Preliminary identification of key miRNAs, signaling pathways, and genes associated with Hirschsprung's disease by analysis of tissue microRNA expression profiles

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Background: **Hirschsprung's disease (HSCR) is a congenital gut motility disorder of infants, and if left untreated, it is fatal to the affected infants. This study aimed to identify key microRNAs (miRNAs), signaling pathways and genes involved in the pathogenesis of HSCR.**

Methods: **The miRNA microarray dataset GSE77296 was downloaded. Nine colon tissue samples were available: six from HSCR patients and three matched control samples. Differentially expressed miRNAs (DEMs) were identified after data preprocessing. Target genes of the selected upregulated and downregulated DEMs were predicted. In addition, functional enrichment analyses for the selected DEMs and target genes were conducted. Finally, interaction networks between the DEMs and target genes were constructed.**

Results: **A total of 162 DEMs (73 upregulated and 89 downregulated) were obtained. A total of 2511 DEM-target gene pairs for the 40 selected DEMs were identified, including 1645 pairs for the upregulated DEMs and 866 pairs for the downregulated DEMs. The upregulated DEM miR-141-3p and down-regulated DEM miR-30a-3p were identified as key miRNAs by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and network analyses. Besides, KEGG pathway enrichment analysis revealed that pathways in cancer and the mitogen-activated protein kinase (MAPK) signaling pathway were key pathways. The key genes frizzled class receptor 3 (*FZD3*) and docking protein 6 (*DOK6*) were obtained through the DEM-target gene interaction networks.**

Conclusion: **Two key miRNAs (miR-141-3p and miR-30a-3p), the MAPK signaling pathway and two key genes (*FZD3* and *DOK6*) were implicated in the pathogenesis of HSCR.**

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Key words: **differentially expressed microRNA; docking protein 6; frizzled class receptor 3; Hirschsprung's disease; mitogen-activated protein kinase signaling pathway**

Introduction

Hirschsprung's disease (HSCR) is a congenital gut motility disorder of infants, presenting as bowel obstruction shortly after birth, and is characterized by a lack of ganglion cells in the distal gut; thus, biopsy of a bowel segment is the most common histochemical diagnostic approach.[1,2] If left untreated, HSCR can be fatal to affected infants exhibiting signs of intestinal obstruction. The current treatment option for HSCR is surgery. Although surgical outcomes are typically good, long-term prognosis remains challenging in most cases.[3] The incidence of HSCR is approximately 1 case per 5000 live births, but significantly differs among races.

Recent genetic studies have shown interracial differences in frequencies of HSCR, which is caused by single or multiple gene mutations.[3-5] Several genes, signaling pathways and microRNAs (miRNAs) associated with HSCR have been widely studied over the past few decades. The proto-oncogene *RET* is reported to be a major gene in the pathogenesis of HSCR, as the successful colonization of enteric neural crest cells requires normal function of *RET*.[6-8] Moreover, *RET*-associated pathways

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have been implicated in the pathogenesis of HSCR.[9] Recently, the slit homologue 2/roundabout homologue 1-miR-218-1-*RET*/pleomorphic adenoma gene 1 (*PLAG1*) zinc finger pathway was also associated with HSCR.[10] The authors previously reported that *RET* and *PLAG1* were downregulated, while miR-218-1 was upregulated in HSCR patients, as compared with controls. In addition, miR-215 was downregulated in HSCR patients, and cell migration and proliferation were inhibited by the loss of miR-215 via the isoleucyl-tRNA synthetase 2-miR-215-sialic acid binding immune globulin-like lectin 8 signaling pathway.[11] Recently, Li et al[9] identified several miRNAs involved in *RET*-related pathways via microarray analysis of colon tissues of HSCR patients. However, no comprehensive bioinformatic analysis of miRNA expression patterns in colon tissues of HSCR patients has been reported so far.

We hypothesized that there may be more genes, miRNAs and pathways involved in HSCR. Therefore, the aim of the present study was to screen candidate miRNAs, target genes and pathways involved in HSCR development. To this end, differentially expressed miRNAs (DEMs) in HSCR patients were identified by screening the dataset GSE77296,[9] followed by prediction of target genes of DEMs. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology (GO) term analyses were performed to identify key HSCR-related pathways. Subsequently, DEM-target gene interaction network analysis was conducted to screen key miRNAs and genes. The results of this study provide additional data to elucidate the mechanisms underlying HSCR development. Findings of this study may serve as biomarkers of HSCR.

Methods

Affymetrix microarray data

The miRNA expression profile dataset GSE77296[9] was downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) based on the platform of the GPL18058 Exiqon miRCURY LNA miRNA array, 7th generation (Exiqon A/S, Vedbaek, Denmark). A total of nine human colon samples were available for analysis, including six stenotic colon segment samples collected from HSCR patients and three age-matched control colon tissues collected from patients with colorectal trauma or that undergoing anorectal colostomy. The HSCR patients were aged 13 days to 4 years. All were diagnosed by barium enema and anorectic manometer evaluation before surgery and pathological analysis for definitive diagnosis. The raw data in txt files were preprocessed using the limma package[12] in R language (version 3.3.2) by performing format conversion, missing value addition, motor assessment scale background correction and quantiles data normalization.

DEM analysis

DEMs present in the HSCR group, but absent in the control group, were identified. Probability values (*P*) of enrichment significance were calculated using the non-paired *t* test in the limma package and then adjusted using the Benjamini-Hochberg procedure. The cutoff thresholds were $P<0.05$ and $|\log_2\text{fold change (FC)}| \geq 1$. Then, a heat map of DEMs was generated with the pheatmap package[13] in R language.

Prediction of target genes

Target genes of the top 20 upregulated and downregulated DEMs with high $|\log_2 FC|$ values were analyzed using the miRWalk2.0 archive[14] with an miRNA information retrieval system. The search criteria were as follows: minimum seed length of 7, $P<0.05$, and 3′-untranslated regions as input parameters. Target genes were selected from the following five commonly used databases: miRWalk (*http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/*), miRDB (*http://mirdb.org/*), miRMap (*http://mirmap.ezlab.org/*), RNA22 (*https://cm.jefferson.edu/rna22/*) and Targetscan (*http://www.targetscan.org/vert_71/*).

Pathway enrichment analysis

The KEGG pathway database (*http://www.kegg.jp/*)[15] is commonly used for systematic analysis of gene functions and GO analysis (*http://www.geneontology.org/*) is commonly used to study the functions of large-scale genomic andtranscriptomic data.[16] In the present study, KEGG pathways and GO-biological processes (BP) terms for the target genes of the selected DEMs were analyzed using the clusterprofiler package[17] in R language. The cutoff threshold was $P<0.5$.

Interaction network analysis

A DEM-target interaction network for the top 20 upregulated and downregulated DEMs was constructed using Cytoscape software (version 3.2.0)[18] based on the predicted interaction pairs. The scores of nodes in the network were obtained using degree centrality.[19] Nodes with the highest degree in the DEM-target interaction network were considered as key DEMs and key target genes.

Results

Analysis of DEMs and target genes

A total of 1962 miRNAs (data not shown) were obtained after annotation, including 162 miRNAs

that were found to be differentially expressed: 73 were upregulated and 89 downregulated. The top 20 upregulated and downregulated DEMs with the highest $|\log_2 FC|$ values are listed in Table 1, which includes miR-142-3p, miR-142-5p, miR-4492 and miR-30a-3p. The target genes of the 40 DEMs were predicted using miRWalk2.0. A total of 2511 DEM-target gene pairs for 25 DEMs were obtained, including 1645 pairs for 16 upregulated DEMs and 866 pairs for nine downregulated DEMs. No target genes were identified for the remaining DEMs with the chosen search criteria.

Pathway enrichment analysis

KEGG pathway enrichment analysis was performed with the top 20 upregulated and downregulated DEMs. The results showed that three of the top 20 upregulated DEMs (miR-141-3p, miR-200a-3p and miR-345-5p) were enriched in 12 KEGG pathways (Fig. 1A), which included ascorbate and aldarate metabolism, the hedgehog signaling pathway and mismatch repair. Four of the top 20 downregulated DEMs (miR-1228-5p, miR-143-5p, miR-30a-3p and miR-3180) were enriched in six KEGG pathways (Fig. 1B), which included selenocompound metabolism, the Ras-proximate-1 signaling pathway and the forkhead box O (FoxO) signaling pathway. KEGG pathway and GO-BP analyses were also performed for the target genes of the 40 DEMs. KEGG pathways and GO-BP terms with the 10 lowest *P* values are shown in Fig. 2. The target genes of the top 20 downregulated DEMs were significantly enriched

Table 1. List of top 20 up-regulated and down-regulated DEMs

Up-regulated DEMs			Down-regulated DEMs		
MiRNA	Log_2FC	*P* value	MiRNA	Log_2FC	*P* value
Hsa-miR-142-3p	3.937	<0.001	Hsa-miR-4492	-2.832	0.0029
Hsa-miR-142-5p	2.848	<0.001	Hsa-miR-30a-3p	-2.684	<0.001
Hsa-miR-488-5p	2.436	0.0016	Hsa-miR-3180	-2.659	<0.001
Hsa-miR-20b-5p	2.254	0.0208	Hsa-miR-2861	-2.401	0.0077
Hsa-miR-192-5p	2.204	<0.001	Hsa-miR-624-3p	-2.294	0.0101
Hsa-miR-194-5p	2.131	<0.001	Hsa-miR-3187-5p	-2.074	<0.001
Hsa-miR-200a-3p	2.131	<0.001	Hsa-miR-378i	-2.038	0.0256
Hsa-miR-146b-5p	2.115	<0.001	Hsa-miR-143-5p	-2.020	0.0034
Hsa-miR-345-5p	2.087	0.0190	Hsv1-miR-H17	-2.017	0.0069
Hsa-miR-451a	2.021	0.0010	Hsa-miR-1228-5p	-1.885	0.0028
Hsa-miR-141-3p	1.976	0.0199	Hsa-miR-4734	-1.874	0.0015
Hsa-miR-4445-3p	1.976	0.0206	Hsa-miR-30c-1-3p	-1.868	0.0063
Hsa-miR-215	1.962	0.0361	Hsa-miR-4488	-1.814	0.0380
Hsa-miR-4694-3p	1.894	0.0011	Hsa-let-7e-3p	-1.798	<0.001
Hsa-miR-382-3p	1.891	0.0092	Hsa-miR-3141	-1.797	0.0094
Hsa-miR-190a	1.883	0.0067	Hsa-miR-3656	-1.768	0.0187
Hsa-miR-33a-5p	1.852	0.0097	Hsa-miR-4433-3p	-1.755	0.0257
Hsa-miRPlus-C1100	1.845	0.0113	Hsa-miR-9-5p	-1.742	0.0054
Hsa-miR-146a-5p	1.835	0.0042	Hsa-miR-5686	-1.740	0.0019
Hsa-miR-144-3p	1.834	0.0050	Hsa-miR-2277-5p	-1.720	0.0178

miRNA: microRNA; DEMs: differentially expressed miRNAs; FC: fold change.

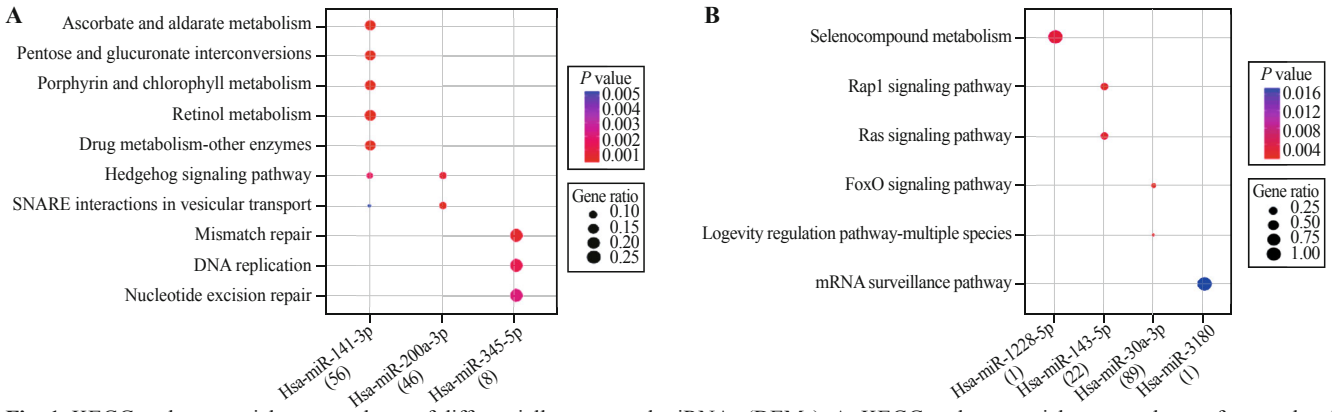

Fig. 1. KEGG pathway enrichment analyses of differentially expressed miRNAs (DEMs). **A**: KEGG pathway enrichment analyses of upregulated DEMs; **B**: KEGG pathway enrichment analyses of downregulated DEMs. KEGG: the Kyoto Encyclopedia of Genes and Genomes; SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Rap1: Ras-proximate-1; FoxO: forkhead box O.

in KEGG pathways, which included pathways in cancer and the mitogen-activated protein kinase (MAPK), Ras and FoxO signaling pathways. The target genes of the top 20 upregulated DEMs were found to be enriched in KEGG pathways, which included pathways in cancer and the MAPK and FoxO signaling pathways (Fig. 2A). The GO-BP terms for the target genes of the top 20 downregulated DEMs included protein phosphorylation, chemical synaptic transmission and homophilic cell adhesion via plasma membrane adhesion molecules. The GO-BP terms for the target genes of the top 20 upregulated DEMs were positively transcriptional regulated by the RNA polymerase II promoter, negatively transcriptional regulated by the RNA polymerase II promoter, and positively transcriptional regulated by the DNA template (Fig. 2B).

Interaction network analysis

DEM-target gene interaction networks were constructed. The upregulated DEM-target gene network included a total of 1193 nodes and 1638 interaction pairs, while the downregulated DEM-target gene network included 798 nodes and 865 interaction pairs. A high topology score means an important role of the node. The degrees of DEMs in the network are listed in Table 2. The upregulated DEM miR-20b-5p had the highest degree of 344 and the downregulated DEM miR-30a-3p had the highest degree of 274. As shown in Table 3, the top

Table 2. Node degrees of differentially expressed microRNAs (DEMs)

MicroRNA	Degree	MicroRNA	Degree
Up-regulated DEMs			
Hsa-miR-20b-5p	344	Hsa-miR-146b-5p	73
Hsa-miR-141-3p	214	Hsa-miR-142-3p	57
Hsa-miR-144-3p	214	Hsa-miR-345-5p	46
Hsa-miR-200a-3p	200	Hsa-miR-190a-5p	37
Hsa-miR-142-5p	95	Hsa-miR-488-5p	36
Hsa-miR-33a-5p	91	Hsa-miR-192-5p	32
Hsa-miR-146a-5p	85	Hsa-miR-215-5p	25
Hsa-miR-194-5p	85	Hsa-miR-451a	4
Down-regulated DEMs			
Hsa-miR-30a-3p	274	Hsa-miR-143-5p	56
Hsa-miR-9-5p	203	Hsa-miR-3180	16
Hsa-miR-30c-1-3p	151	Hsa-miR-1228-5p	7
Hsa-miR-2861	86	Hsa-miR-3656	2
Hsa-miR-624-3p	70		

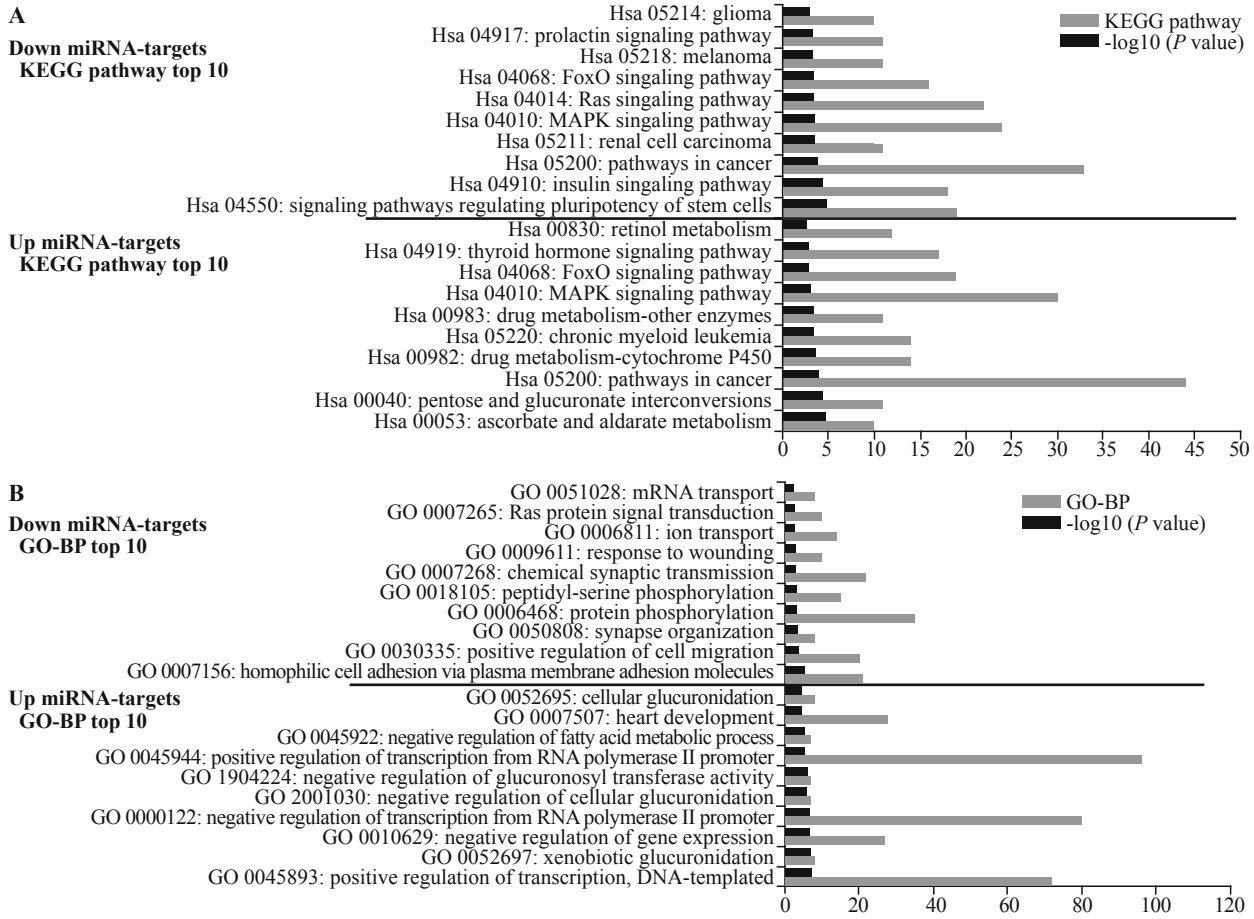

Fig. 2. KEGG and GO enrichment analyses of target genes. **A**: KEGG pathway enrichment analyses of target genes of downregulated and upregulated DEMs; **B**: GO-BP terms of target genes of downregulated and upregulated DEMs. KEGG: the Kyoto Encyclopedia of Genes and Genomes; GO-BP: gene ontology-biological processes; miRNA: microRNA; FoxO: forkhead box O; MAPK: mitogen-activated protein kinase.

Table 3. Node degrees of top 10 target genes

Target genes of up-regulated DEMs	Degree	Target genes of down-regulated DEMs	Degree
FZD3	8	*DOK6*	4
KIAA1244	6	*CXCL9*	3
QKI	6	*FOXP4*	3
SPOPL	6	*HIPK2*	3
GRSF1	5	*MME*	3
JAZF1	5	*SLC26A2*	3
LCOR	5	*TEAD1*	3
PPARA	5	*ANKH*	2
ZNF148	5	*AR*	2
BACH2	4	*ATF1*	2

DEM: differentially expressed microRNA; *FZD3*: frizzled class receptor 3; *KIAA1244*: ARFGEF family member 3; *QKI*: QKI, KH domain containing RNA binding; *SPOPL*: speckle type BTB/POZ protein like; *GRSF1*: G-rich RNA sequence binding factor 1; *JAZF1*: JAZF zinc finger 1; *LCOR*: ligand dependent nuclear receptor corepressor; *PPARA*: peroxisome proliferator activated receptor alpha; *ZNF148*: zinc finger protein 148; *BACH2*: BTB domain and CNC homolog 2; *DOK6*: docking protein 6; *CXCL9*: C-X-C motif chemokine ligand 9; *FOXP4*; forkhead box P4; *HIPK2*: homeodomain interacting protein kinase 2; *MME*: membrane metalloendopeptidase; *SLC26A2*: solute carrier family 26 member 2; *TEAD1*: TEA domain transcription factor 1; *ANKH*: ANKH inorganic pyrophosphate transport regulator; *AR*: androgen receptor; *ATF1*: activating transcription factor 1.

10 target genes with the highest scores included frizzled class receptor 3 (*FZD3*), ARFGEF family member 3 (*KIAA1244*), docking protein 6 (*DOK6*), and C-X-C motif chemokine ligand 9 (*CXCL9*).

Discussion

Although HSCR is the most common congenital gut motility disorder in infants, its pathogenesis remains unclear. In this study, comprehensive bioinformatics analysis was conducted to identify alterations to miRNA expression profiles of HSCR patients. The results of this study indicated that two key miRNAs, miR-141-3p and miR-30a-3p, were involved in HSCR development. Besides, pathways in cancer and the MAPK signaling pathway were found to play important roles in HSCR. Moreover, *FZD3* and *DOK6* were identified as key genes that might participate in the pathogenesis of HSCR. Interestingly, these miRNAs and genes were newly identified to be associated with HSCR.

In this study, 73 miRNAs were significantly upregulated and 89 were significantly downregulated in HSCR patients, compared with the controls. Among these miRNAs, expression of miR-141-3p was significantly increased, while that of miR-30a-3p was significantly decreased. In addition, these data showed that miR-141-3p was significantly enriched in seven KEGG pathways, which included ascorbate and aldarate metabolism and mismatch repair. Besides, the degree of miR-141-3p was the second highest in the DEM-target gene interaction network. miR-141-3p, a member of the miR-200 family, is related to cell migration and proliferation.[20,21] Also, miR-200a and miR-141 are associated with cell migration and proliferation in HSCR.[22,23] In addition, previous studies have demonstrated that upregulation of miR-141-3 pinhibits the proliferation and differentiation of stromal (mesenchymal) stem cells in humans and neurogenesis of neural stem cells in rats.[24,25] Therefore, miR-141-3p might play a key role in HSCR development via disrupting the formation of enteric neural crest cells (ENCCs). The results of the present study showed that miR-30a-3p was the second most significantly downregulated DEM. In addition, miR-30a-3p was significantly enriched in two KEGG pathways, including the FoxO signaling pathway, which is involved in cell survival and apoptosis of the retinal ganglion cell.[26] Moreover, the degree of miR-30a-3p in the DEM-target gene interaction network was the highest among the downregulated DEMs. These results indicate that miR-30a-3p plays a role in HSCR development.

KEGG pathway enrichment analyses of the target genes of the selected DEMs were conducted, which revealed that both targets of upregulated and downregulated DEMs were significantly enriched in two common pathways, including pathways in cancer and the MAPK signaling pathway. These results were in accordance with the findings of a previous study that pathways in cancer and the MAPK signaling pathway were involved in HSCR.[9] To date, numerous pathways have been implicated in human cancers, including the MAPK signaling pathway, which regulates proliferation, differentiation, apoptosis, and survival of cells in the nervous system.[27,28] Besides, the MAPK signaling pathway can be activated by *RET*, which is a major candidate gene responsible for HSCR and is required for the successful colonization of ENCCs in the embryonic gut.[6-9,29] Thus, the MAPK signaling pathway might play a key role in the pathogenesis of HSCR under *RET* regulation.

DEM-target gene interaction networks for the upregulated and downregulated DEMs identified a total of 1991 nodes and 2503 interaction pairs included in the conducted networks. These data indicated that *FZD3* had the highest degree in the upregulated DEM network and the node *DOK6* had the highest degree in the downregulated DEM network. Human *FZD3*, a member of the frizzled family located on chromosome 8p21,[30] is required for the formation of neural crest cells, which is regulated by the Wnt signaling pathway.[31] Besides, proper expression of *FZD3* and *FZD6* is of great importance for midbrain morphogenesis[32]. The expression level

of *FZD3* was higher in aganglionic tissues than in normal tissues in HSCR patients.[33] Therefore, *FZD3* dysregulation may contribute to HSCR development. DOK6, a neuronal adapter protein that is a member of the DOK family of intracellular adaptors, is involved in the *RET* signaling pathway associated with neurite outgrowth.[34-36] Given the important position of *DOK6* in the DEM-target gene interaction network, *DOK6* may also be involved in HSCR development.

As a limitation to the present study, several miRNAs and genes were found to play roles in HSCR development, but the expression levels in colon tissues of HSCR patients as well as the regulatory relationships between miRNAs and downstream target genes were not detected. However, due to the lack of experimental materials (colon tissues of HSCR patient), these results should be further verified.

In conclusion, these data revealed that miR-141-3p and miR-30a-3p are key miRNAs involved in HSCR and that miR-141-3p might act as a negative regulator for ENCC formation. Besides, the MAPK signaling pathway was indicated to play a vital role in *RET*-regulated HSCR pathogenesis. In addition, *FZD3*, which is regulated by the Wnt signaling pathway, and *DOK6* were identified as key genes in the *RET* signaling pathway and might participate in the pathogenesis of HSCR.

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Ethical approval: The dataset used in this study is downloaded from GEO database. Our study is not involved in animal or human experiment, so there is no ethical approval.

Competing interest: None.

Contributors: Gao ZG wrote the first draft of this paper, all authors contributed to the intellectual content and approved the final version of the manuscript.

References

1 Kapur RP. Practical pathology and genetics of Hirschsprung's disease. Semin Pediatr Surg 2009;18:212-223.
2 Best KE, Addor MC, Arriola L, Balku E, Barisic I, Bianchi F, et al. Hirschsprung's disease prevalence in Europe: a register based study. Birth Defects Res A Clin Mol Teratol 2014;100: 695-702.
3 Leenders E, Sieber WK, Kiesewetter WB. Hirschsprung's disease. Surg Clin North Am 1970;50:907-918.
4 Emison ES, McCallion AS, Kashuk CS, Bush RT, Grice E, Lin S, et al. A common sex-dependent mutation in a *RET* enhancer underlies Hirschsprung disease risk. Nature 2005;434:857-863.
5 Pan WK, Zhang YF, Yu H, Gao Y, Zheng BJ, Li P, et al. Identifying key genes associated with Hirschsprung's disease based on bioinformatics analysis of RNA-sequencing data. World J Pediatr 2017;13:267-273.
6 Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, et al. Mutations of the *RET* proto-oncogene in Hirschsprung's disease. Nature 1994;367:378-380.
7 Sribudiani Y, Metzger M, Osinga J, Rey A, Burns AJ, Thapar N, et al. Variants in *RET* associated with Hirschsprung's disease affect binding of transcription factorsand gene expression. Gastroenterology 2011;140:572-582.e2.
8 Carter TC, Kay DM, Browne ML, Liu A, Romitti PA, Kuehn D, et al. Hirschsprung's disease and variants in genes that regulate enteric neural crest cell proliferation, migration and differentiation. J Hum Genet 2012;57:485-493.
9 Li S, Wang S, Guo Z, Wu H, Jin X, Wang Y, et al. miRNA profiling reveals dysregulation of RET and RET-regulating pathways in Hirschsprung's disease. PLoS One 2016;11: e0150222.
10 Tang W, Tang J, He J, Zhou Z, Qin Y, Qin J, et al. SLIT2/ ROBO1-miR-218-1-RET/PLAG1: a new disease pathway involved in Hirschsprung's disease. J Cell Mol Med 2015;19: 1197-1207.
11 Lei H, Li H, Xie H, Du C, Xia Y, Tang W. Role of miR-215 in Hirschsprung's disease pathogenesis by targeting SIGLEC-8. Cell Physiol Biochem 2016;40:1646-1655.
12 Smyth GK. Limma: linear models for microarray data. In: Gentleman R, Carey VJ, Huber W, Irizarry RA, Dudoit S, eds. Bioinformatics and computational biology solutions using R and bioconductor. New York: Springer New York, 2005:397-420.
13 Kolde R. Pheatmap: pretty heatmaps 2015. R package version 1.0.8. https://CRAN.R-project.org/package=pheatmap (accessed December 11, 2015).
14 Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nat Methods 2015;12:697.
15 Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28:27-30.
16 Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25-29.
17 Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 2012;16:284-287.
18 Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13: 2498-2504.
19 Opsahl T, Agneessens F, Skvoretz J. Node centrality in weighted networks: generalizing degree and shortest paths. Social Networks 2010;32:245-251.
20 Korpal M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors *ZEB1* and *ZEB2*. J Biol Chem 2008;283:14910-14914.
21 Uhlmann S, Zhang JD, Schwäger A, Mannsperger H, Riazalhosseini Y, Burmester S, et al. miR-200bc/429 cluster targets PLCgamma1 and differentially regulates proliferation and EGF-driven invasion than miR-200a/141 in breast cancer. Oncogene 2010;29:4297-4306.
22 Li H, Tang J, Lei H, Cai P, Zhu H, Li B, et al. Decreased miR-200a/141 suppress cell migration and proliferation by targeting PTEN in Hirschsprung's disease. Cell Physiol Biochem 2014; 34:543-553.
23 Tang W, Qin J, Tang J, Zhang H, Zhou Z, Li B, et al. Aberrant

reduction of miR-141 increased CD47/CUL3 in Hirschsprung's disease. Cell Physiol Biochem 2013;32:1655-1667.
24 Jiang Q, Wang Y, Shi X. Propofol inhibits neurogenesis of rat neural stem cells by upregulating microRNA-141-3p. Stem Cells Dev 2017;26:189-196.
25 Qiu W, Kassem M. miR-141-3p inhibits human stromal (mesenchymal) stem cell proliferation and differentiation. Biochim Biophys Acta 2014;1843:2114-2121.
26 Yan P, Tang S, Zhang H, Guo Y, Zeng Z, Wen Q. Palmitic acid triggers cell apoptosis in RGC-5 retinal ganglion cells through the Akt/FoxO1 signaling pathway. Metab Brain Dis 2017;32:453-460.
27 Takeda K, Ichijo H. Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. Genes Cells 2002;7:1099-1111.
28 Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 2010;1802:396-405.
29 Tansey MG, Baloh RH, Milbrandt J, Johnson EM Jr. GFRalpha-mediated localization of RET to lipid rafts is required for effective downstreamsignaling, differentiation, and neuronal survival. Neuron 2000;25:611-623.
30 Sala CF, Formenti E, Terstappen GC, Caricasole A. Identification, gene structure, and expression of human frizzled-3 (*FZD3*). Biochem Biophys Res Commun 2000;273:27-34.
31 Deardorff MA, Tan C, Saint-Jeannet JP, Klein PS. A role for frizzled 3 in neural crest development. Development 2001;128:3655-3663.
32 Stuebner S, Faus-Kessler T, Fischer T, Wurst W, Prakash N. *Fzd3* and *Fzd6* deficiency results in a severe midbrain morphogenesis defect. Dev Dyn 2010;239:246-260.
33 Su L, Zhang Z, Gan L, Jiang Q, Xiao P, Zou J, et al. Deregulation of the planar cell polarity genes *CELSR3* and *FZD3* in Hirschsprung disease. Exp Mol Pathol 2016;101:241-248.
34 Crowder RJ, Enomoto H, Yang M, Johnson EM Jr, Milbrandt J. Dok-6, a novel p62 Dok family member, promotes Ret-mediated neurite outgrowth. J Biol Chem 2004;279:42072-42081.
35 Li WQ, Shi L, You YG, Gong YH, Yin B, Yuan JG, et al. Downstream of tyrosine kinase/docking protein 6, as a novel substrate of tropomyosin-related kinase C receptor, is involved in neurotrophin 3-mediated neurite outgrowth in mouse cortex neurons. BMC Biol 2010;8:86.
36 Dahal GR, Wang JX, Guo LH. Long-term outcome of children after single-stage transanal endorectal pull-through for Hirschsprung's disease. World J Pediatr 2011;7:65-69.

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