STUDY ON THE CORRELATION BETWEEN TLRS SIGNALLING PATHWAY-MEDIATED IRAK-M GENE AND SEPSIS

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ABSTRACT

Objective*: To explore the correlation between the interleukin-1 receptor-associated kinase M (IRAK-M) gene mediated by the Toll-like receptor (TLR) signalling pathway and sepsis.*

Methods*: In this study, 68 patients with sepsis treated in our hospital from February 2014 to February 2015 were selected as the study objects (observation group), and 68 normal individuals were selected as the control group. The relationship between IRAK-M gene mediation by the TLR signalling pathway and sepsis was studied by fluorescence quantitative PCR, enzyme-linked immunosorbent assay (ELISA), Western blotting and immunohistochemistry.*

Results*: The mRNA expression levels of TLRs and IRAK-M in blood of patients with sepsis were remarkably increased, with significant differences compared to the normal group (P<0.05). The protein expression levels of TLRs and IRAK-M in the blood of the observation group and control group were detected by ELISA and Western blotting. This study found that the expression of TLRs and the IRAK-M protein in the blood of patients from the observation group (0.96 μg/L, 7.3 μg/L) was significantly higher than in the control group (0.32 μg/L, 0.54 μg/L) and there was a significant difference between the two groups (P<0.05). The immunohistochemical results of the observation group and the control group showed that expression of the IRAK-M gene in the pathological site of the patients in the observation group was significantly increased; the number of positive cells (94.3%) was significantly higher than that in the control group (18.4%) (P<0.05).*

Conclusion: *The IRAK-M gene may be involved in the pathogenesis of sepsis in severely burned patients through the TLR signalling pathway.*

Keywords*: TLR signalling pathway, IRAK-M gene, sepsis, diagnosis.*

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Introduction

Sepsis is a systemic inflammatory reaction caused by infection. It is a common complication caused by trauma, burns, postoperative infection and critical disease[1], which may lead to severe sepsis, septic shock, multiple organ injury and other diseases[2]. Statistical data showed that the incidence of sepsis in China is increasing rapidly, at a rate of nearly 1.2% per year[3]. Therefore, it is of great practical significance to strengthen research into the pathogenesis of sepsis[4]. However, as a systemic inflammatory response caused by infection, the mechanism of sepsis is still unclear.

It has been shown that the research on sepsis is divided into three main stages[5]: the priming stage based on inflammatory reaction, in which many factors that induce inflammatory reaction, such as bacterial infection, virus infection, etc. are studied[6]; the study of inflammatory-related cells and viscosity factors[7]; and the study of genes associated with the inflammatory response. Increasing evidence indicates that the Toll-like receptor (TLR) signalling pathway, as an important signalling pathway related to the body's immune response, is involved in the identification of pathogens and in mediating the inflammatory response before initiating the immune response and many other immune response mechanisms[8, 9].

As a kind of interleukin-1 receptor-associated kinase, IRAK-M is also involved in the immune response to external stimuli[10]. However, there are few reports on the relationship between IRAK-M mediation by the TLR signalling pathway and sepsis. In this study, we studied the correlation between IRAK-M mediation by the TLR signalling pathway and sepsis for the first time in order to reveal the relationship between them and to provide a theoretical and experimental basis for the further diagnosis and treatment of sepsis.

Materials and methods

General information

In this study, 68 patients with sepsis admitted to our hospital from February 2014 to February 2015 were selected as the observation group, including 47 males and 21 females, with an average age of 29.7±18.6 years. Sixty-eight normal individuals were selected as the control group at the same time, and included 34 males and 34 females, with an average age of 28.4±18.3 years. Venous blood (5 mL) was extracted from the observation group and the control group on an empty stomach. After centrifugation at 5000 rpm, the supernatants of both groups were stored at -80°C for further experiments. Meanwhile, the pathological skin tissues of the patients with sepsis and normal skin tissues were sampled for immunohistochemistry. The subjects in the study were not allergic to antibiotics, etc. The procedure was accepted by the patients and their family and approved by the ethics committee.

Methods

The common molecular reagents, RNA extraction kit and reverse transcription kit used in this study were purchased from Takara Biomedical Technology Co., Ltd. (Dalian, China); Immunohistochemical kits were purchased from QIAGEN (Germany) and ELISA kits were purchased from Tiangen Biotechnology Co., Ltd. (Beijing).

Fluorescent quantitative reverse transcript-PCR (qRT-PCR)

Total RNA extraction

Approximately 0.3 g of the research samples were removed from -80°C storage and quickly melted on the prepared ice. After thawing of the samples, 0.35 mL of RNA plus (TAKARA) was added, and the mixture was blended quickly and vigorously. Then, 0.25 mL of RNA plus and 200 μL of chloroform was added, shaken quickly for 15 seconds, and left to stand for 15 minutes at room temperature. The slurry was centrifuged at 12000 rpm for 15 min at 4°C and the supernatants were removed into the RNase-free EP tube. The same amount of isopropanol was added, and the mixture was quickly blended, and then left to stand for 10 minutes at room temperature. The slurry was centrifuged at 12000 rpm for 10 min at 4°C and the supernatants were discarded. Then, 1000 μL of 75% ethanol was added, mixed gently and centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was discard and as much residual ethanol as possible was removed. The quality of the extracted RNA was determined by adding proper amount of RNase-free water, and the rest was used for the qRT-PCR assay.

qRT-PCR

The fluorescent quantitative reverse transcription kit used in the study was purchased from TAKARA; the related operations were performed according to the manufacturer's protocol. The fluorescence quantitative reaction system was as follows: 5 μL of SYBR Premix Ex Taq II (2×), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 1 μL of cDNA, and 3 μL of dH2O. The gene-specific primer sequences for qRT-PCR analyses are shown in Table 1.

Primer	Sequence
TLR-F	AGTCGTGCTGATGCTACG
TLR-R	CGTAGCTGATCGCGATCGAC
IRAK-M-F	TGCTAGCTCGCGTACGTCAGC
IRAK-M-R	CGATCGTAGCCCGTACG
GAPDH-F	TGACTTCAACAGCGACACCCA
GAPDH-R	CACCCTGTTGCTGTAGCCAAA

Table 1: Primers for qRT-PCR.

Enzyme-linked immunosorbent assay (ELISA)

The total protein extracted from the samples of the experimental group and the control group was used as the research object. The expression of NF-Kb and STAT3 in different samples was determined by ELISA, and the operation was carried out according to the instructions supplied with the ELISA kit (QIAGEN, German).

In the study, the standard curve was made with reference to the ELISA standard curve step; that is, the protein samples were diluted in Elution Buffer at a 1:100 dilution; after obtaining different concentrations of diluents, standard curves were made according to the manufacturer's instructions. The study samples of the observation group and the control group were diluted with sterilised PBS (pH 7.2) at a dilution of 1:200 dilution and 100 μL of the test sample was added to the 96-well plate. Then, 50 μL of detection solution was added to each well, and after incubating at room temperature for 2 hours, the TMB chromogenic substrate was added. The absorbance was measured at 495 nm, and the expression levels and concentrations of NF-Kb and STAT3 were calculated according to the standard curve.

Immunohistochemical analysis

The tissues of the experimental and control groups were selected and an aliquot of 10% formaldehyde was added as the immobilisation treatment, before embedding samples in paraffin "Molecular cloning: A laboratory manual. The third edition".

• The thickness of each paraffin slice was about 4μm; the prepared slice was fixed onto the glass slide and baked at 70°C for 1 h.

• After dewaxing with xylene, the sample was eluted with anhydrous alcohol, washed with ultrapure water to remove residual alcohol, and finally washed with PBS (pH7.2) 5 times for 5 minutes each time. Then, samples were placed in the autoclave at 121°C for 2 min; after cooling, the samples were placed in PBS solution at room temperature for 30 min.

• After evaporation of the PBS solution, 50 μL peroxidase blocker was added to the above slices at 37°C for about 10 minutes, and then washed with PBS solution 5 times for 5 minutes each. Then, the PBS solution was removed and 45 μL of the non-immune animal serum was added before incubating at room temperature for 10 min.

• The primary antibody was added to the above slices and incubated at room temperature for about 2 hours (or 4°C overnight), before being washed with PBS solution 5 times for 5 minutes each.

• Next, 50μl of streptomycin was added to the above slices and incubated at 37°C for 2 h; samples were then washed with PBS solution 5 times for 5 minutes each.

• Then, 100 μL of chromogenic solution A was added and the slices were observed under a microscope.

• Slices were washed with distilled water after 10 min and re-stained with haematoxylin for 5 min, before being washed again. Samples were dehydrated and dried with anhydrous alcohol, and sealed with neutral gum.

Western blotting assay

The extraction of total protein from experimental samples stored at -80°C was performed as follows: amounts of 150mg of tissue samples were taken from the -80°C storage; these were ground in a mortar containing liquid nitrogen and collected into 1.5mL EP tubes. To this, 300 μL of protein extract and 10μL of protease inhibitor was added. After incubating in ice water for 30 min, the mixture was centrifuged at 12000 r/min for 15 min and the supernatant was collected and analysed. The detection of protein expression levels by Western blotting: after 10 μL of supernatant and sample buffer was mixed, SDS-PAGE electrophoresis was performed, and then the membrane was routinely transferred. After blocking for 1 hour at room temperature, they were incubated with DDR1 antibody at a 1:250 dilution at 4°C overnight, and then incubated with the secondary antibody labelled with horseradish peroxidase at a 1:250 dilution; the mixture was shaken at room temperature for 1 h. After 3 membrane washes, diaminobenzidine was used for colour development and the Fluorchem 9900 imaging system was used for photographing. The relative protein content of DDR1 was calculated by measuring the integral optical density of each protein band.

Data processing

The experimental data were processed by SPSS 20.0 statistical software and expressed in the form of mean±standard deviation ($\bar{x}\pm s$). Data analysis between different groups was carried out by the single factor analysis method. $P<0.05$ represents a significant difference.

Results

The transcriptional level of TLR and IRAK-M in the observation and control groups

Total RNAs were extracted from normal and septic samples. The relative expression of TLRs and IRAK-M mRNA was determined by fluores-

cence quantitative PCR. As shown in Fig. 1, the results of RNA extraction showed that the RNA had three distinct bands; the brightness of the 28S band was about twice that of the 18S band, which indicated that the extracted RNA can be used in further quantitative experiments. The fluorescence quantitative PCR results are shown in Fig. 2.

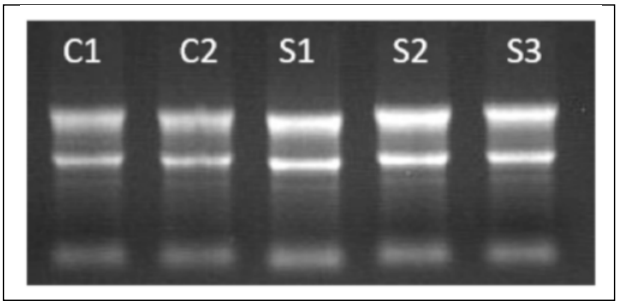

Fig. 1: Electropherogram of RNA extraction from different tissues. C1 and C2 represent RNA extracted from the control group; S1, S2 and S3 represent RNA extracted from the pathological tissues of the observation group.

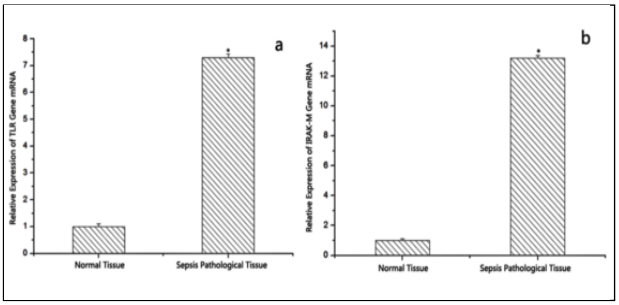

Fig. 2: The expression level of TLR and IRAK-M gene mRNA in the observation group and control group. a: mRNA expression level of the TLR gene; b: mRNA expression level of the IRAK-M gene. Asterisks (*) indicate significant difference between two groups (P<0.05).

It can be seen from Fig. 2a that the transcriptional level of TLR in patients with sepsis was significantly higher than in the normal control group and that there was a significant difference between the two groups (P<0.05). Fig. 2b shows the relative expression level of IRAK-M mRNA in the normal group and in patients with sepsis. As shown in Fig. 2b, the relative expression of IRAK-M gene mRNA in sepsis patients is significantly higher than that in the normal control group, with a significant difference between the two groups (P<0.05).

Detection of TLRs and IRAK-M protein expression levels in the observation and control groups by ELISA

The total protein was extracted from the control and observation groups. The protein expression level of TLRs and IRAK-M was determined by ELISA and the results are shown in Fig. 3. It can be seen from Fig. 3a that the protein expression level of TLR in patients with sepsis (0.96 μg/L) was significantly higher than that in the normal control group (0.32 μg/L), with a significant difference between the two groups (P<0.05). Fig. 3b showed the relative expression level of the IRAK-M protein in the normal group and in patients with sepsis. As shown in Fig. 3b, the relative expression level of the IRAK-M protein in sepsis patients (7.3 μg/L) is significantly higher than that in the normal control group (0.54 μg/L), with a significant difference between the two groups (P<0.05).

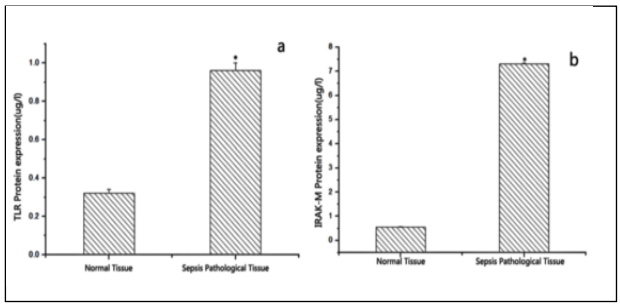

Fig. 3: The expression levels of TLR and IRAK-M proteins in the observation and control groups. a: TLR protein expression levels; b: IRAK-M protein expression levels. Asterisks (*) indicate significant difference between two groups (P<0.05).

Detection of TLRs and IRAK-M protein expression level in the observation and control groups by Western blotting assay

The total protein was extracted from the tissue samples of the normal individuals and patients with sepsis using the animal cell total protein extraction kit. The protein expression level of TLRs and IRAK-M was determined by western blotting; the results are shown in Fig. 4.

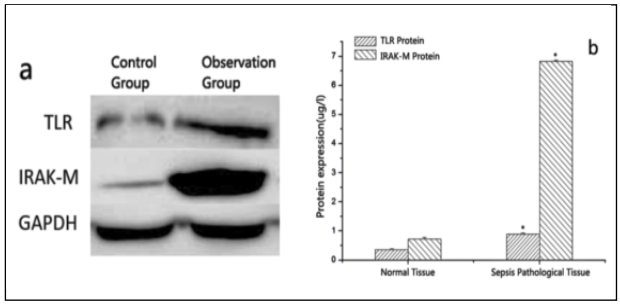

Fig. 4: The expression level of the TLR and IRAK-M proteins in the observation and control groups. a: TLR protein expression level; b: IRAK-M protein expression level. Asterisks (*) indicate significant difference between two groups (P<0.05).

It can be seen from Fig. 4a that the expression levels of TLR and IRAK-M proteins in patients with sepsis was significantly higher than that in the normal control group. The quantitative results in Fig. 4b also showed that the expression level of the TLR and IRAK-M proteins in patients with sepsis was higher than that in normal subjects (P<0.05).

Immunohistochemical results of the IRAK-M gene in the observation and control groups

In the immunohistochemical experiment, we selected the pathological tissues of patients with sepsis, normal tissues of patients with sepsis and normal skin tissues of normal individuals as the research objects; the expression level of IRAK-M was measured.

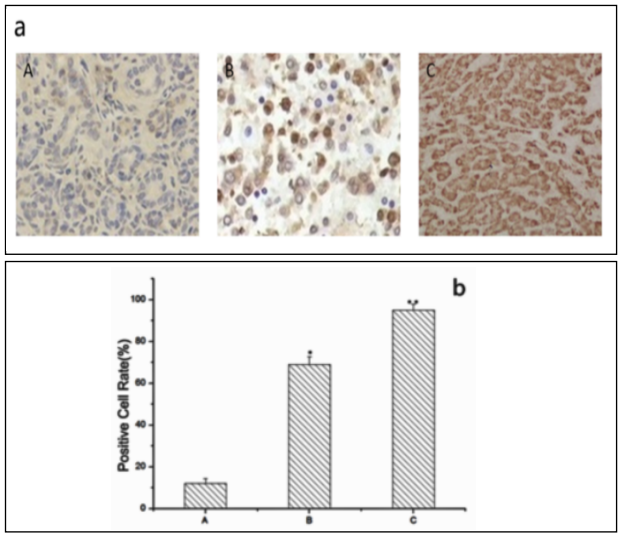

Fig. 3: The results of IRAK-M gene immunohistochemistry and the number of IRAK-M-positive cells in the observation and control groups. A: Normal tissues from a normal individual; B: Normal tissues of patients with sepsis; C: Pathological tissues of patients with sepsis. a: Immunohistochemical results; b: Results of positive cell counts. Asterisks (*) indicate significant difference between the two groups (P<0.05).

As shown in Fig. 5a, the expression level of IRAK-M was the highest in sepsis tissues, followed by in the normal skin tissues of septic patients, while it was the lowest in normal skin tissues. The positive rate of IRAK-M in the above three types of study samples is shown in Fig. 5b. The positive rate of IRAK-M in the pathological tissues of patients with sepsis was the highest, and there was a significant difference between the pathological tissues and the other two groups (P<0.05). This was followed by the normal skin tissues of patients with sepsis, and there was also a significant difference in the number of IRAK-M positive cells compared to in normal skin tissues from normal individuals (P<0.05).

Discussion

It has been demonstrated that the TLR signalling pathway plays an important role in mediating the invasion of exogenous pathogens[11], inducing monocytes, macrophages and lymphocyte proliferation[10]. For example, the TLR signalling pathway can induce the above-mentioned immune system to produce and activate IL-1 and TNF-α inflammatory factors[12], thus promoting the immune response mechanism of the body[13]. The results showed that the expression of TLR and TLR2 mRNA in the serum of mice treated with lipopolysaccharide (LPS) for 24 hours was significantly increased. It has been shown that the expression of TLR and TLR2 mRNA in the serum of mice treated with lipopolysaccharide (LPS) for 24 hours was increased significantly[14].

Clinical studies have shown that lipopolysaccharides can also induce the increased expression of TLR4 and TLR2 in monocytes[15]. Meanwhile, genetic polymorphism of TLR2, such as the Arg753Gln, Cys159Thr, etc. mutations can lead to different resistances to sepsis in different patients to some extent[16, 17].

In this study, we found that the expression of key proteins in the TLRs signalling pathway in sepsis tissues increased in comparison with the normal tissue. However, comparing the expression of key proteins in the TLR signalling pathway in normal tissues of patients with sepsis and normal tissues of normal individuals, it was found that the expression of key proteins in the TLR signalling pathway was also increased in the normal tissues of patients with sepsis; there was a significant difference between the two groups (P<0.05). These results indicate that activation of the TLR signalling pathway induced by sepsis is not only confined to the lesion tissues, but can also significantly promote activation of the TLR signalling pathway in the normal tissues of patients with sepsis, thus enhancing their immune ability to sepsis and other inflammation.

As the main immune proteins of cells deal with foreign invading pathogens, it has been shown that IRAK-M participates in the treatment of related inflammatory reactions through the TLR signalling pathway in influenza and other inflam-

matory responses[18]. However, the correlation between IRAK-M and the TLR signalling pathway in sepsis is rarely reported. In this study, it was indicated for the first time that IRAK-M can participate in the degradation progression of sepsis through the TLR signalling pathway. However, it is not clear how the TLR signalling pathway activates IRAK-M and promotes its related immune activity. It has been shown that IRAK-M can inhibit the phosphorylation of IRAK-I and prevent it from binding to other factors, thus promoting its interaction with related proteins such as the SOCS1 protein and participating in the immune responses[19].

References

1) Roland CL, May CD, Watson KL, Sannaa GAA, Dineen SP, et al. Analysis of Clinical and Molecular Factors Impacting Oncologic Outcomes in Undifferentiated Pleomorphic Sarcoma. Ann Surg Oncol 2016; 23: 2220-2228.
2) Roland CL, May CD, Watson KL, Sannaa GAA, Dineen SP, et al. Analysis of Clinical and Molecular Factors Impacting Oncologic Outcomes in Undifferentiated Pleomorphic Sarcoma. Ann Surg Oncol 2016; 23: 2220-2228.
3) Yu D, Tan Y, Chakraborty M, Tomchik S, Davis RL. Elongator complex is required for long-term olfactory memory formation in Drosophila. Learn Mem 2018; 25: 183.
4) Itzhak VB, Tsilia L, Dror M, Ronit Z, Elizabeth T, et al. Usefulness of Serum Procalcitonin as a Marker for Coexisting Infection in Patients with Acute Myocardial Infarction. Am J Cardiol 2018.
5) Wang Y, Xintai Peoples Hospital. Analyzing the Determination Results of D-Dimer and Hemorheology for COPD Patients in Acute Aggravating Period. Chin Continuing Med Educ 2017.
6) Barbera B A, Emery J L, Recino A, F SW, Anne C, et al. Inhibition of Phosphoinositide 3-Kinase p110delta Does Not Affect T Cell Driven Development of Type 1 Diabetes Despite Significant Effects on Cytokine Production. Plos One 2016; 11: 0146516.
7) Camps-Bossacoma M, Pérez-Cano F J, Franch À, Untersmayr E, Castell M, et al. Effect of a cocoa diet on the small intestine and gut-associated lymphoid tissue composition in an oral sensitization model in rats. J Nutr Biochem 2017; 42: 182-193.
8) Long H, Prescott S, Wysocka J. Ever-Changing Landscapes: Transcriptional Enhancers in Development and Evolution. Cell 2016; 167: 1170-1187.
9) Zhu Y, Zhang M, Ouyang M, Zhou D, Li L. Roles of MyD88 and TRIF in cardiac dysfunction during sepsis. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 2017; 29: 684.
10) Wolf D, Barreras H, Bader C S, Copsel S, Lightbourn CO, et al. Marked in vivo Donor Treg Expansion via IL-2 and TL1A-Ig Stimulation Ameliorates GVHD but Preserves GVL in Recipients Post-HSCT. Biol Blood Marrow Transplant 2017; 23.
11) Lo J, Xia C Q, Peng R, Salzler MJC. Immature Dendritic Cell Therapy Confers Durable Immune Modulation in an Antigen-Dependent and Antigen-Independent Manner in Nonobese Diabetic Mice. J Immunol Res 2018; 2018:1-13.
12) Chu Q, Gao Y, Bi D, Xu T. MicroRNA-148 as a negative regulator of the common TLR adaptor mediates inflammatory response in teleost fish. Sci Rep 2017; 7: 4124.
13) Kawasaki Y, Maeda R, Kanno S, Suzuki Y, Ohara S, et al. Long-term follow-up outcome of pediatric IgA nephropathy treated with tonsillectomy plus methylprednisolone pulse therapy. Pediatr Int 2017; 59: 41-47.
14) Yao C, Dan W, Wang B, Li H, Xiong J, et al. HMGB1 translocation and release mediate cigarette smoke–induced pulmonary inflammation in mice through a TLR4/MyD88-dependent signaling pathway:. Mol Biol Cell 2017; 28: 201-209.
15) Yujuan D, Junhong Z, Chung-Wah W, Lijing Z, Xiaodong L, et al. Tumour suppressor functions of miR-133a in colorectal cancer. Mol Cancer Res 2013; 11: 1051-1069.
16) Vahed S Z, Omidi Y, Ardalan M, Samadi N. Dysregulation of urinary miR-21 and miR-200b associated with interstitial fibrosis and tubular atrophy (IFTA) in renal transplant recipients. Clin Biochem 2016; 50: 32.
17) Tirandaz N, Dehghan M, Valipour MS. Heat and fluid flow through a helical annulus enhanced by a porous material: A perturbation study. Appl Therm Eng 2017; 112: 1566-1574.
18) Maria ED, Elisa L, Licia P. TOll-like receptors, immunoproteasome and regulatory T cells in children with Henoch-Schonlein purpura and primary IgA nephropathy. Ped Nephrol 2014; 29: 1545-1551.
19) Shen P, Shen J, Sun C, Yang X, He L. A system biology approach to understanding the molecular mechanisms of Gubentongluo decoction acting on IgA Nephropathy. BMC Complement Altern Med 2016; 16: 312.

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