai, China) were used in transfection experiments.

Dual-luciferase reporter assay

The wild-type and mutated 3'-UTR sequences of SIGLEC-8 (pGL3-SIGLEC-8 and pGL3-SIGLEC-8-mut, respectively), which were predicted to interact with miR-215, were inserted into the KpnI and SacI sites of a luciferase reporter vector controlled by a pGL3 promoter (Genscript, Nanjing, China). The 24-well plates were evenly seeded with cells and transfected using Lipofectamine 2000 (Invitrogen Corp, CA, USA) with 100 ng of pGL3-SIGLEC-8, pGL3-SIGLEC-8-mut, 50 nM miR-215 mimic and negative control, respectively. A renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected to normalize the differences in transfection efficiency. Cells were harvested after 48 h and the dual-luciferase Reporter Assay System (Promega, WI, USA) was used to measure luminous intensity. All experiments were independently repeated three times.

Cell transwell assays

Forty-eight hours after either miR-215 inhibitor or siRNA was transfected into cells, 100 μl of cell suspension containing serum-free medium was seeded into the upper chamber while 600 μl medium with 10% FBS was added to the lower chamber as a chemotaxin. After 48 h, cells were stained with crystal violet staining solution (Beyotime, Nantong, China) for 20 min, then counted and photographed under 40x magnification (five views per well). Migrated cells were counted by using Image-pro Plus 6.0 software. Counts of migrated cells observed in the negative control samples were normalized to 1. All experiments were independently repeated three times.

Cell proliferation assays

After miR-215 inhibitor or siRNA was transfected into cells for 48 h, a cell counting kit-8 (CCK-8) assay was used to test proliferative capability of these cells. The TECAN infinite M200 Multimode microplate reader (Tecan, Mechelen, Belgium) was employed to measure the absorbance at 450 nm. All experiments were independently repeated three times.

Cell cycle and apoptosis analysis

Cells were transfected for 48 h with either miR-215 inhibitor or negative controls. For cell cycle detection, cells were fixed with 75% alcohol and stained with propidium iodide (Sigma, MO, USA). For apoptosis detection, cells were washed with PBS and subsequently tested using the annexin V-FITC Apoptosis Detection Kit (BD Biopharmingen, NJ, USA). All experiments were analyzed by BD Biosciences FACS Calibur Flow Cytometry (BD Biosciences, NJ, USA). All experiments were independently repeated three times.
Statistical analysis

Experimental data of tissue samples are presented as box plots of the median and range of log-transformed relative expression level, which was analyzed by Wilcoxon rank-sum (Mann-Whitney) test. The results obtained from in vitro experiment assays are presented as mean ± SEM from three separate experiments conducted in triplicates per experiment. The data were analyzed by double-sided Student’s t-test. Pearson correlation analysis was used to analyze the relationship of expression level of tissues between case and control groups. Statistical analysis was performed using STATA 9.2 and presented with GraphPad Prism software. Results were considered statistically significant at P < 0.05.

Results

Results of sample information analysis

A total of 132 human colon tissue specimens, including 70 HSCR patient colon tissues and 62 matched controls, were analyzed in this study. Clinical information, including gender (Male/Female), age and weight, was obtained from patients both with and without HSCR. However, there was no significant difference in the distribution of age (4.11 ± 0.28 and 3.74 ± 0.23 months old) and body weight (5.4 ± 0.35 and 5.1 ± 0.19 kg) between HSCR patients and control patients. Furthermore, the gender rate (Male/Female) of HSCR and control patients was 55/15 and 48/14, respectively, which matched the gender rate of this disease.

MiR-215 was significantly down-regulated in HSCR patient colon tissues

The expression levels of miR-215 in HSCR patient colon tissues were significantly lower than that of controls, P = 0.0001 (Fig. 1B). This finding suggested that down-regulation of miR-215 might be important in the pathogenesis of HSCR.

Fig. 1. IARS2 and miR-215 are down-regulated, while SIGLEC-8 is up-regulated, in HSCR patient colon tissues. (A, B, C) Relative expression levels of IARS2, miR-215 and SIGLEC-8 were detected in HSCR patient colon tissues (n = 70) and controls (n = 62). Data are presented as a box plot of the median and range of log-transformed relative expression level. The top and bottom of the box represent the seventy-fifth and twenty-fifth percentile, respectively. The whiskers indicate the 10th and 90th points. (D) The protein level of IARS2 and SIGLEC-8 in HSCR tissues. (E, F) The correlation between the host gene IARS2 and miR-215. MiR-215 and target gene SIGLEC-8 were analyzed in HSCR tissues (P = 0.0001; R = 0.40 and P = 0.0001; R = -0.37, respectively). Data were analyzed using the Pearson’s correlation analysis with natural log transformed expression levels.
MiR-215 inhibitor suppressed cell migration and proliferation

We then examined the functional of miR-215 on cell migration, cell proliferation, cell apoptosis and the cell cycle. When we transfected the 293T and SH-SY5Y cell lines with miR-215 inhibitor/mimics and control, the expression of miR-215 change correspondingly (Fig. 2B), we observed that both the number of migrated cells and the proliferation rate were significantly lower relative to the control when transfecting the 293T and SH-SY5Y cell lines with miR-215 inhibitor and significant increased after overexpression of miR-215 (Fig. 2A). Additionally, by the flow cytometry analysis, we found that the cell cycle and cell apoptosis progression of cells transfected with miR-215 inhibitor were very similar to that of the negative control (Fig. 2C). We concluded that miR-215 plays an important role in HSCR pathogenesis by affecting cell migration and proliferation ability.

SIGLEC-8 was up-regulated in HSCR patient colon tissues

Consistent with the mechanism and function of miRNA, we predicted that miR-215 regulates one or more genes to produce biological effects. We searched DIANA LAB (www.microrna.gr) and TargetScan (www.targetscan.org) databases to predict target genes that may potentially be regulated by miR-215. The results showed that miR-215 binds to the 3’-UTR of the mRNA sequences of SIGLEC-8, ARFGEF1, CCNT2 and ACPP. Then SYBR Green qRT-PCR was used to detect mRNA expression levels of these targeting genes in HSCR and control tissues. Among the aforementioned predicted genes, we found that the expression level of SIGLEC-8 was significantly higher in HSCR tissues relative to controls (P = 0.0001) (Fig. 1C). Moreover, linear analysis showed a negative correlation between the expression levels of miR-215 and SIGLEC-8 (P = 0.0001; R = -0.37) (Fig. 1F), indicating that SIGLEC-8
might be regulated by miR-215. However, there was no difference in the expression levels of ARFGEF1, CCNT2 and ACPP between HSCR and control tissues. Protein levels of SIGLEC-8 were consistent with mRNA expression levels (Fig. 1D). These results suggested that the SIGLEC-8 gene might be the target gene of miR-215.

**SIGLEC-8 was the target gene of miR-215**

We transfected human 293T and SH-SY5Y cell lines with either miR-215 inhibitor or a negative control. SIGLEC-8 mRNA expression levels were evaluated 48 h after transfection. This showed that the relative expression levels of SIGLEC-8 mRNA were increased with miR-215 inhibitor transfection. Western blot analysis was used to confirm changes in protein level (Fig. 3A). To investigate whether SIGLEC-8 is regulated by miR-215 binding to the 3’UTR of its mRNA sequence, both the wild-type and mutated luciferase reporter plasmids (containing the binding region of the 3’UTR of SIGLEC-8 mRNA) were constructed and a miRNA luciferase reporter assay was performed (Fig. 3B). Results showed that co-transfection of miR-215 mimics and the pGL3-SIGLEC-8 promoter vector significantly decreased luciferase activity, relative to the control, in cell lines suggesting that miR-215 directly targets SIGLEC-8 (Fig. 3C).

**SIGLEC-8 siRNA may partly reverse biological changes induced by miR-215 inhibitor**

A set of rescue experiments were performed to investigate whether the increased level of SIGLEC-8 is directly contributing to the cell migration and cell proliferation induced by the miR-215 inhibitor. When we transfected cell lines with only miR-215 inhibitor, as mentioned before, we found that cell migration and proliferation were decreased. However, when we co-transfected SIGLEC-8 siRNA with miR-215 inhibitor, this partially rescued the functional changes to cell biology that resulted from the miR-215 inhibitor (Fig. 4).

**IARS2, the host gene of miR-215, was also down-regulated**

The miR-215 gene resides in intron 12 of its host gene, IARS2. We found that IARS2 expression levels were distinctly lower in HSCR tissues than control tissues (Fig. 1A). Correlation analysis showed a significant positive correlation between the expression levels of IARS2 and miR-215 (P = 0.0001; R = 0.40) (Fig. 1E), indicating that miR-215 was
transcribed together with its host gene IARS2. The protein levels of IARS2 showed the same tendency (Fig. 1D) suggesting IARS2 down-regulation might play a vital role in the pathogenesis of HSCR.

Low expression of IARS2 suppressed cell migration and proliferation in cell lines

To detect whether IARS2 plays an important role in the pathogenesis of HSCR, we conducted transwell and CCK-8 assays to detect cell migration and proliferation potential in human 293T and SH-SY5Y cells that have been transfected with IARS2 siRNA. Our findings showed that the number of migrated cells transfected with IARS2 siRNA was significantly fewer compared to the negative control (Fig. 5A, C). On the other hand, as shown in Fig. 5B and D, down-regulation of IARS2 inhibited cell proliferation, which was consistent with the cell migration assay.

Discussion

Hirschsprung's disease causes an uncommon congenital digestive tract in newborns characterized by an absence of enteric neurons in myenteric and submucosal plexus along a variable portion of the distal gut [14]. Failure of ENCCs to colonize the entire gut plays a leading role in ENS dysfunction. The migration and proliferation of ENCCs, neuronal and glial differentiation, formation of ganglia, axon pathfinding and synaptogenesis were previously reported as the main process involved in the development of the ENS [15]. ENCCs go through a several week long journey to reach the distal intestinal tract during embryogenesis [16]; therefore factors that affect migration, proliferation, survival or differentiation of ENCCs could lead to aganglionosis, followed by HSCR.
including tumorigenesis. Furthermore, evidence indicates that miRNAs play a regulatory role during embryo development [17]. Among these, miR-215, a member of the miR-192/215 family, was reported to be altered in various human diseases, especially malignant tumors. For instance, miR-215 was reported to be decreased in colon cancer [18]. MiR-215 is capable of suppressing tumor progress [19] and inducing cell cycle arrest [20]. In our study, siglec-8, which was predicted as the target gene of miR-215 by bioinformatics tools, is a member of the CD33-like subgroup of SIGLECs. It was mostly reported to be involved in the apoptosis of human eosinophil cells [21] such as mast cells [22]; however, there are no previous studies about its role in nerve cells. The luciferase assay showed that miR-215 could inhibit the expression of SIGLEC-8 by combining directly to the 3'-UTR of SIGLEC-8, while inhibition of miR-215 expression resulted in the up-regulation of SIGLEC-8. In order to determine whether miR-215 could deter HSCR pathogenesis by targeting SIGLEC-8, we performed a set of rescue experiments in this study. We co-transfected miR-215 inhibitor and SIGLEC-8 siRNA and observed that the inhibition of the migration and proliferation of these cells could partly be reversed by SIGLEC-8 siRNA. The same was not true of cells that were only transfected with miR-215 inhibitor. This result suggests that miR-215 inhibits cell migration and proliferation by targeting SIGLEC-8 in human 293T and SH-SY5Y cells.

Previous studies have shown that miRNAs have functions during disease processes, including tumorigenesis. Furthermore, evidence indicates that miRNAs play a regulatory role during embryo development [17]. Among these, miR-215, a member of the miR-192/215 family, was reported to be altered in various human diseases, especially malignant tumors. For instance, miR-215 was reported to be decreased in colon cancer [18]. MiR-215 is capable of suppressing tumor progress [19] and inducing cell cycle arrest [20]. In our study, we demonstrated that the decreased expression level of miR-215 significantly suppressed cell migration and proliferation in enteric nervous system, which in contrast with the study in cancer, implies that miR-215 has a different role in the pathogenesis of HSCR. SIGLEC-8, which was predicted as the target gene of miR-215 by bioinformatics tools, is a member of the CD33-like subgroup of SIGLECs. It was mostly reported to be involved in the apoptosis of human eosinophil cells [21] such as mast cells [22]; however, there are no previous studies about its role in nerve cells. The luciferase assay showed that miR-215 could inhibit the expression of SIGLEC-8 by combining directly to the 3'-UTR of SIGLEC-8, while inhibition of miR-215 expression resulted in the up-regulation of SIGLEC-8. In order to determine whether miR-215 could deter HSCR pathogenesis by targeting SIGLEC-8, we performed a set of rescue experiments in this study. We co-transfected miR-215 inhibitor and SIGLEC-8 siRNA and observed that the inhibition of the migration and proliferation of these cells could partly be reversed by SIGLEC-8 siRNA. The same was not true of cells that were only transfected with miR-215 inhibitor. This result suggests that miR-215 inhibits cell migration and proliferation by targeting SIGLEC-8 in human 293T and SH-SY5Y cells.
was down-regulated in HSCR patient colon tissues. We verified that knockdown of IARS2 repressed cell migration and proliferation in human 293T and SH-SY5Y cells. We propose that IARS2 down-regulation may act in concert with miR-215 to play an important role in the pathogenesis of HSCR by affecting ENCC cell migration and proliferation.

In this study, we have discovered a novel mechanism in that down-regulation of IARS2 inhibits cell migration and proliferation; meanwhile, co-expression of miR-215 inhibits cell migration and proliferation via targeting SIGLEC-8 in the pathogenesis of HSCR. But, our research is still insufficient. Much more work is needed in future.

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**Disclosure Statement**

There are no conflicts of interest.

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