Down-regulation of miR-206 is associated with Hirschsprung disease and suppresses cell migration and proliferation in cell models

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Hirschsprung disease (HSCR) is a well-known congenital digestive disease that originates due to the developmental disorder of neural crest cells. MiR-206 is known to have a relationship with digestive malfunctions. Therefore, we investigated whether or not miR-206 was involved in the pathogenesis of HSCR. qRT-PCR and Western blot assays were used to detect the expression levels of miRNA and mRNAs, and proteins in case and control tissue samples and two cell lines (293T and SH-SYSY). The functions of miR-206 in vitro were measured by transwell assay, CCK8 assay and flow cytometry. Finally, we conducted dual-luciferase reporter assay to verify the connections between miR-206 and the target mRNAs SDPR.

Down-regulation of miR-206 was found in HSCR case tissue samples compared with controls, which was validated to be connected with the increased level of mRNA and protein of SDPR by qRT-PCR and dual-luciferase reporter assay. Moreover, miR-206 suppressed the cell migration and proliferation and silencing of SDPR could rescue the extent of the suppressing effects by miR-206 inhibitor. The findings suggest that miR-206 may play a significant role in the pathogenesis of HSCR, as well as inhibiting the cell migration and proliferation by targeting SDPR in disease models.

Hirschsprung disease (HSCR) is a common congenital digestive malformation which is characterized by the absence of the ganglion cells in the sub-mucosal and mesenteric plexuses. The incidence of HSCR is two in 10 000 live births worldwide with significant differences in various ethnic groups (1.5, 2.1 and 2.8 per 10 000 live births in Caucasians, African-Americans and Asians, respectively)1. Still, the mechanism of the pathogenesis of HSCR remains unclear except that during the 5th to 12th week in fetal period, the enteric neural crest cells, strongly associated with the bowel functions, fails to migrate to the hindgut2. HSCR is proven to have a complex genetic etiology involving several genes, including RET, EDNRB, SOX10 and PHOX2B3–6. However, there are few reports on the roles of non-coding genes, such as miRNA, in the pathogenesis of HSCR.

MicroRNAs (miRNAs) are endogenous 20 ~ 24 nt RNAs that play significant role in regulating gene expression post-transcriptionally in animals and plants by binding to the 3’UTR of the mRNA of the target genes7,8. MiRNAs are reported to have strong association with diverse diseases, including malignancies, such as gastric cancer, breast cancer, colon cancer and lung cancer, by affecting the cell migration, metastasis, proliferation and apoptosis9–12. Recent studies have demonstrated that miR-206 is responsible for various cancers due to its impact on the cell biological processes, such as cell proliferation, cell differentiation and apoptosis13–15. However, until now, there are no reports on the involvement of miR-206 in the early pathogenesis of HSCR.

In this study, we conducted experiments to unravel how miR-206 interacts with its target gene that contributes to the pathogenesis of HSCR in disease models.

Methods

Ethics Statement and subject tissue samples. The study was approved by the Institutional Ethics Committee of Nanjing Medical University. All of the experiments in the research were in compliance with the government policies and defined protocols which are accepted in current
practices. In total, 80 HSCR case samples were enrolled into the research, which were
early diagnosed by barium enema and anorectal manometry evaluation after
surgery between 2009 and 2013 (NJMU Birth Cohort). Also, the entire group of
control was 80 matched subjects that were confirmed HSCR-free. Written informed
consent was obtained from patients’ guardians after full explanation of the
experiment. All tissue samples were stored at ~80°C immediately after surgery.

RNA extraction and quantitative real-time PCR (RT-qPCR). Total RNAs, including
miRNAs, were extracted from 80 matched controls, HSCR-stenosed segments
(HSCR-S) and 80 HSCR-dilated segments (HSCR-D) colon tissue samples and two
clones by the method of Trizol reagent (Life Technologies, CA, US). TaqMan®
MicroRNA Assays (Applied Biosystems, CA, US) was applied for the detection of
expression level of miR-206 in tissue samples with the normal endoscopic control.
Meanwhile, the mRNA of SDPR was measured by ABI 7900HT with SYBR (Takara,
Tokyo, Japan) along with the GAPDH as the internal control. Details of the probes
and primers are given in Supplementary Table 1.

Protein extraction and Western blot. The tissue samples and cells were lysed by
RIPA buffer (Beyotime, Nantong, China). The incubation of the primary antibody
(cat. # AP9935c, Abgent, SanDiego, US) against SDPR proteins with Polyvinylidene
Fluoride (PVDF) membranes was performed at 4°C overnight. After rinsing, the
secondary antibody (Beyotime, Nantong, China) was incubated with the PVDF
membranes for 1 hour at room temperature. During the whole process, GAPDH was
regarded as the normal control. Image J software was applied for the detection and
quantification of the protein level in Western Blot.

Cell culture and transfection. In this study, due to the limitation of ENS cell model of
HSCR, we searched many papers and found two appropriate cell lines, namely,
human 293T and SH-SY5Y cells, which were acquired from American Type Culture
Collection (ATCC, Manassas VA, US) were employed for the experiments in vitro.
Both cell lines were cultured in DMEM medium (Hyclone, UT, US), which contained
10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and
streptomycin (100 µg/ml). The experiments of transfection reagents (Invitrogen, CA, US) was used as the vector for transfection reagents during the
procedure according to the manufacturers’ protocols.

Cell transwell and proliferation assay. Cell transwell assay, which was designed to
evaluate the capacity of cell migration, was mainly performed with the application of
the Transwell migration chambers (8 µm pore size, Millipore Corporation, Billerica,
MA). Firstly, two cell lines were cultured in six-well plates and transfected with miR-
206 inhibitor/mimics or SDPR siRNA. After 48 h of transfection, cells were harvested
with serum-free medium as single-cell suspension and 100 µl of cell suspension was
seeded in the upper chamber (1 × 10^5) cells/ml, along with the lower chamber filled
with the 600 µl DMEM medium with 10% FBS. After incubation from 24 h to 48 h,
the cells were stained with crystal violet staining solution (Beyotime, Nantong, China). Lipofectamine 2000 Reagent (Invitrogen, CA, US) was used as the vector for the firefly luciferase activity. A cell lysate was prepared with
10% homogenization solution (Beyotime, Nantong, China) along with the GAPDH as the internal control. Image J software was applied for the detection and
quantification of the protein level in Western Blot.

Table 1 | Clinical characteristics of the study population

| Variable                  | HSCR (n = 80) | Control (n = 80) | P     |
|---------------------------|--------------|-----------------|-------|
| Age (months, mean, SE)    | 4.25(0.30)   | 4.77(0.83)      | 0.57a |
| Sex                       |              |                 |       |
| Male                      | 70           | 55              | 0.17c |
| Female                    | 10           | 25              |       |
| Disease Classification    |              |                 |       |
| S-HSCR                    | 29           |                 |       |
| L-HSCR                    | 51           |                 |       |

aStudent t-test
bTwo-sided y2 test
1S-HSCR: Short-segment HSCR; L-HSCR: Long-segment HSCR.

Results

Study population. In total, 160 colon tissue specimens were recruited from
Department of Pediatric Surgery, Nanjing Children’s Hospital
affiliated Nanjing Medical University, including 80 HSCR-confirmed cases and 80 matched controls. The clinical characteristics of the study subjects are shown in Table 1,ranging over age, sex and
disease classification. As displayed in Table 1, there were no
statistically significant differences in terms of age and sex between
HSCR cases and controls. Moreover, according to the length of
ganglionosis in colon, HSCR was divided into two main types, short-segment HSCR (S-HSCR) and long-segment HSCR (L-HSCR).

Down-regulation of miR-206 in HSCR. Figure 1A shows that the
expression level of miR-206 in HSCR cases was significantly
lower as compared with matched controls. This implies that miR-206
might have connections with the pathogenesis of HSCR. Another
experiment was conducted to examine the miR-206 expression level in
80 matched controls, HSCR-stenosed segments (HSCR-S) and 80
HSCR-dilated segments (HSCR-D). The results showed that the
expression level of HSCR-D and HSCR-S were both much lower
than controls (Supplement Figure C).

miR-206 inhibitor suppressed cell migration and proliferation
without impacting cell cycle and apoptosis. In order to confirm
the functional performance of miR-206 in vitro, we examined how
miR-206 impacted cell migration, cell proliferation, cell cycle process
and apoptosis. To achieve this, 293T and SH-SY5Y cell lines were
transfected with miR-206 inhibitor and then subjected to transwell
and CCK8 assays. Both the cell lines showed reduction in number of
migrating and proliferating cells suggesting that the down-regulation

FlowJo V7 software (Tree Star, Oregon, US). Experiments of cell cycle and apoptosis
were also performed in triplicate independently.

Dual-luciferase reporter assay. Dual-luciferase reporter assay was used to validate
whether or not miR-206 regulated SDPR by binding to the 3’UTR region of SDPR
mRNA. Thus, the sequence of 3’UTR region of SDPR predicted to have interaction
with miR-206 was inserted into the KpnI and SalI sites of pG3 promoter vector
(Genscript, Nanjing, China). These constructs were named pG3-SDPR and pG3-SDPR-
mut, respectively. According to the manufacturers’ protocols, after
transfection with negative control, miR-206 mimics, pG3-SDPR and pG3-SDPR-
mut, cells were collected to measure fire fly and renilla luciferase activities by Dual
Luciferase Assay (Promega, Madison, WI). Experiments of dual-luciferase reporter
assay were also performed in triplicate independently.

Statistical analysis. Statistical analyses were performed by using Stata 9.0 statistical
software package (Stata Corp. Texas, US) and presented by Graphpad software
(GraphPad Software, Inc., CA, US). Data of the relative expression level of miR-206
and SDPR in human tissue samples were presented as a box plot of the median and
range of log-transformed expression level accessed by Wilcoxon rank-sum test. The
data for the experiments in vitro that were repeated three times, were plotted as mean ± SEM via double-sided Student’s t-test. Results were considered to have statistically
significant differences if p < 0.05.
of miR-206 had a suppressive affection on cell migration and proliferation (Figure 1B). Furthermore, flow cytometry analysis was performed to investigate the impact of miR-206 on cell cycle and apoptosis. The results show no statistical difference in the percentage of apoptotic cells between cells transfected with miR-206 inhibitor and the negative control. Likewise, there were no changes in the cell cycle process (Figure 1C,D).

Bioinformatics prediction of target gene for miR-206. We applied three main databases (DIANA LAB, Targetscan and Pictar) to predict the underlying target genes, which may be regulated by miR-206. Finally, after the prediction and function analysis, the three common target genes, SDPR (serum deprivation response), FN1 (fibronectin 1) and PAX3 (paired box 3), were selected. SDPR and FN1 are admitted for the confirmed remarkable associations with plasma membrane, which contributes to the dysfunction of caveolae, cell adhesion and migration. PAX3 is generally accepted to have a key role in the fetal development and pathogenesis of colonic aganglionosis.

Up-regulation of SDPR in HSCR patients. To determine whether all of three target genes were involved in HSCR, qRT-PCR was used for the examination of the mRNA level in 80 HSCR case and matched control tissue samples. SDPR was the only candidate gene that showed significant up-regulation between HSCR cases and matched controls. Moreover, in order to reveal whether expression level of SDPR had a relationship with the diseases classification, we checked the expression level of SDPR in two main types of HSCR. The results indicate that SDPR expressed much more in L-HSCR than S-HSCR (p = 0.0241) (Figure 2A). Immediately, correlation analysis was conducted between miR-206 and SDPR in match controls and cases, respectively. The findings demonstrated that compared with the poor correlation in controls, there were evident associations between miR-206 and SDPR in HSCR cases (Figure 2B). Simultaneously, via western blot, the protein expression level of SDPR was consistent with the mRNA expression level (Figure 2C).

In contrast, FN1 and PAX3 were invariant between HSCR cases and controls (Supplementary Figure A, B). In addition, we evaluated the changes of expression level of SDPR in 293T and SH-SY5Y cell lines after transfection with miR-206 inhibitor. After 48 hours, the expression level of SDPR mRNA and the protein level were detected by qRT-PCR and western blot, respectively. As expected, SDPR expression was remarkably up-regulated at both mRNA and protein levels in 293T and SH-SY5Y cell lines (Figure 2D, E).

SDPR was target gene for miR-206. To verify the relationship between miR-206 and SDPR, two independent methods were applied to validate the miRNA-target gene interaction. Firstly, we constructed the wild and mutant type luciferase plasmids with the binding area of 3’UTR of SDPR mRNA, which was referred to as pGL3-SDPR and pGL3-SDPR-mut, respectively (Figure 3A). Transfection of miR-206 mimics with pGL3-SDPR into 293T cell line and SH-SY5Y cell lines significantly inhibited the luciferase activity as compared with the control. Meanwhile, there was no significant alteration in luciferase activity for cell lines transfected with negative control, miR-206 mimics and pGL3-SDPR-mut (Figure 3B). The findings demonstrate that miR-206 regulated SDPR by combining the 3’UTR region of its mRNA.

Silencing of SDPR partially rescued the cell migration and proliferation with miR-206 inhibitor mediation. We had
The regulation of cellular and biological processes is a critical aspect of cell behavior. Changes in gene expression, particularly those leading to aberrant protein expression, can significantly alter cellular function. SDPR mRNA is a target of miR-206, the expression of which was analyzed using western-blotting. The results indicated that miR-206 is connected with SDPR in HSCR cases.

**Discussion**

In our study, three potential target genes of miR-206 were predicted by DIANA LAB, Pictar and Targetscan, while the results showed SDPR was the only one that had significant differences between HSCR cases and matched controls. In the following step, we detected the expression level of miR-206 and SDPR mRNA in HSCR case and control tissue samples. The results show that miR-206 was down-regulated in HSCR with concomitant up-regulation of SDPR. Therefore, dual-luciferase reporter assay was conducted to reveal the underlying relationships between miR-206 and SDPR. As expected, the results illustrate that miR-206 had an inverse regulatory relationship with SDPR by directly binding to the 3'UTR region of SDPR mRNA, which might have caused degradation or structural changes leading to aberrant expression of proteins.

There are many reports suggesting that miR-206 had strong association with regulation of cellular and biological processes. Especially miR-206 was observed to regulate cell movement during zebrafish gastrulation by regulating mitogen-activated protein kinase (MAPK) JNK signaling. In order to confirm the functional effect of miR-206 on cell biological processes in HSCR, assays such as, cell transwell, cell proliferation, apoptosis and cell cycle were used for validation. The results indicate that the down-regulation of miR-206 suppressed cell migration and proliferation. Moreover, the successful rescue in transwell assay by SDPR siRNA implied that miR-206 performed the suppression by up-regulating SDPR.

SDPR (serum deprivation response) is a key substrate for protein kinase C (PKC) phosphorylation and this interaction determines the compartimentalization of PKC to caveoleae. SDPR was further verified to play a key role in inducing membrane curvature and participate in the formation of caveoleae. Caveoleae is a calcium channel related to gut electrophysiological pacing function, which has also been identified to have impacts on cell migration and proliferation. Recently, it has been demonstrated that the absence of the caveoleae on the membrane surface contributed to the formation of fibroblast-like ICC (Interstitial Cajal Progenitors Cells) in the narrow segment of HSCR compared with the normal adult colon, which was shown to have surface caveoleae. Meantime, fibroblast-like ICC was observed in Igf1r+/CD34+ ICC in Ws/Ws rat colon. Accordingly, the over-expression of SDPR was validated to play a key role in inducing deformation of caveoleae and extensive tubulation of the plasma membrane. In our study, the expression level of SDPR in HSCR cases was much higher than the normal matched controls. Thus, we speculate that through negative regulation, down-regulation of miR-206 led to the up-regulation of SDPR inducing the deformation of caveoleae of ENCCs (enteric neural crest cells) in colon, which would contribute to the pathogenesis of HSCR. Further research needs to be performed to validate the hypothesis, especially the caveoleae of ENCCs.

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**Figure 2** SDPR was up-regulated in HSCR cases and two cell lines. (A): The mRNA expression levels of SDPR in human HSCR case and control tissues and the relative expression level of SDPR in S-HSCR and L-HSCR, respectively. The upper correlation was performed in controls and it showed poor relationships between miR-206 and SDPR, while the lower correlation analysis indicated that miR-206 is connected with SDPR in HSCR cases. (B): The protein expression levels of PTEN in human HSCR tissues and controls (3 representative samples from both groups are shown) (above). Quantization of Western-blotting was done by Image J software (blow). (D–E): Cells were transfected with 100 nM miR-206 inhibitor for 48 h, qRT-PCR was performed to evaluate the mRNA level of SDPR. SDPR protein expression levels were analyzed by western-blotting.

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In conclusion, our research reveals that miR-206 plays an important role in the pathogenesis of HSCR and suppresses cell proliferation and migration by regulating SDPR in disease models. Our study provides a new approach for understanding the pathogenesis of HSCR and might contribute to a novel approach to the therapy of HSCR in the future.

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Author contributions

A.S., H.L. and Y.X. designed the experiments; A.S., H.Z. and H.X. performed the experiments; W.T., H.L., J.T. and H.W.Z. contributed essential technical assistance; H.Z. and A.S. wrote the paper.
**Additional information**

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Corrigendum: Down-regulation of miR-206 is associated with Hirschsprung disease and suppresses cell migration and proliferation in cell models

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This Article contains errors in Figure 3C. The image depicting the 293T cell line co-infected with SDPR siRNA is a duplicate of the Control image of the SH-SY5Y cell line. In addition, the image depicting the 293T cell line co-infected with SDPR siRNA + miR-206 inhibitor is a duplicate of the image depicting the SH-SY5Y cell line co-infected with miR-206-inhibitor. The correct Figure 3C appears below as Figure 1.

Figure 1.

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