

## Lentiviral mediated overexpression of NR4A1 in mononuclear cells ameliorates lupus nephritis

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**Abstract:** Objective: to investigate the mechanism of lentivirus-mediated overexpression of NR4A1 in the alleviation of lupus nephritis in lupus mice. Methods: Ten male MRL/lpr mice (LN group) and ten CS7BL mice (normal control) aged 4 months were selected. The expression level of NR4A1 in peripheral blood of two groups was compared, and the pathological changes of kidney tissue were compared. The expression level of NR4A1 in peripheral blood of LN mice with different severity of illness was compared, and the overexpression of NR4A1 inhibited cell proliferation activity. Results: Among MRL/lpr mice, type IV LN mice were the most, accounting for 7 cases, the others were type II, type III and type V respectively. The renal tubulointerstitial lesions of CS7BL mice and 2 non-IV LN mice were mild. The expression of NR4A1 in peripheral blood of active LN mice was significantly lower than that of stable LN mice, and the difference was statistically significant ( $p < 0.05$ ). At 48h after transfection, the proliferation activity of monocytes in transfected group was lower than that in untransfected group. At 72h after transfection, the cell activity of transfected group was still lower than that of untransfected group. Conclusion: Overexpression of NR4A1 monocytes can help alleviate lupus nephritis in lupus mice, and is expected to be a molecular marker for predicting the pathogenesis and progression of lupus nephritis.

### 1. Introduction

Lupus nephritis (LN) is one of the most common organ manifestations of systemic lupus erythematosus (SLE), which constitutes the main risk for morbidity and mortality of SLE patients. The exact etiology and pathogenesis of SLE are still unclear. The pathogenesis of SLE is very complex, which is generally considered to be related to a variety of factors including epigenetic, sex hormones, viral infection, environmental and immune regulatory factors [1]. Pathologically, almost all SLE patients have renal involvement. The severity of kidney disease was associated with the prognosis of SLE [1]. SLE is an autoimmune disease characterized by polyclonal lymphocyte activation, autoantibody production, and increased release of inflammatory cytokines. In renal tissues of SLE, the increased monocyte infiltration is common in most glomerulonephritis and tubulointerstitial lesions. Activated monocytes cause renal lesions by secreting cytokines, inflammatory mediators and producing oxygen free radicals, and contribute to glomerular sclerosis and tubulointerstitial fibrosis.

NR4A1 (Nuclear receptor subfamily 4, group A-1, NR4A1), a member of nuclear hormone receptor superfamily, can participate in a series of physiological and pathological processes such as regulating cell biological behavior, inflammatory response, stress response, energy metabolism, abnormal accumulation of blood lipids, and tumor angiogenesis[2]. Previous studies have shown that NR4A1 is abnormally expressed in lupus nephritis patients, but its specific correlation is not clear [3]. In this study, lentivirus was used as an intermediary to independently verify the selected NR4A1, and the correlation between the expression level of NR4A1 and its target molecules and

disease activity, immunological indexes and renal injury indexes was analyzed, and the possible role of nr4a1 in the pathogenesis of LN was explored, which provided theoretical basis for seeking biomarkers for diagnosis and potential targets for treatment of LN.

Epigenetic factors play an important role in the pathogenesis of SLE. With the development of genome sequencing and microarray technology, studies have found that non-coding RNA represented by micro-RNA (miRNA), long non-coding RNA (LNC RNA) and cyclic RNA (CIRCULAR RNA) plays an important role in the occurrence and development of disease [3]. At present, there is little research on ceRNA in LN in the literature both in China and abroad, based on the view that circ RNA can participate in the pathogenesis of LN as a target gene that ce RNA competitively binds to miRNA [4–6].

NR4A1, a member of nuclear hormone receptor superfamily, can participate in a series of physiological and pathological processes such as regulating cell biological behavior, inflammatory response, stress response, energy metabolism, abnormal accumulation of blood lipids, and tumor angiogenesis. Previous studies have shown that NR4A1 is abnormally expressed in lupus nephritis patients, but its specific correlation is not clear. In this study, lentivirus was used as an intermediary to independently verify the selected NR4A1, and the correlation between the expression level of NR4A1 and its target molecules and disease activity, immunological indexes and renal injury indexes was analyzed, and the possible role of nr4a1 in the pathogenesis of LN was explored, which provided theoretical basis for seeking biomarkers for diagnosis and potential targets for treatment of LN [7].

Using lentivirus as the mediator, we independently verified selected NR4A1, analyzed the correlation between the expression levels of NR4A1 and its target molecules and disease activity, immunological indicators and kidney injury indicators, and explored its possible role in the pathogenesis pathway of LN, in order to provide a theoretical basis for seeking biomarkers for the diagnosis of LN and potential targets for treatment.

## **2. Materials and methods**

### **2.1. Animal**

Ten four-month-old male MRL/lpr mice (LN group), weighing about 25g–28 g, and 10 four-month-old CS7BL mice (normal control), weighing about 22g–25 g, were introduced from Shanghai Slack Experimental Animal Co., Ltd. and reared in the clean animal room of the Animal Experimental Center of Medical College.

### **2.2. Research technique**

#### **2.2.1. Pathological diagnosis of renal tissue**

The pathological types of LN were classified into six according to the WHO classification criteria. The degree of interstitial lesions is divided into mild, light–moderate, moderate and severe according to the degree of interstitial cell infiltration and interstitial fibrosis. The degree of interstitial fibrosis is mild when it is less than 25%, mild–moderate when it is 25%–50%, moderate when it is 50%–75%, and severe interstitial lesions when it is higher than 75%.

#### **2.2.2. Image analysis**

Five glomeruli were randomly selected for each section. The ratio of the IOD (Integrated Optical Density) of the antibody-positive region in the transverse section of each glomeruli to the transverse section area of glomeruli was calculated, and then the mean value, i.e., the mean optical density, was calculated as the comparison index. Twenty high-power fields were randomly selected from the tubulointerstitial area of each section that did not contain glomeruli and blood vessels. The ratio of the IOD in the antibody-positive staining area of the selected area to the whole field area was calculated (the area occupied by the removal of renal blood vessels and renal tubular lumen). The average value, i.e., the average optical density, was then calculated as the comparison index.

### 2.2.3. Quantitative analysis

According to the amplification curve, the number of cycles (Ct) was read, GAPDH was used as an internal reference gene, and the expression level of NR4A1 was analyzed by  $2^{-\Delta\Delta C_t}$  with relative quantitative method. The NanoDrop2000 spectrophotometer was used to detect the concentration and purity of samples: the concentration was  $\geq 50\text{ng}/\mu\text{L}$ , and the total amount was  $\geq 1\text{ }\mu\text{g}$ ; The OD260/280 is between 1.8 and 2.2. The completeness of agarose gel electrophoresis requires that the 28S/18S band be clearly visible without obvious degradation.

Procedures were performed by the same physician in strict accordance with the instructions. The expression levels of NR4A1 in peripheral blood of two groups were compared, and the expression levels of NR4A1 in peripheral blood of LN mice with different severity levels were compared. The correlation between NR4A1 and the occurrence and severity of LN was analyzed, as well as the predictive value of NR4A1 for LN.

### 2.2.4. Packaging, production and titer determination of lentivirus

After 24 hours, the cells were in good condition and suitable for transfection. The cells were cotransfected with 20  $\mu\text{L}$  lentivirus packaging plasmid mixture (500 ng /  $\mu\text{L}$ ) and recombinant plasmid, and packaged according to the virus packaging instructions; The cell supernatant was collected after 48 h, and the titer of recombinant lentivirus was determined by gradient dilution method. 223 cells were inoculated into 86-well plates from  $4 \times 10^3$  cells per well, and 100  $\mu\text{L}$  cell suspension was added into each well. Ten sterile centrifuge tubes were collected after 24 h, and 90  $\mu\text{L}$  fresh culture medium free of antibiotics was added into each tube. Then 10  $\mu\text{L}$  of the stock solution of the virus to be tested was added into the first tube, and after uniform mixing, 10  $\mu\text{L}$  was added into the second tube, and so on to the last tube. After they were infected with 283 cells respectively, fluorescence cell count was performed under a microscope, and the titer value of the virus stock solution was obtained by dividing the obtained value by the corresponding dilution multiple. Subpackaging the virus solution  $-60^\circ\text{C}$ , and the collected virus was named LV-NR4A1.

### 2.2.5. Statistical treatment

Counting data is expressed as (n), which is tested by  $\chi^2$ . The measurement data is expressed by  $\bar{x} \pm s$ , and the comparison between groups is tested by t; The correlation was analyzed by linear regression.  $P < 0.05$  means significant difference, and  $P < 0.01$  means extremely significant difference.

## 3. Result

### 3.1. Pathological changes of renal tissue

According to the WHO classification standard of lupus nephritis, among the MRL/lpr mice, the type IV LN mice were the most in number, accounting for 7 cases, and the rest were respectively type II and type III, 1 case each, and type V, 1 case. The renal tubulointerstitial lesions in the two non-IV LN mice of CS7BL were mild, and the interstitial lesions in two of the eight IV LN mice were mild. Three cases were between mild to moderate; one case was moderate; two cases were more severe.

### 3.2. Expression levels of NR4A1 in peripheral blood of LN mice is associated with different disease severity

Table 1 Expression of NR4A1 in peripheral blood of LN mice is associated with different severity of disease ( $\bar{x} \pm s$ )

Severity of illness	Number of mice	NR4A1
stationary phase	10	$0.07 \pm 0.02$
active stage	10	$0.04 \pm 0.04$
t value	-	8.362
P value	-	$< 0.001$

The expression of NR4A1 in peripheral blood of active LN mice was significantly lower than that of stable LN mice, and the difference was statistically significant ( $P < 0.05$ ) (Table 1).

### 3.3. Overexpression of NR4A1 inhibits cell proliferative activity

The effect of LV-NR4A1 infection on the proliferation and activity of monocytes was detected rapidly by CCK-8. The results are shown in Fig. 1. At 48h after transfection, the proliferation activity of monocytes in the transfected group was lower than that in the monocyte non-transfected group. The cell activity of the transfected group was still lower than that of the non-transfected group at 72h after transfection. These results indicated that overexpression of NR4A1 could inhibit the proliferative activity of monocytes.

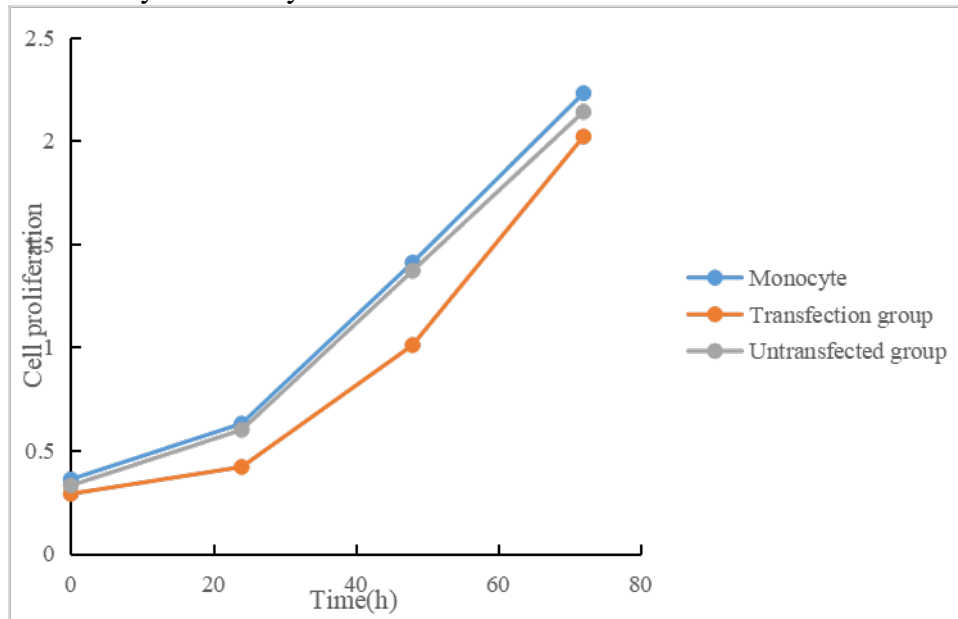


Figure 1 Effect of NR4A1 overexpression on cell proliferation inhibition

## 4. Discussion

SLE is a common autoimmune disease, and the lesions can affect tissues and organs throughout the body, with kidney involvement being the most common. LN is one of the most important organ damage and the main cause of death in SLE, and its immunological characteristics are mainly the production of polyclonal lymphocyte activated autoantibody and the increased release of inflammatory cytokines. The infiltration and activation of inflammatory cells in the glomeruli is an important feature of the progression of LN, and chemokines play a vital role in regulating the infiltration of inflammatory cells into renal tissues and are closely related to the severity of kidney disease.

Glomerular mesangial cells, tubular epithelial cells, vascular smooth muscle cells, and renal tissue-infiltrating cells in renal tissue may all produce NR4A1 and participate in the infiltration of inflammatory cells. High-dose immunosuppressive agents may affect the expression of NR4A1 in renal tissue under pathological conditions. It has been reported in the literature [8] that NR4A1 in the renal tissue of LN mice treated with CTX pulse therapy was decreased. In LN mice, urine NR4A1 excretion was also significantly reduced after methylprednisolone pulse therapy [9]. In our study, all 18 mice showed varying degrees of NR4A1 expression in renal interstitium, which was mainly distributed in tubular epithelial cells and interstitial small vessel walls, but NR4A1 staining was not positive in interstitial infiltrating cells. The above results indicated that in LN mice, the production of renal interstitium NR4A1 was mainly derived from tubular epithelial cells and renal interstitium vessel wall cells.

Nr4A1, a member of NHRS superfamily, is a bidirectional transcription regulator with transcriptional activation and transcriptional inhibition. It is located on the 12q13 chromosome and

widely distributed in different cell types. It is able to participate in the regulation of a series of physiological and pathological processes such as cell biological behavior, inflammatory response, stress response, energy metabolism, atherosclerosis, vascular endothelial cell injury, dyslipidemia, tumor angiogenesis, and cardiovascular disease occurrence [10–11]. Some study [12] has shown that NR4A1 can affect or aggravate the occurrence and progression of diabetic nephropathy. This study showed that the expression level of NR4A1 in the peripheral blood of the normal control group was significantly lower than that of the LN group, and the expression level of NR4A1 in the peripheral blood of the LN group was significantly lower than that of the normal control group, suggesting that NR4A1 was low expressed in the peripheral blood of mice with lupus nephritis. This study showed that the expression level of NR4A1 in the peripheral blood of active LN mice was significantly lower than that of stable LN mice, suggesting that the lower the expression level of NR4A1 in the peripheral blood, the more serious the disease of lupus nephritis mice would be.

In this experiment, the NR4A1 is efficiently transfected and the expression level in mononuclear cells is increased by the method of lentivirus infection of cells; the system has the characteristics of stable transfection, high infection efficiency, capability of effectively infecting non-periodic cells and cells after mitosis, and increased biological safety; and the carrier is provided with a fluorescent marker, so that the detection is easier. In this experiment, the target gene NR4A1 was correctly inserted into the lentivirus expression vector and effectively overexpressed NR4A1 in monocytes. At the same time, it was verified that overexpression of NR4A1 gene could inhibit monocyte activity and promote its apoptosis.

## 5. Conclusions

In summary, Overexpression of NR4A1 monocytes can help alleviate lupus nephritis in lupus mice, and is expected to be a molecular marker for predicting the pathogenesis and progression of lupus nephritis. However, its specific mechanism needs further study.

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