MicroRNA-339-5p inhibits lipopolysaccharide-induced rat mesangial cells by regulating the Syk/Ras/c-Fos pathway

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Abstract

Purpose: Chronic glomerulonephritis (CGN) is a disease that occurs in the glomeruli. The mechanism of CGN is thought to be involved in a range of inflammatory responses. MicroRNA-339-5p (miR-339-5p) has been reported to be involved in inflammatory responses in many diseases. However, the role of miR -339-5p in CGN remains unclear. The purpose of this study was to investigate the role of miR-339-5p in lipopolysaccharide (LPS) -induced nephritis injury in vitro.

Methods: The RNA expression of miR-339-5p and Syk/Ras/c-Fos pathway was detected by qRT-PCR, the protein expression and localization of Syk/Ras/c-Fos pathway was detected by western blot and immunofluorescence (IF), and the targeted binding of miR-339-5p to Syk was detected by double luciferase. Cell viability and cell cycle were detected by cell counting kit-8 (CCK-8) and flow cytometry. The concentrations of inflammatory cytokines IL-1β, IL-10, IL-6 and TNF-α were detected by enzyme linked immunosorbent assay (ELISA).

Results: LPS increased HBZY-1 cell viability, decreased G2 phase, promoted cell proliferation and inflammatory cytokine release. Overexpression of miR-339-5p can inhibit HBZY-1 cell viability, decreased the expression of Syk/Ras/c-Fos signaling pathway, down-regulate the expression level of inflammatory cytokines, increase G2 phase, and inhibit cell proliferation.

Conclusion: miR-339-5p inhibits the proliferation and inflammation of rat mesangial cell through Syk/Ras/c-Fos signal pathway.

Introduction

Chronic glomerulonephritis (CGN) is one of the most important non-communicable diseases in the world. About 10% of people worldwide suffer from chronic kidney disease [1]. Similarly, the prevalence rate of chronic kidney disease in Chinese is 10.8%, and the incidence of CGN in central China is as high as 14.2% [2]. The occurrence of CGN is mainly due to glomerular pathological damage mediated by immune inflammatory response. Patients will develop symptoms such as edema proteinuria and hypertension [3]. At present, the specific pathogenesis is not clear, and there is no effective targeted therapy [4]. If the treatment is not timely, end-stage renal disease (ESRD) will be developed [5–6]. Kidney transplantation will be the only choice [7–8], which increases the economic burden of patients and affects their quality of life, and has become a global public health problem.

MicroRNAs (miRNAs), which usually contains 21-25 nucleotides [9–10], is a kind of short-stranded non-coding RNA. It regulates gene expression by targeting mRNAs for translation inhibition or degradation, mainly in post-transcriptional horizontal regulation-related protein expression [11–12]. More and more evidence show that miRNAs play important roles in the regulation of inflammation-mediated disease [13], and participates in a series of important processes such as disease development, inflammation, immune response and so on [14–15]. In addition, studies have found that miRNA can affect the process of cell proliferation and apoptosis [16]. miR-339-5p has been proved to be a tumor suppressor that can reduce

cisplatin resistance in laryngeal cancer [17]. It can exert tumor inhibition and induce apoptosis of glioblastoma [18]. It has been reported that miR-339 promotes apoptosis injury by inhibiting proliferation and invasion of hepatocellular carcinoma cells [19]. However, the role of miR-339-5p in CGN remains unclear. This study investigated the relationship between miR-339-5p and CGN, aiming to provide reference for the pathogenesis of CGN.

Materials And Methods

Cell Lines and Cell Culture

The HBZY-1 cell line was obtained from the Cell Bioscience Inc (Shanghai, China). After receiving the cells, the cells were placed in the incubator for 3 hours to stabilize the cell state. After washing with PBS (Hyclone, Logan, UT, USA), 1mL trypsin (Beyotime Biotechnology, Shanghai, China) was added for digestion for 2min. After centrifugation, the suspension was transferred to T-25 culture flask (KIRGEN, Shanghai, China) and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone) containing 10% fetal bovine serum, penicillin (100 U/mL, Invitrogen, Carlsbad, CA, USA), and streptomycin (100 μg/mL, Invitrogen). The HBZY-1 cell line was cultured in a humidified incubator containing 5% CO_2 at 37°C.

Cell transfection

HBZY-1 cells were divided into groups after synthesis of miR-339-5p inhibitors, miR-339-5p mimics and negative control (NC) sequences. The transient transfection sequence of Lipofectamine 2000 kit was used to transfect HBZY-1 cells, 24 hours after transfection, the gene expression of each group was detected, and the cells of each group were collected for cell cycle and slice climbing experiments. At the same time, the cells were collected and put in the refrigerator at -80℃ for follow-up Western blotting and qRT-PCR experiments.

Quantitative Real-Time Polymerase Reaction

Trizol reagent (Life Technologies, Carlsbad, CA, USA) was used to extract total RNA in each group. PrimeScript™RT Reagent Kit with gDNA Eraser Kit (TaKaRa, Kyoto, Japan) for reverse transcription into cDNA. SYBR qPCR SuperMix Plus (NovoProtein, Shanghai, China) is used for real-time quantitative polymerase chain reaction in fluorescent quantitative PCR apparatus (Thermo Fisher Scientific, Waltham, MA, USA). The reaction conditions were predenaturated for 1min at 95 °C in the first step. The second step is denaturation at 95 °C for 20s, annealing at 60 °C for 1min, 40 cycles. Using β-actin or U6 as control, the relative expression levels of related RNAs were calculated by $2^{-\Delta\Delta CT}$ method. Primer sequences of each indicator were shown in Tab. 1.

Western blotting assay

The cell samples were collected and the total cell proteins were extracted with RIPA lysis buffer (Beyotime), After electrophoretic separation and transferred to polyvinylidene fluoride (PVDF) membrane

(Millipore, Billerica, MA, USA). Seal at room temperature for 2 hours in a Tris buffer containing 5% skim milk. The primary antibody was incubated overnight. The dilution ratio was Syk (1:500), p-Syk (1:1000), Ras (1:1000), MEK (1:1000), p-MEK (1:1000), ERK (1:1000), p-ERK (1:500) and c-Fos (1:500). After washing, secondary antibody (Abcam, Shanghai, Chnia) labeled with horseradish peroxidase was incubated at room temperature for 1h. Protein bands were obtained by automatic exposure apparatus (Shanghai Peiqing, China). The bands were quantified by Image J software (1.8.0).

Cell Counting Kit-8 Proliferation Assay

The HBZY-1 cells in the culture bottle were washed with aseptic PBS and digested with trypsin (Beyotime) for 2 min. After centrifugation, the cell count was adjusted to 5×10^4 cells/well, and inoculated in a 96-well plate, and 5% CO_2 was incubated overnight in an incubator at 37 ℃. The cells of each group were transfected as required, and 10μL of CCK-8 solution (Bioss, Beijing, China) was added to each well of the 96-well plate. After 4 hours of culture, the absorbance of each hole was measured at the OD450 nm of enzyme-linked immunosorbent assay (Rayto, Shenzheng, China).

Immunofluorescence

The cells were inoculated in 6-well plates. 4% paraformaldehyde (Ebiogo, Anhui, China) was fixed for 20 min, goat serum (Zsbio, Beijing) was closed for 30 min, antibody was incubated for 60 min, the dilution ratio was Syk (1:300), p-Syk (1: 300), Ras (1:200), ERK1/2 (1:200), p-ERK1/2 (1: 200) and c-Fos (1:50). DAPI (Beyotime) was stained for 5 min and sealed with fluorescence quenching agent (Beyotime). Fluorescence microscope was used to observe and photograph was taken. Light repellent drip plus secondary antibody (1:400) (abcam), incubated at 37℃ for 30 min (Beyotime) and stained at room temperature for 5 min. The fluorescence intensity of each group was observed under fluorescence microscope (Motic,Xiamen,China).

Flow Cytometry Assay

The cells of each group were washed with aseptic PBS, digested with 1mL trypsin for 2 minutes, centrifuged with 1000rpm 5min, removed the supernatant, fixed with precooled anhydrous ethanol in the refrigerator at -20℃ for one hour, the fixed cells were washed twice with precooled PBS, added 20uLRNase, resuscitated cells, digested 30min in 37℃ water baths, then added PI staining solution 400ul resuscitated cells, stained 40min at 4℃. Flow cytometry (BECKMAN, Brea, CA, USA) was used to observe the changes of cell cycle in each group.

Luciferase Reporter Assay

The 3'-UTR mutation sequence and 3'-UTR wild sequence of Syk were inserted into pSI-Check2 (Hanbio Biotechnology, Shanghai, China) luciferase reporter vector respectively. The vectors containing Syk mutant and wild sequence were named Syk- mutant (Syk-mut) and Syk-wild type (Syk-wt). Syk-mut and Syk-wt, together with miR-399-5p mimic or miR-NC, were co-transfected into 293T cells by Opti-MEM low-

serum medium and liposome 2000, respectively. The cells were collected 48 hours after transfection, and the relative luciferase activity was detected by double luciferase analysis kit (Promega, Beijing, China).

Statistical analysis

All experiments were repeated three times and data were presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software, and the figures were drawn by GraphPad Prism 8.0. Aoneway analysis of variance (ANOVA) was used to calculate the P-values, which < 0.05 was considered to be statistically significant.

Results

miR-339-5p was significantly downregulated in HBZY-1 cells

In order to clarify the relationship between miR-339-5p and CGN, HBZY-1 cells induced by LPS were used as CGN experimental model in this study [20]. qRT-PCR was used to detect the expression levels of miR339-5p in the Control group and the Model group. qRT-PCR results showed that miR-339-5p expression level was significantly down-regulated in the model group compared with the control group (Fig.1) (Supplementary 5,6,7).

Overexpression of miR-339-5p decreased the activity of HBZY-1 cells and inhibited cell proliferation

To further explore the role of miR-339-5p in HBZY-1 cells. qRT-PCR results showed that overexpression of miR-339-5p or inhibition of miR-339-5p level would cause changes in the expression level of miR-339-5p RNA (Fig.2A)(Supplementary 5,6,7). CCK-8 detection showed that transfection of miR-339-5p mimic could effectively inhibit the viability of HBZY-1 cells, and transfection of miR-339-5p inhibitor could effectively increase the viability of HBZY-1 cells (Fig.2B)(Supplementary 1) . The following results can be obtained from the cell cycle change diagram: miR-339-5p inhibitors reduce the proportion of G2 phase cells and promote cell proliferation. However, under the intervention of miR-339-5p overexpression, the proportion of cells in G2 phase increased, the cell cycle stagnated at the later stage of DNA synthesis, and the cell proliferation ability decreased (Fig.2C-D) (Supplementary 2).Therefore, overexpression of miR-339-5p can inhibit the activity and proliferation of HBZY-1 cells, while inhibition of miR-339-5p results in the opposite.

miR-339-5p reduces the levels of inflammatory cytokines in HBZY-1 cells

In order to determine whether miR-339-5p affects the level of inflammatory factors in HBZY-1 cells. Western blot (Fig.4A-D)(Supplementary 3,4) and qRT-PCR (Fig.4E-H) (Supplementary5,6,7) were used to detect the expression levels of inflammatory cytokines. The results showed that overexpression of miR-339-5p significantly reduced the expression levels of inflammatory cytokines IL-1β, IL-6 and TNF-α, and significantly increased the expression level of pro-inflammatory cytokines IL-10. Inhibition of the expression of miR-339-5p resulted in the opposite result. Therefore, miR-339-5p reduced the level of inflammatory cytokines in HBZY-1 cells.

Syk was the direct target gene of miR-339-5p

The dual luciferase reporting assay was used to confirm whether miR-339-5p could bind to Syk. The results showed that the number of cells cotransfected with miR-339-5p mimics and Syk-wt was significantly lower than that of miR-339-5p and Syk-mut (Fig.4A). The luciferase activity of the cells transfected with mimics NC had no significant change in Syk-wt and Syk-mut, which confirmed that Syk was the direct target gene of miR-339-5p (Fig.4B)(Supplementary 9) . In addition, the regulatory relationship between miR-339-5p and Syk was studied by Western blot (Fig.4C-D)(Supplementary 3,4), qRT-PCR (Fig.4E) (Supplementary5,6,7) and IF (Fig.4F-G) (Supplementary 8,10,11) experiments. The results showed that by inhibiting the expression of miR-339-5p, the expression of Syk in HBZY-1 cells was significantly increased, while the expression of overexpression miR-339-5p was significantly decreased in HBZY-1 cells, so miR-339-5p could directly negatively regulate the expression of Syk in HBZY-1 cells.

miR-339-5p inhibits Syk/Ras/c-Fos signal pathway

The relationship between miR-339-5p and Syk/Ras/c-Fos signaling pathway was verified. The results showed that overexpression of miR-339-5p inhibited the expression levels of Syk/Ras/c-Fos signaling pathway related indicators in each group of cells, while inhibition of miR-339-5p resulted in the opposite result. Therefore, it can be concluded that miR-339-5p overexpression can inhibit Syk/Ras/c-Fos signaling pathway(Fig.5) (Supplementary 3,4,5,6,7.8,12,13,14,15) .

Syk downregulation reversed the effects of miR-339-5p on HBZY-1 cells

This study confirmed the inhibitory effect of miR-339-5p on Syk and speculated that Syk was involved in the regulation of miR-339-5p on HBZY-1 cells. The results showed that R406 could down-regulate the expression levels of inflammatory cytokines IL-1β, IL-6 and TNF-α, and up-regulate the expression levels of anti-inflammatory cytokines IL-10(Fig.6A-H) (Supplementary 3,4,5,6,7) . As can be seen from the changes in cell cycle diagram, R406 can also regulate the changes of cell cycle, stagnating the cell cycle in G2 phase, reversing the decrease in the G2 phase of HBZY-1 cells caused by miR-339-5p inhibitors, and reducing the cell proliferation ability (Fig.6J-I)(Supplementary 2) . In addition, CCK-8 assay showed that R406 inhibited HBZY-1 cell viability (Fig.6K)(Supplementary 1) . Thus, Syk inhibitor R406, in contrast to miR-339-5p's effect on the inflammatory factors, cycle and cell viability of cells.

Discussion

GMC (glomerularmesangialcell,GMC) is the main cellular component in the glomerulus and participates in many important biological functions in the kidney. The proliferation and inflammation of GMC can lead to renal damage and a series of renal diseases [21–22]. In addition, the decrease of GMC biological activity can stimulate glomerular endothelial cell apoptosis, affect renal senescence, cause glomerulosclerosis and other pathological conditions [23–24]. After acting on cells, LPS can promote the secretion of various inflammatory cytokines, which is consistent with the pathological manifestation of CGN [25]. Therefore, in

this paper, rat Mesangial HBZY-1 cells were selected and proliferated by LPS to establish a HBZY-1 cell model.

With the discovery of more and more miRNA, people have a deeper understanding of their functions. At present, there are thousands of miRNA, which constitute the most abundant class of gene regulatory system in cells [26]. Most of the miRNA expressions are highly tissue-specific and cell-specific, so they can be used in disease diagnosis and treatment, which has become a new research focus [27, 28, 29, 30]. The main function of miRNA is the post-transcriptional regulation of protein-coding genes, which binds to the target mRNA and inhibits its translation in a sequence-dependent manner [31, 32, 33]. Our group screened a series of glomerulonephritis-related miRNA by consulting the literature, and further predicted through bioinformatics prediction and analysis that there may be a complementary sequence between miR-339-5p and the central gene Syk of glomerulonephritis verified by our group [34], which plays an important role in cell proliferation, apoptosis, inflammation and so on [35]. In this paper, double luciferase experiments showed that there was a targeted binding relationship between miR-339-5p and Syk, and overexpression of miR-339-5p could negatively regulate the relative expression of Syk proteins and genes. Studies have shown that overexpression of miR-339-5p in prostate cancer [36] and breast cancer [37] decreased cell proliferation activity and caused cell-related inflammatory response. This study showed that the expression level of miR-339-5p in HBZY-1 cells decreased significantly, and compared with the model group, overexpression of miR-339-5p significantly decreased cell viability, decreased the level of inflammatory factors in cells, and changed the composition of cell cycle. It significantly increased the G2 phase of the cells. Therefore, we think that the molecular mechanism of miR-339-5p in CGN may be related to the inhibition of cell proliferation and inflammation.

Syk is a kind of intracellular tyrosine protein kinase, which mainly activates downstream signal pathways by phosphorylating substrate proteins involved in signal pathways, promotes the secretion of inflammatory factors and mediates inflammatory immune response [38–39]. Syk has been found to activate inflammatory bodies and promote the release of inflammatory factors in diabetic cardiomyopathy and diabetic nephropathy [40]. In the process of aureococcus aureus infection, Syk is phosphorylated rapidly, which reduces the defense function of the immune system and releases pro-inflammatory factors IL-1β and IL-18 [41]. In addition, Syk is an important part of the middle Syk/Ras/c-Fos signal pathway. Through bioinformatics prediction and analysis in the early stage, our research group found that miR-339 had the highest binding degree with Syk, and studied the influence of Syk/Ras/c-Fos signaling pathway on HBZY-1 cells [20], but did not discuss the pathogenesis of CGN from the genetic level. Therefore, through the establishment of LPS-induced HBZY-1 cell model, this paper expounds the pathogenesis of CGN from the point of view of miR-339-5p and Syk/Ras/c-Fos signal pathway.

In this study, we found that in HBZY-1 cells, the expression of pro-inflammatory factors such as IL-1β, IL-10 and 1L-6 increased, while the expression of anti-inflammatory factor IL-10 decreased. The Syk/Ras/c-Fos signal pathway was activated, and the expression levels of Syk, Ras, MEK1, MEK2, ERK1, ERK2 and c-Fos were significantly increased, and the fluorescence intensity and average optical density in IF were also significantly increased. Overexpression of miR-339-5p can effectively reverse the up-regulated expression

of genes and proteins related to Syk/Ras/c-Fos pathway in chronic glomerulonephritis model established by LPS, and reduce the level of inflammatory factors in HBZY-1 cells. Then we carried out the recovery experiment, and found that Syk inhibitor R406 could reverse the increase of cell viability and the decrease of G2 phase caused by miR-339-5p inhibitor, as well as the increase of inflammatory factors. Based on the findings presented herein, we proposed a schematic representation of the relationship between miR-339-5p and CGN (Fig. 7). In conclusion, miR-339-5p can down-regulate the expression level of Syk in HBZY-1 cells, inhibit the activation of Syk/Ras/c-Fos signaling pathway, decrease the expression of inhibitory inflammatory factors IL-1β, IL-6 and TNF-α in HBZY-1 cells, and increase the expression of inflammatory factor IL-10. Improve the inflammatory state of HBZY-1 cells.

Conclusion

In conclusion, this study suggests that overexpression of Mir-339-5P can regulate the Syk/Ras/c-Fos signaling pathway by targeting Syk, thus reducing the cell viability of LPS-induced HBZY-1 cells, inhibiting the expression of inflammatory factors and inhibiting cell proliferation. It not only provides a new idea for us to further explore the molecular mechanism of CGN, but also provides a new target for the clinical treatment of CGN.

Declarations

Acknowledgements Not applicable.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by HJ, XLZ, XJQ and LBW. The first draft of the manuscript was written by JRG and MMS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability The data used and analyzed to support the findings of this study are available from the corresponding author upon request.

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

Consent to participate Not applicable.

Consent to Publish All authors declare that they have seen and approved the submitted version of this manuscript.

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Tables

Tab.1 primer sequence

Gene	Amplicon Size	Forward primer	Reverse primer
	﹙bp﹚	﹙5'→3'﹚	﹙5'→3'﹚
β-actin	150	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
U6	94	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
miR-339-5p	69	ACACTCCAGCTGGGTCCCTGTCCTCCAGGAG	TGGTGTCGTGGAGTCG
Syk	110	ATGGGAATGAAGTATTTGGAG	GAAAGACCGAAGTCACTGATC
c-Fos	189	CTCTAGTGCCAACTTTATCCC	CCGCCTGACATGGTCTTC
Ras	194	GTGGTAGTTGGAGCTGGTG	TACTGGTCCCTCATTGCA
ERK1	182	CCAGAGTGGCTATCAAGAAGA	CTCCATGAGGTCCTGAACAA
ERK2	167	ATCCCAGCATTCAGAAGTCA	GAGGACCATGTCAGATTCATTT
MEK1	111	TGACGCAGAAGCAGAAGGTG	CAGATGGCTTGTGGGAGACC
MEK2	102	CTCACCATCAACCCTACCATC	GTCCAGCTCTTCCAACTTCTTC
IL-6	98	GCCTTCTTGGGACTGATGTT	ACTGGTCTGTTGTGGGTGGT
IL-10	149	GTCATCGATTTCTCCCCTGT	TTTGAGTGTCACGTAGGCTT
TNF-α	152	GGGCCACCACGCTCTTCTGT	GGCTACGGGCTTGTCACTCG
IL-1β	94	TCCACCTCAATGGACAGAAC	AAGGCCACAGGGATTTTGT

Figures

Figure 1

miR-339-5p was downregulated in model group. The relative expression level of miR-339-5p was measured by qRT-PCR. $^{**}P < 0.01$ vs. Control.

Figure 2

Overexpression of miR-339-5p decreased HBZY-1 cell activity and proliferation. (A) The expression of miR-339-5p was detected by qRT-PCR. (B) CCK-8 was used to detect the activity of HBZY-1 cells in each group.

(C-D) The periodic changes of each group were detected by flow cytometry. $^{**}P < 0.01$ vs. Control, $^{\#\#}P < 0.01$ vs. Model.

Figure 3

miR-339-5p reduces the levels of inflammatory cytokines in HBZY-1 cells. (A-D) The expression levels of IL-1β, IL-6, TNF-α and IL-10 proteins were detected by Western blot. (E-H) The mRNA expression levels of IL-1β, IL-6, TNF-α and IL-10 were detected by qRT-PCR. $^{**}P < 0.01$ vs. Control, $^{\#\#}P < 0.01$ vs. Model, $^{\#}P < 0.05$ vs. Model.

Figure 4

miR-339-5p directly targets Syk and negatively regulates Syk. (A) Binding sequence of miR-339-5p and Syk. (B) Bouble luciferase reporter gene analysis was used to detect the binding of miR-339-5p to Syk. (C-D) The relative protein expression levels of Syk and p-Syk were detected by Western blot. (E) The relative expression level of mRNA in Syk was detected by qRT-PCR. (F-G) The expression of Syk and p-Syk and the average optical density were observed by immunofluorescence (×200). $^{**}P < 0.01$ vs. Control, $^{\#\#}P < 0.01$ vs. Model.

Figure 5

miR-339-5p inhibits the Syk/Ras/ c-Fos signaling pathway. (A) The relative expression levels of Ras, c-Fos, ERK1/2, p-ERK1/2, MEK1/2 and p-MEK1/2 detected by Western blot. (B) The relative mRNA expression levels of Ras, c-Fos, ERK1, ERK2, MEK1 and MEK2 were detected by qRT-PCR. (C) The expression of Ras, c-Fos, ERK1/2 and p-ERK1/2 was observed by immunofluorescence and the average optical density was analyzed (×200). $^{**}P < 0.01$ vs. Control, $^{\#\#}P < 0.01$ vs. Model, $^{\#}P < 0.05$ vs. Model.

Figure 6

Syk downregulation reversed the effects of miR-339-5p on HBZY-1 cells. (A-D) The expression of IL-1β, IL-6, TNF- α and IL-10 protein was detected by Western blot. (E-H) The mRNA expression levels of IL-1 β, IL-6, TNF- α and IL-10 were detected by qRT-PCR. (I-J) flow cytometry was used to detect the cycle changes. (K) CCK-8 was used to detect the viability of HBZY-1 cells. $^{**}P < 0.01$ vs. Control, $^{\#\#}P < 0.01$ vs. Model, $^{*}P < 0.05$ vs. Control. $^{\#}P < 0.05$ vs. Model.

Figure 7

Molecular signaling pathways underlying CGN.

Supplementary Files

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