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Effects of miR-150-5p on cerebral infarction rats by regulating the Wnt signaling pathway *via* p53

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Abstract. – OBJECTIVE: The aim of this study was to screen differentially expressed micro ribonucleic acids (miRNAs) in the plasma of patients with cerebral infarction (CI). In addition, the role of miR-150-5p in the incidence of CI is mainly explored via animal models and molecular biology experiments.

PATIENTS AND METHODS: Blood samples were collected from hospitalized patients diagnosed with CI, including 15 CI patients and 15 non-CI patients as negative controls. Differentially expressed miRNAs in the plasma of these subjects were screened by microarray analysis. TargetScan was applied to predict the target genes of miR-150-5p, which were subjected to GO and pathway enrichment analyses using WebGestalt. Sprague-Dawley rats were randomly divided into Sham group (n=20), Control group (n=20), and Experimental group (n=20). CI model in rats was established in the latter two groups. Rats in Experimental group and Control group were intravenously injected with miR-150-5p mimics or miR-negative control (NC), respectively. The expressions of vital genes in the Wnt signaling pathway, including p53, Cyclin D1 (CCND1), c-Myc, β-catenin (CTNNB1) and Survivin were detected by Western blot in rats at 3 d after injection.

RESULTS: A total of 3,568 differentially expressed miRNAs were detected in the peripheral blood between CI patients and controls, whose 2,100 were upregulated, including miR-150-5p ($p<0.05$). The target genes of miR-150-5p were involved in molecular pathways, such as the Wnt signaling pathway, carcinogenesis, endocrine regulation, and infection. Compared with rats in Control group, the protein expression of p53 was downregulated ($p<0.05$), while CCND1, c-Myc, CTNNB1 and Survivin were upregulated ($p<0.05$) in Experimental group.

CONCLUSIONS: MiR-150-5p regulates the Wnt signaling pathway and participates in cell proliferation and apoptosis by downregulating p53, which may be a potential mechanism of CI induction.

Key Words:
Cerebral infarction, MiR-150-5p, p53, Wnt signaling pathway, Target gene.

Introduction

Cerebral infarction (CI), also known as ischemic stroke or apoplexy, is not only a severe brain disease but also one of the most common death causes in the world. It accounts for about 10% of all the causes of death[1]. CI and cerebral hemorrhage are two common types of cerebrovascular diseases, which usually lead to permanent disability and even death[2-6]. Some scales for the nervous system (e.g., NIHSS and mRS) are capable of predicting the prognosis of CI patients, but they can hardly evaluate the prognosis of patients with apraxia, aphasia or misdirection[7]. Large quantities of animal and human studies[8-10] have reported that inflammation is a leading cause of CI. Cerebral infarction, CI can trigger oxidative stress, neuronal excitotoxicity, blood-brain barrier dysfunction, microvascular injury and ischemic inflammation, resulting in irreversible damage to the brain tissues[11,12]. In addition, the nerve cell apoptosis has been widely recognized as a major pathophysiological mechanism of CI[13,14]. Currently, there are limited treatments for CI due to a lack of accurate and reliable blood biomarkers[15]. Therefore, discovering potential molecules with diagnostic significance will help with the early diagnosis and risk prediction of the disease.

Micro ribonucleic acids (miRNAs), a category of non-coding RNAs generally with 18 nucleotides in length, are able to modulate gene expressions. MiRNAs can bind to RNA-induced silencing complexes and perform post-transcriptional regulation by affecting the stability of target mRNAs or repressing the translation of target mRNAs into proteins[16]. Differentially expressed miRNAs have been reported in multiple cardiovascular and neurological diseases, including CI and myocardial infarction. A growing number of miRNAs have been discovered and deeply studied for their potentials as novel sensitive,

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high-efficient and highly specific biomarkers. MiR-26a, miR-497 and miR-451 are differentially expressed in CI profiling. These miRNAs can be used as biomarkers in the early screening of CI[17-19]. It has been reported[20,21] that miR-150-5p plays a major role in the proliferation and invasion of cancer cells. Scherrer et al[22] manifested that miR-150-5p serves as a new prognostic marker in patients with ischemic stroke, which is highly correlated with the 90-day mortality, but investigations of its mechanism have not been conducted. In the present study, miR-150-5p was differentially expressed in CI patients, according to the sequencing of plasma RNA samples. Furthermore, the mechanism of miR-150-5p in CI was explored in the rat model of CI.

The pathogenesis of CI involves various signaling pathways, among which the Wnt signaling pathway is considered to be implicated in the proliferation and apoptosis of nerve cells in the case of CI. Therefore, regulating the activity of the Wnt signaling pathway becomes an effective target of CI treatment. MiRNAs can conjugate with target genes to suppress their transcriptional activities. MiR-140-5p enhances the cerebral protective effect of dexmedetomidine against hypoxic-ischemic brain damage by modulating the Wnt/β-catenin (CTNNB1) signaling pathway. Though miR-150-5p has been proven to have an association with CI, no report has denoted whether miR-150-5p participates in the pathogenesis of CI *via* the Wnt signaling pathway. In this study, sequencing technique was applied to investigate the differential expression profiles of plasma miRNAs in CI patients, and the differential expression of miR-150-5p was verified, so as to further determine the impact of miR-150-5p on CI injury. Additionally, using the rat model of CI. It was found that overexpression of miR-150-5p exerts its effects on CI by activating the Wnt signaling pathway by downregulating p53 expression. It participated in CI progression by influencing cell growth and apoptosis, thereby providing more adequate theoretical bases for miR-150-5p as a marker and target of CI.

Patients and Methods

Animals and Reagents

Four-week-old Sprague-Dawley (SD) rats (Beijing SPF Center, Beijing, China), miR-150-5p mimics (Shanghai GenePharma Co, Ltd., Shanghai, China), Roswell Park Memorial Institute-1640 (RPMI-1640) medium and fetal bovine serum (FBS; Nanjing BioChannel Biotechnology Co., Ltd., Nanjing, China), TransFast transfection reagent (Promega Corporation, Madison, WI, USA), small interfering RNA (siRNA) and miRNA reagents (Shanghai Ke Lei Biological Technologr Co., Ltd., Shanghai, China), antibodies of β-actin, p53, c-Myc, Cyclin D1 (CCND1) and Survivin (CST, Danvers, MA, USA), and Image J software (NIH, Bethesda, MD, USA) for gray analysis of proteins.

Selection of Research Subjects and Acquisition of Plasma Samples

This study was approved by the Ethics Committee of Affiliated Hospital of Weifang Medical University. Signed written informed consents were obtained from all participants before the study. Plasma samples were collected from patients diagnosed with CI from August 2016 to May 2018, and healthy subjects were selected as controls. Those accompanied other diseases were strictly excluded. The plasma samples were centrifuged at 12,000 rpm for 6 min, at room temperature to separate the plasma. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract plasma RNA.

Sequencing Analysis of MiRNAs

Four samples were taken from both Experimental group and Control group for sequencing analysis of miRNAs, which was accomplished by KangChen Biotechnology (Shanghai, China). In short, small interfering RNA (siRNA) was isolated from total RNA first and then purified, and it was ligated to the 5'-linker and purified, followed by ligation to the 3'-linker and purification again. Next, the siRNA was synthesized into first strand and second-strand complementary deoxyribonucleic acids (cDNAs), and Illumina Genome Analyzer IIx sequencing analysis was performed finally. FC>2.0 and $p<0.05$ suggested that the miRNAs were differentially expressed.

Prediction and Analysis of Target Genes

The intersection of the target genes of miR-150-5p predicted by TargetScan was uploaded to WEB-based Gene SeT AnaLysis Toolkit (WebGestalt)[23] for Genome Ontology (GO) and Pathway enrichment analyses. WebGestalt, an online web committed to enrichment analyses, supports formats of 12 species and 324 gene

IDs. Its functional annotations not only contain the commonly used GO and KEGG, but also cover the protein-protein and miRNA-miRNA interaction, disease annotation, drug targets, and other annotation information. There are three available algorithms of enrichment analysis: Overrepresentation Enrichment Analysis, Gene Set Enrichment Analysis, and Network Topology-based Analysis.

Preparation of CI Model in Rats

This study was approved by the Animal Ethics Committee of Weifang Medical University Animal Center. A total of 60 male SD rats with 4 weeks old were adaptively housed. Until they were aged about 10 weeks old and weighing approximately 220 g, the rats were randomly divided into Sham group (n=20), Control group (n=20), and Experimental group (n=20). After injection for consecutive 3 days, the rats were sacrificed for collecting brain tissues. The specific modeling processes were as follows: 1) the rats were first anesthetized by inhaling a certain dose of diethyl ether and then fixed on an insulation board; 2) after exposure and separation of the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA), the distal and proximal ends of CCA and ECA were ligated; 3) the ICA was clipped temporarily using a micro-artery clamp, and the proximal ends of CCA and ECA were ligated; 4) a small incision was made at 4 mm away from the junction of CCA, and the thread was inserted into the ECA and gently tied using the thread wound around the distal CCA; 5) the incision was sutured. The rats in Sham group were operated without performing suture occlusion and injection. After suture occlusion, the rats in the Control group and Experimental group were injected with 300 μg of miR-NC or miR-150-5p mimics *via* the tail vein, respectively.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from the brain tissues through TRIzol method, quantified using NanoDrop ND-2000 (Thermo, Waltham, MA, USA) and synthesized into cDNA *via* RT kits. QRT-PCR was conducted using the SYBR Green PCR kits and relative level was detected on a 7500-Fast Real Time-PCR system (Applied Biosystems, Foster City, CA, USA). Finally, the relative expression levels of miR-150-5p and genes were calculated through $2^{-\Delta\Delta Ct}$ method, with U6 (small nuclear RNA) as an internal reference of miRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference of genes. The primer sequences for qRT-PCR were shown in Table I.

Western Blotting

After homogenization, the brain tissues were ultrasonicated and centrifuged to obtain the protein samples. Later, the protein concentration was measured by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), which was prepared into the same concentration. Then, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and the proteins in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated with anti-p53, c-Myc, CCND1, and Survivin antibodies at 4°C overnight, followed by incubation with horse radish peroxidase (HRP)-coupled secondary antibodies at room temperature for 1 h. Finally, band exposure was achieved by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was employed for statistical analysis. The numer-

Table I. Primer sequence.

Index	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
MiR-150-5p	TCTCCCAACCCTTGTACCAGTG	TGGTGTCGTGGAGTCG
P53	CCAGGGCAGCTACGGTTTC	CTCCGTCATGTGCTGTGACTG
CCND1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
CTNNB1	ATGGAGCCGGACAGAAAAGC	CTTGCCACTCAGGGAAGGA
C-Myc	GGAGAAACGAGCTGAAGCGTAG	CAGCCAAGGTTGTGAGGTTAGG
Survivin	CTTCATCCACTGCCCTACCG	CAGGGGAGTGCTTCCTATGC

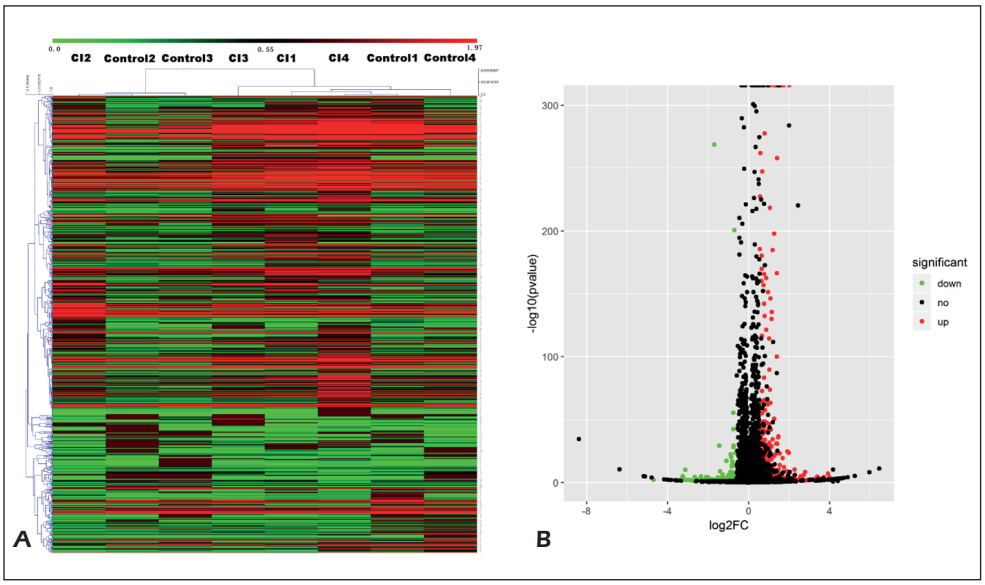

Figure 1. Differentially expressed miRNAs in CI patients. **A**, Heatmap of differentially expressed miRNAs in CI patients, **B**, Volcano plots of differentially expressed miRNAs in CI patients, where the red dots and green dots represent up-regulated miRNAs and down-regulated miRNAs, respectively.

ical variables were expressed as mean ± standard deviation (SD). The *t*-test was used for comparison between two groups, and $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-150-5p was Upregulated in CI Patients

It was found through miRNA sequencing analysis that plasma level of miR-150-5p increased in CI patients compared with that in controls ($p<0.05$) (Figure 1). Moreover, its expression pattern was further verified in the samples we collected by qRT-PCR (Figure 2).

Predicted Target Genes of MiR-150-5p

A total of 351 target genes of miR-150-5p were obtained through TargetScan prediction, and the top 30 target genes were shown in Table II. Among them, 8mer, 7mer-m8, and 7mer-A1 stood for the number of conservative binding sites.

GO and Pathway Enrichment Analyses on Target Genes of miR-150-5p

The target gene sets of miR-150-5p were uploaded to WebGestalt online tool for enrichment analysis. 340 out of the 351 target genes were successfully enriched, and a series of target genes involved in biological processes (BPs) and pathways were acquired (Figure 3). These BPs covered abundant categories, including response to endogenous stimulus and regulation of cell growth, proliferation and metabolism (Figure 4). The Wnt signaling pathway was prominently enriched among the signaling pathways obtained. In addition, the thyroid hormone signaling pathway, cancer-related signaling pathway, and infection-related signaling pathway were also manifested in the enrichment list (Table III).

The Expression of MiR-150-5p was Upregulated After Injection of MiR-150-5p Mimics into CI Rats

After animal procedures, qRT-PCR assay was adopted to determine the expression of miR-150-5p in the brain tissues of rats.

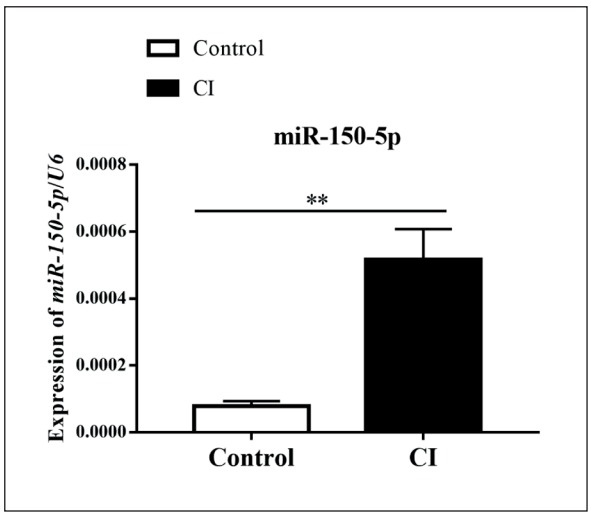

Figure 2. Plasma level of miR-150-5p increases in CI patients. Note: The expression of plasma miR-150-5p is higher in CI patients than that in Control group (**$p<0.01$).

Table II. Former 30 target genes of miR-150-5p predicted by TargetScan.

Target	8mer site (n)	7mer-m8 site (n)	7mer-A1 site (n)	Weighted relevance score (point)
MYB	2	0	1	-1.14
MDM4	0	0	1	-1.04
ENSA	1	0	0	-0.87
ADIPOR2	1	0	0	-0.72
HILPDA	1	0	0	-0.66
TADA1	1	0	0	-0.58
MTCH2	0	0	1	-0.54
DCAF6	1	0	0	-0.5
ELOVL3	1	0	0	-0.48
IKBIP	1	0	0	-0.45
WTAP	1	0	0	-0.45
CMTM6	1	0	0	-0.45
MBD6	0	2	1	-0.4
BASP1	0	1	0	-0.38
SKP1	1	0	0	-0.38
CCNT1	1	0	0	-0.38
ZBTB4	1	1	0	-0.38
CNPPD1	0	1	0	-0.36
ZCCHC17	0	1	0	-0.34
ZMAT2	1	0	0	-0.34
PLP2	1	0	0	-0.34
STAT1	1	0	0	-0.34
TP53	1	0	0	-0.32
CAST	1	0	0	-0.32
SHISA4	1	0	0	-0.32
OSBPL9	1	0	0	-0.31
GRIPAP1	1	0	0	-0.31
FOXD3	1	0	0	-0.31

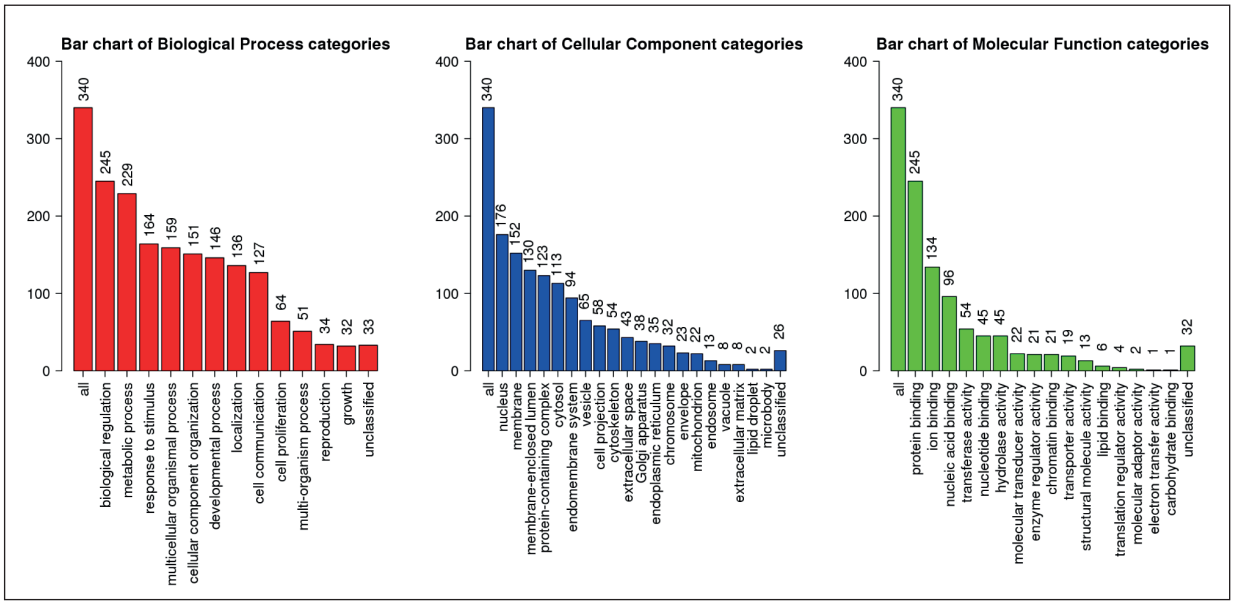

Figure 3. GO entry statistics enriched for target genes of miR-150-5p. Note: Biological Process (BP): enrichment of biological processes, Cellular Component (CC): enrichment of cellular components, Molecular Function (MF): enrichment of molecular function.

Table III. Pathways enriched for target genes of miR-150-5p.

Pathway	Size	Overlap	Enrichment ratio	p	FDR
MicroRNAs in cancer	150	16	5.7027451	1.51E-08	4.92E-06
Wnt signaling pathway	146	15	5.4927981	7.30E-08	1.19E-05
Thyroid hormone signaling pathway	116	12	5.5306795	1.48E-06	1.60E-04
Proteoglycans in cancer	198	15	4.0502451	3.77E-06	3.07E-04
Hepatocellular carcinoma	167	13	4.1618087	1.29E-05	8.44E-04
Breast cancer	147	12	4.3643457	1.76E-05	9.54E-04
Pathways in cancer	524	24	2.4486978	3.26E-05	0.0015202
Prostate cancer	97	9	4.9605064	7.63E-05	0.0026676
Human T-cell leukemia virus 1 infection	255	15	3.1448962	7.64E-05	0.0026676
Gastric cancer	148	11	3.9736188	9.42E-05	0.0026676

The results indicated that miR-150-5p was upregulated in brain tissues of Experimental group than that of Control group ($p<0.01$), suggesting the successful intervention of miR-150-5p in rats with CI (Figure 4).

MiR-150-5p Regulated the Wnt Signaling Pathway

Subsequently, the activity of the Wnt signaling pathway in rats was determined by examining mRNA levels of p53, c-Myc, CCND1, CTNNB1, and Survivin in the brain tissues. It was revealed that the mRNA expression of p53 declined ($p<0.05$) (Figure 5), while those of c-Myc, CCND1, CTNNB1, and Survivin were upregulated in Experimental group than controls, and the differences were statistically significant ($p<0.05$) (Figure 6). Similar trends on their protein levels were observed by Western blot as well (Figure 7 and 8).

Discussion

CTNNB1 is involved in the regulation of cell proliferation as a vital transcription factor in cells. It is well known that CTNNB1 promotes cancer progression depending on the Wnt signal[24,25]. In general, Wnt receptors inhibit the phosphorylation, disable the activation, and enhance the stability of CTNNB1, as well as facilitate the translocation of CTNNB1 to the nucleus[26]. In this study, the research team first analyzed differentially expressed miRNAs in the plasma of CI patients

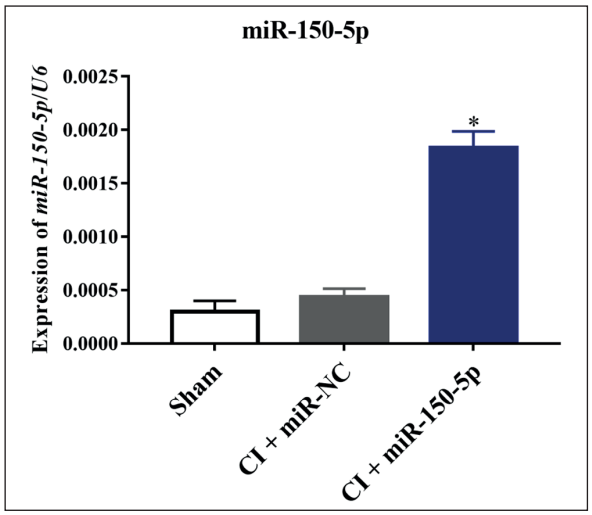

Figure 4. Changes in miR-150-5p expression after injection. Note: The expression of miR-150-5p is elevated notably in Experimental group compared with that in Control group (*$p<0.01$).

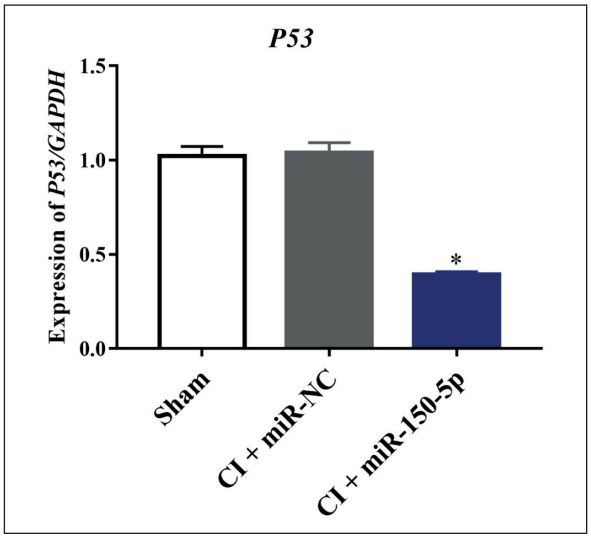

Figure 5. MRNA expression of p53 after injection of miR-150-5p. The mRNA expression of p53 declines in Experimental group in comparison with that in Control group (*$p<0.05$).

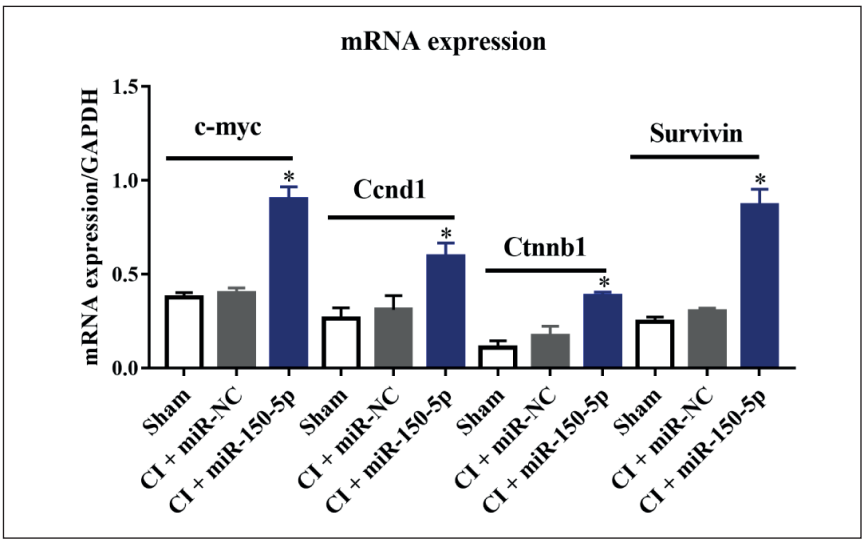

Figure 6. MRNA expressions of c-Myc, CCND1, CTNNB1 and Survivin after injection of miR-150-5p. The mRNA expressions of c-Myc, CCND1, CTNNB1 and Survivin rise in Experimental group in contrast with those in Control group ($p<0.05$).

by microarray analysis. WebGestalt online tool for GO and pathway functional enrichment analyses were subsequently conducted. It was found that the downstream target genes of miR-150-5p were associated with the Wnt signaling pathway ($p<0.001$).

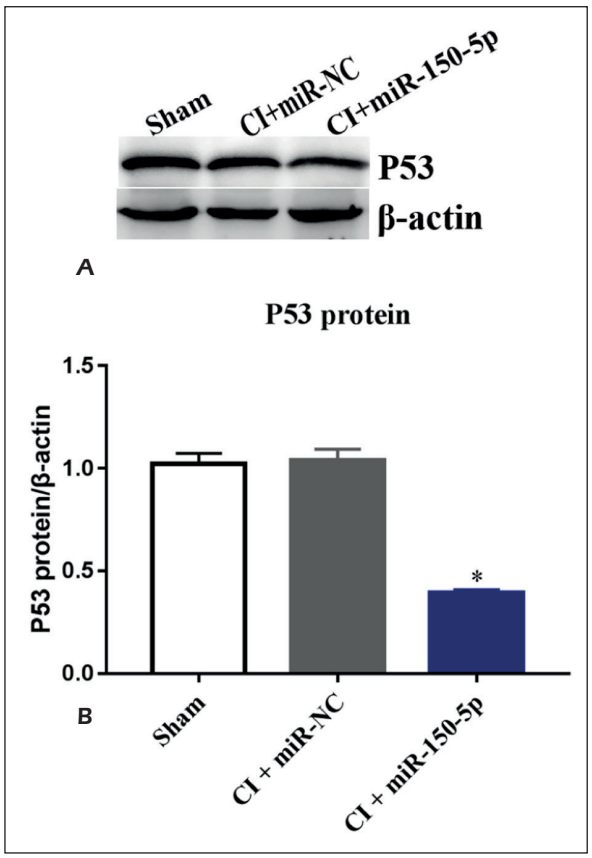

Figure 7. Protein expression of p53 after injection of miR-150-5p. Experimental group has a lower protein expression level of p5 than Control group (*$p<0.05$).

P53 is the most important suppressor gene correlated with the occurrence and development of tumors, and its biological effects are implicated in the regulation of senescence and metabolism mainly by modulating the transcription of downstream target genes[27,28]. It regulates cell cycle proteins and it is maintained at a relatively low level and degraded by ubiquitin pathway under normal conditions[29]. Once cells are exposed to the pressure of genetic toxicity, p53 will become stable and induce cell cycle arrest and/or cell death after post-translational modification *via* phosphorylation and acetylation. When the activity of p53 is lost due to gene deletion or mutation, the normal cells will be deprived of the ability to control their growth and death, thus triggering immortalization and finally resulting in cancers. As is well known, the mutation rate of p53 in cancer tissues is fairly high, while some studies reported that wild type p53 can negatively regulate the Wnt signaling pathway by downregulating CTNNB1[30,31]. It can be conjectured that p53 probably performs the same functions in the CI model. P53 expression level repressed by miR-150-5p may also be a crucial reason for accumulation of CTNNB1 and subsequent activation of the Wnt signaling pathway in the rat CI model. Wellenstein et al[32] reported that the knockdown of p53 in breast cancer cells can stimulate the secretion of Wnt ligands, thereby remarkably enhancing the activity of the Wnt signaling pathway. In the present study, it was discovered that the expression level of p53, an essential target gene regulated by miR-

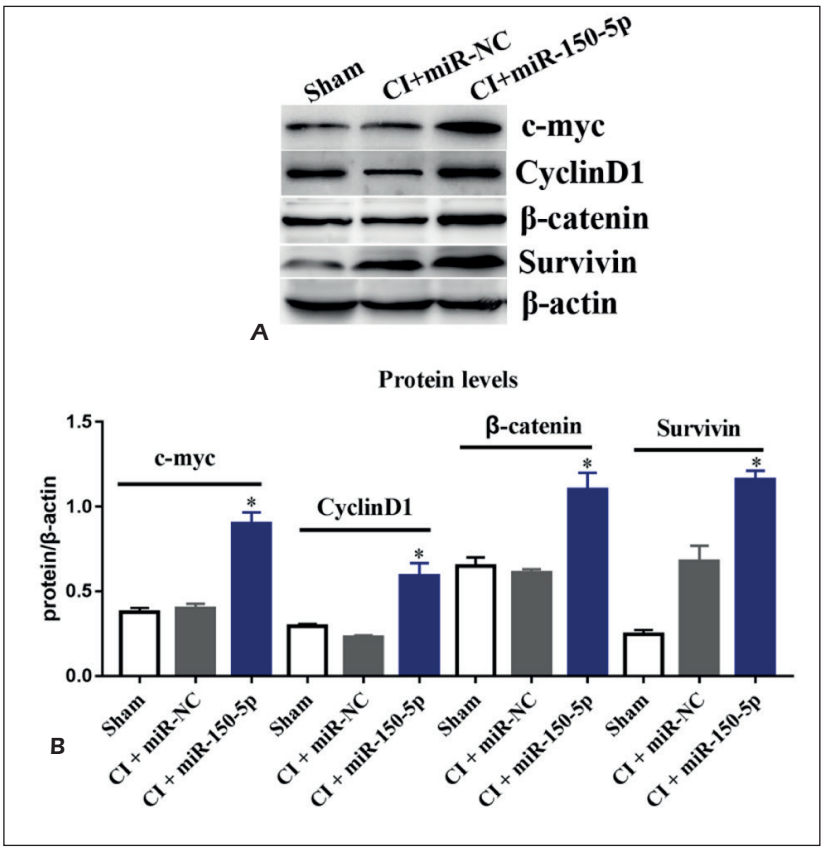

Figure 8. Protein expressions of c-Myc, CCND1, CTNNB1 and Survivin after injection of miR-150-5p. Experimental group exhibits higher protein expression levels of c-Myc, CCND1, CTNNB1 and Survivin rise in than Control group (*p<0.05).

150-5p, declined in Experimental group, which might cause the abnormal accumulation of CTNNB1 and activation of the Wnt signaling pathway. Furthermore, it was revealed that miR-150-5p significantly downregulated the protein expression level of p53 in Experimental group compared with Sham group and Control group (p<0.05). Meanwhile, relevant changes in the downstream target genes (c-Myc, CCND1 and Survivin) in the Wnt signaling pathway were observed. It is believed that miR-150-5p may induce CI through the p53-modulated Wnt signaling pathway.

However, the complexity of the mechanism of miRNAs is worth thinking[33,34]. The specific molecular mechanism of p53 in downregulating CTNNB1 remains unclear at present, which is also a biological issue and a research direction requiring attention. Solving this problem is of important significance for revealing the mechanism of miR-150-5p in inducing CI through the Wnt signaling pathway regulated by p53. The novelty of this study was that further elaborating the mechanism of miR-150-5p can set theoretical bases for development of novel therapies, such as miR-150-5p inhibitor therapy and provide more suitable treatment protocols for CI treatment.

Conclusions

In summary, the expression profiles of differentially expressed miRNAs in CI patients were screened in this study, and it is expected to further explore and define more miRNAs with diagnostic significance in CI. Furthermore, it was clarified that miR-150-5p was differentially expressed in the plasma of CI patients, laying a foundation for its subsequent functions in CI diagnosis, as well as theoretical and scientific bases for the treatment of CI with miR-150-5p. The possibility of miR-150-5p acting as a potential therapeutic target of CI was proposed, and opinions about miR-150-5p as a promising therapeutic target of some other relevant diseases were also provided.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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