Original Article
Mitofusin 2 expression in IgA1 induced rat glomerular mesangial cells and IgA nephrology animal model

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Abstract: Aim: The present study is aimed to observe the relationship between glomerular mesangial cell (GMC) proliferation and the expression of Mitofusin 2 (Mfn2), then to further confirm the underlying signal pathway take part in regulating mesangial cell proliferation in IgA nephropathy (IgAN). Methods: GMCs stimulated with IgA1 were cultured *in vitro*. To detect cell proliferation, the Cell Counting Kit-8 Cell activity assay (CCK8) was used to determine the best induction time of IgA1. Concentration-dependent changes in Mfn2 and relevant proteins (Bcl-2, caspase 3, p-Akt) induced by IgA1 were detected by western-blot and real-time PCR. Making IgAN animal model, detected IgA deposition, GMCs proliferation by immunofluorescence and light microscope in HE stain. Western-blot and immunohistochemical is used for detecting the expression of Mfn2 and relevant protein. Results: IgA1 induced GMCs proliferation in a concentration-dependent manner. Along with GMCs proliferation in vivo or in vitro, Mfn2 expression obviously decrease. Changes in Mfn2 expression accompany with down-regulated caspase 3 expression up-regulate Bcl-2 and p-Akt. Conclusions: In IgAN, GMCs proliferation is associated with Mfn2 abnormal expression this mechanism may be related to activation of Ras-PI3k/Akt signal pathways. These findings may provide a new direction for the mechanism of GMCs proliferation in IgAN.

Keywords: IgAN, Mfn2, Bcl-2, caspase-3, p-Akt

Introduction

IgA nephropathy (IgAN), the most common form of primary glomerulonephritis, is characterized by predominant deposition of IgA in the renal mesangium [1, 2]. Nearly 25-30% of all patients go on to developed-stage renal disease. The disease is characterized by mesangial deposition of polymeric IgA1 (pIgA) [3], followed by proliferation of mesangial cells, increased synthesis of extracellular matrix, and infiltration of immune cells [4]. Thus, inhibition of mesangial cell proliferation plays an important role in alleviating glomerular sclerosis and delaying the progression of disease.

Mitofusin 2 (Mfn2) is a recently discovered multifunctional protein widely expressed in the heart, skeletal muscle, liver, brain, kidney, and other tissues and organs [5, 6]. In 2004, Chen et al. demonstrated that Mfn2 was a powerful regulator of cell proliferation *in vivo* and *in vitro*. It is an endogenous Ras inhibitor; overexpres-

sion of rat Mfn2 overtly suppresses the mitogenic stimulus-evoked vascular smooth muscle cell (VSMC) proliferation in culture and blocks balloon injury-induced restenosis *in vivo* via inhibiting the Ras-Raf-MEK-ERK/MAPK signaling pathway [5]. In 2007, Chen et al. further demonstrated that Mfn2 displays a profound proapoptotic effect in VSMCs. Upregulation of rMfn-2 triggers marked apoptosis in cultured VSMCs via inhibition of the Ras-PI3K-Akt cell survival pathway and subsequent activation of the mitochondrial death pathway. Notably, the profound anti-proliferative and proapoptotic effect of Mfn2 is independent of its functional role in mitochondrial fusion [7]. Bach et al. reported that the expression of Mfn2 was decreased in diabetic patients [8]. Tang et al. demonstrated that Mfn2 expression was inhibited significantly in diabetic rats and further showed that Mfn2 overexpression was associated with decreased kidney volume (as indicated by KW/BW) and reduced proteinuria and ACR in diabetic rats, indicating that Mfn2 slo-

wed the progression of diabetic nephropathy (DN) and suggesting that it might be a potential therapeutic target for the treatment of early-stage DN [9]. It was also reported that abnormal expression of Mfn2 occurs in high glucose-induced rat glomerular mesangial cells, while overexpressed Mfn2 could alleviate glomerular mesangial cell (GMC) proliferation and increase apoptosis, and that Mfn2 may exert its activities through the MAPK/ERK and PI3K/Akt signaling pathways in connection with proliferation and apoptosis.

Given this background, we conclude that Mfn2 is associated with the proliferation and apoptosis of GMCs in DN, while studies on the relationship between Mfn2 and IgAN are limited. Therefore, we sought to investigate the effects of Mfn2 on the GMC proliferation and apoptosis in IgAN and to explore whether Mfn2 is a new therapeutic target for IgAN.

Methods

Cell culture

The rat GMC strain was provided by the China Center for Culture Collection (Peking Union Medical College, Peking, China). The cells were cultured in minimal essential medium (MEM) containing Earle's balanced salt solution (MEM/EBSS) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), streptomycin (100 µg/mL), and penicillin (100 IU/mL) at 37°C in 95% air/5% CO_2.

Cell proliferation analyses

Cells were seeded in 96-well plates with a final total volume of 100 µL/well, and were treated with different interventions for 48 h. Then, 10 µL of Cell Counting Kit-8 (CCK8) solution was added to each well. The plates were incubated at 37°C for 1.5 h in the dark. The absorbance at 450 nm was measured using a microplate reader (Omega), and cell proliferation was calculated using the following equation: cell proliferation rate = (meanA_{450} of interfered cells/mean A_{450} of non-interfered cells) ×100%. To induce the proliferation of GMCs, cells were incubated with IgA1 as a test group. According to the results of the CCK8 assay, we defined the optimal time IgA1 for culture with GMC to be 24 h.

Real-time quantitative PCR

Mfn2 RNA was extracted from GMCs using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. After DNase treatment (Promega, Madison, WI, USA), RNA was reverse-transcribed to first-strand cDNA using a RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). Cycling and real-time detection were performed with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were designed using Vector NTI (Invitrogen). The primers for Mfn2 were: forward GAGTGTCAAGACCGTGAACCA and reverse CATCCAGGCAAAACTTATCAATCCA, and for GAPDH: forward CAACGGGAAACCCATCACCA and reverse ACGCCAGTAGACTCCACGACAT.

Transcription abundance was expressed as fold increase over a control value calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Antibodies against Mfn2 and GAPHD were obtained from Sigma (St. Louis, MO, USA). Antibodies against caspase-3, Bcl-2, Mfn2 and P-Akt were obtained from Cell Signaling Technologies (Danvers, MA, USA). The Mfn2, Bcl-2, caspase-3, and p-Akt protein levels were examined by Western blotting. Total protein was isolated from cells and tissues after 30 min on ice. After centrifugation (12,000 rpm, 15 min, 4°C), the supernatant was collected, and the protein concentration was measured using the bicinchoninic acid protein assay (Thermo Scientific, Waltham, MA, USA). Equal amounts of protein (50 µg) were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking non-specific binding sites for 3 h with 5% non-fat milk, the membranes were incubated overnight on ice with primary antibodies against Mfn2 (1:500), caspase-3 (1:400), p-Akt (1:400), Bcl-2 (1:500), and GAPHD (1:250). After washing, the membranes were probed with appropriate secondary antibodies for 90 min on ice. Then, membrane enhanced chemiluminescence was conducted using a chemiluminescence detection kit.

IgAN animal model

Twenty-five healthy male SD rats weighing 180-220 g (200 ± 15 g) were purchased from the experimental animal center of Zhejiang Chinese
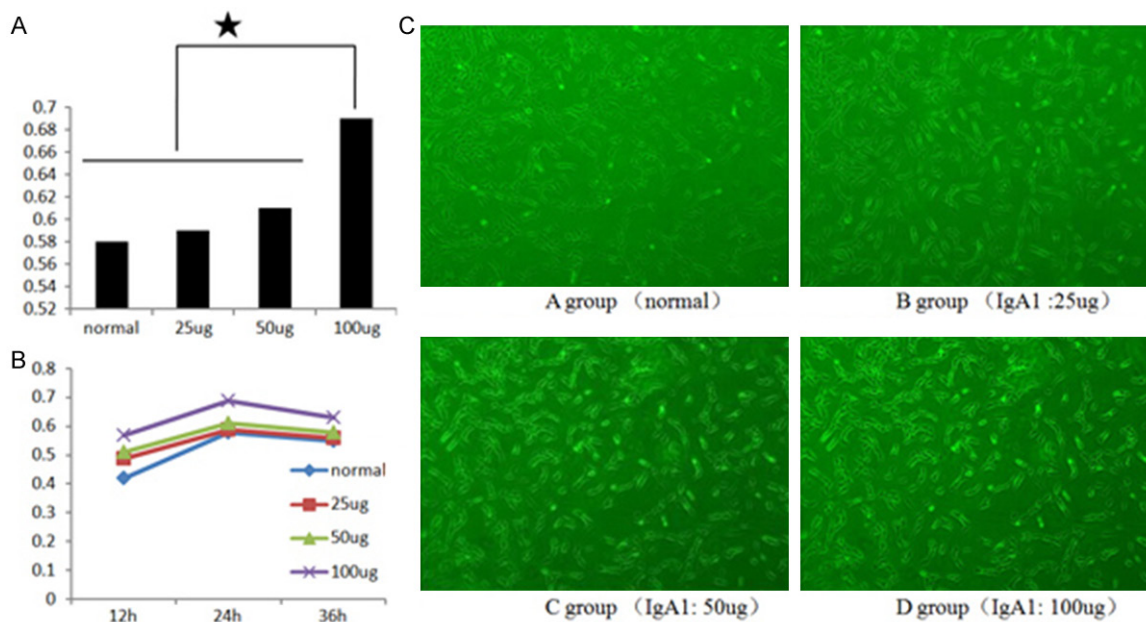
Figure 1. IgA1 induced GMCs proliferation in different concentration at different time. A: IgA1-induced GMCs proliferation in different concentration, ★Compared with D group (IgA1 in 100 ug), P<0.05. B: IgA1 induced GMCs for different time. C: ×200 GMCs proliferation.

medical university. The rats were randomly divided into an IgAN group (N = 21) and normal control group (N = 10). Rats in the model group were administered 200 mg/kg bovine serum albumin by gavage every other day for 12 weeks. Additionally, 0.05 mg lipopolysaccharide was injected into the caudal vein during the 6th and 8th weeks. Twice per week, 0.1 ml carbon tetrachloride and 0.5 mL castor oil were subcutaneously injected, totally for 12 weeks. Rats in the control group were administered sterile distilled water by gavage. An equal volume of saline was injected into the caudal vein and subcutaneous.

Renal pathology and immunohistochemical detection

Immunofluorescence and optical microscopy were used to examine renal tissue. Kidney slices were fixed in 10% formalin, embedded in paraffin wax, cut into 5 μm sections and stained with hematoxylin and eosin. The tissues were evaluated by light microscopy. The other portion of each fixed specimen was used for immunofluorescence detection of IgA.

For immunohistochemical stains, in brief, sections were rehydrated and antigens retrieved using heated citrate. After the incubation with blocking buffers, tissue sections were exposed

sequentially to the primary antibody, the horseradish peroxidase-coupled secondary antibodies. The signals were developed with DAB Peroxidase Substrate Kit (Vector Laboratories). All immunohistochemical analyses were repeated at least three times and representative images were presented.

Statistical analyses

Data are reported as means ± SD. The densitometry analysis for protein and mRNA levels was performed using the Image-Pro Plus software (ver. 5.0.2; Media Cybernetics, Bethesda, MD, USA). Statistically significant differences among the three groups were analyzed by one-way analysis of variance with the Student-Newman-Keuls test applied for multiple comparisons. Statistical analyses were performed using the SPSS for Windows software (ver. 19.0; SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were deemed to indicate statistical significance.

Result

IgA1 promoted the proliferation of GMCs in a concentration and time dependent manner

The proliferation of GMCs were detected by CCK8. We stimulated GMCs with IgA1 in differ-
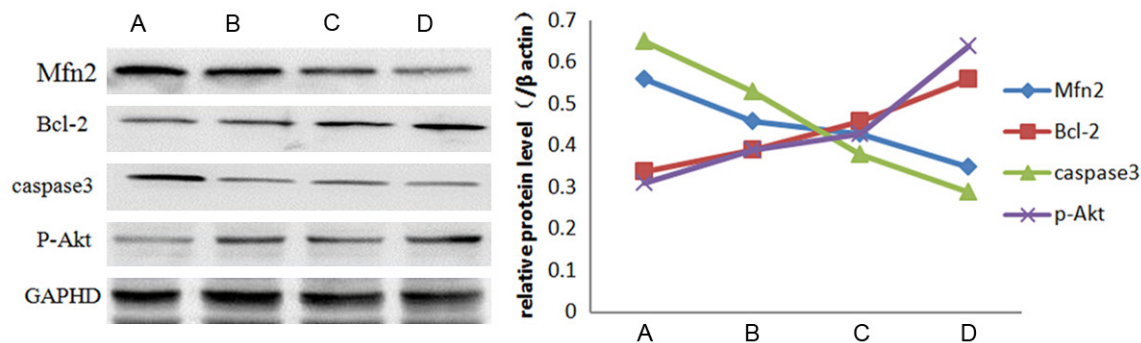
Figure 2. Mfn2 and relevant protein expression are associated with proliferation of GMCs induced by IgA1 in different concentration. A: IgA1 in 25 ug, B: IgA1 in 50 ug; C: IgA1 in 75 ug; D: IgA1 in 100 ug. Compared with A Group, Mfn2 and caspase 3 obviously decreased, Bcl-2 and p-Akt increased in D group ($P<0.05$), there were no different between other group.

ent concentration (25 ug, 50 ug, 100 ug) and for different time (12 h, 24 h and 36 h), the result show that, with increasing of time, IgA1-induced GMCs proliferation gradually increased, peaked at 24 h, slightly decrease when last for 36 h, so we defined the optimal time at 24 h for further research (**Figure 1B**). In our study IgA1 in 100 ug (D group) induced GMCs proliferation was significant higher compared with other concentrations (control: A group; IgA1 in 25 ug: B group; in 50 ug: C group) ($P<0.01$), while there was no different between other concentrations ($P>0.05$) (**Figure 1A**, **1C**). These findings indicated that, IgA1 promoted the proliferation of GMCs in a concentration and time dependent manner.

Expression of Mfn2 and relevant signaling proteins associated with the Ras-PI3K-Akt signaling pathway

Real-time PCR was used to detected Mfn2 expression in RNA level. The result revealed that, Mfn2 expression decreased with proliferation of GMCs. Compared with the A group, Mfn2 obviously decrease in 100 ug IgA1 group (D group) (0.447 ± 0.045) ($P<0.05$). The result was further confirmed by western blot. Proving that IgA1 stimulated GMCs proliferation accompanied with the inhibition of Mfn2 expression in an IgA1 concentration-dependent manner. Mfn2 slightly decreased in B and C group, compared with control A group ($P>0.05$), while obviously decrease was found in stimulated with IgA1 in 100 ug (D group) ($P<0.01$).

To further explore the underlying signal transduction mechanisms associated with GMCs proliferation. We detected the expression of relevant signaling proteins such as Bcl-2, caspase 3, p-Akt. We all know that Ras-PI3K-Akt signaling pathway is vital for cell survival. Activation of the Ras-PI3K-Akt signaling blocks apoptotic. It has been demonstrated that overexpression of antiapoptotic members of Bcl-2 is able to inhibit cell apoptotic, vice-versa [7]. We found out that compared with the A group, Bcl-2 and p-Akt significantly increase, while caspase 3 obviously decreased in D group ($P<0.05$) (**Figure 2**), no different in B and C group ($P>0.05$). So Bcl-2 and p-Akt were increased by IgA1 stimulated (in 100 ug), while Mfn2 and caspase 3 were inhibited. The similar was found out in Diabetic Nephropathy [9].

Mfn2 expression in IgAN animal model

In addition of cell experiments, we also made animal model for tissue experiment. In our research, all of the rats in IgAN group had IgA deposition in mesangial region, detected by immunofluorescence. According to HE stain, there was obvious mesganila cell proliferation in IgAN group, compared with control group. Then we detected the Mfn2 expression by western-blot, the results show that, there was a obviously decrease of Mfn2 expression in IgAN group, compared with control group ($P<0.05$), then we used immunohistochemistry to confirm the position of Mfn2, there was a dramatic difference of mfn2 expression in mesangial area, while no difference in tubulointerstitial area (**Figure 3**). The present study demonstrated that, in IgAN, GMCs proliferation along with Mfn2 expression decrease.
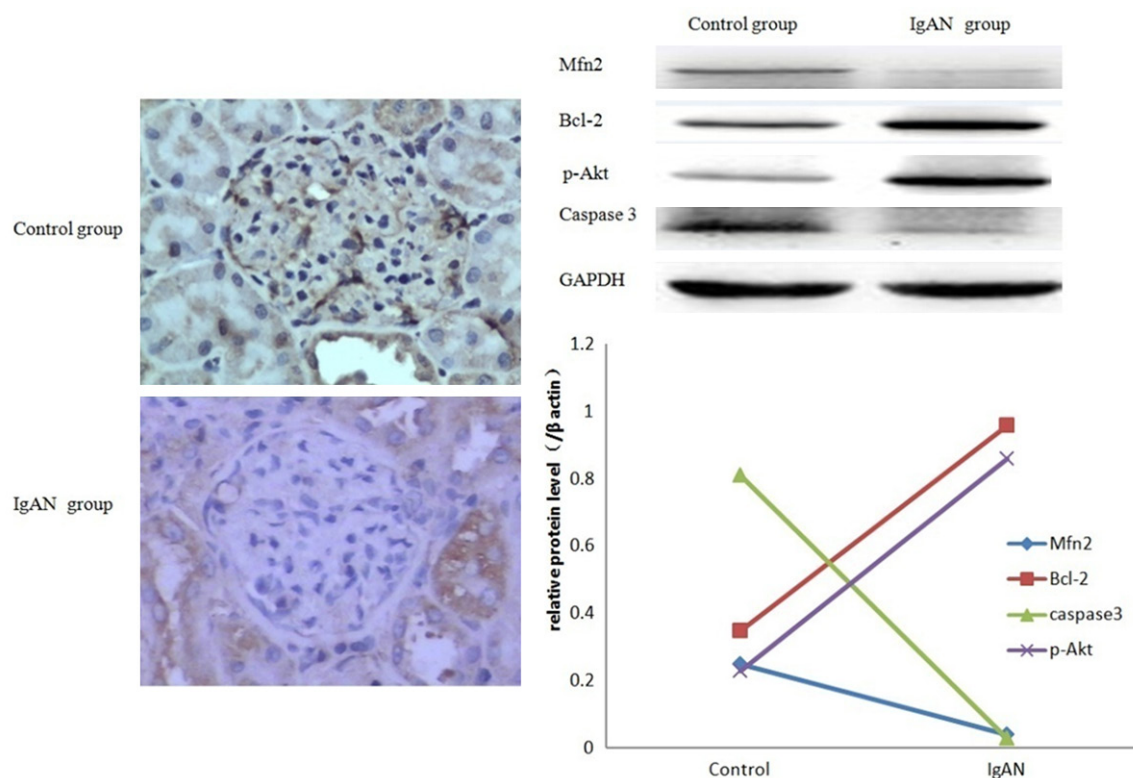
Figure 3. Mfn2 and relevant protein expression in control and IgAN group. In IgAN group, Mfn2 expression significantly decreased in mesangial area, while no difference in tubulointerstitial area. Accompany with the change of Mfn2, Bcl2 and p-Akt increased and caspase 3 decrease.

The expression of relevant signaling proteins associated with the Ras-PI3K-Akt signaling pathway in IgAN animal model

Our research show that, Compared with control group, Bcl-2 and p-Akt significantly increased while caspase 3 decreased in IgAN group (P<0.05) (**Figure 3**). These results proved that, In IgAN, Mfn2 expression decrease, activatedRas-PI3K-Akt signaling pathway, then induced GMCs proliferation.

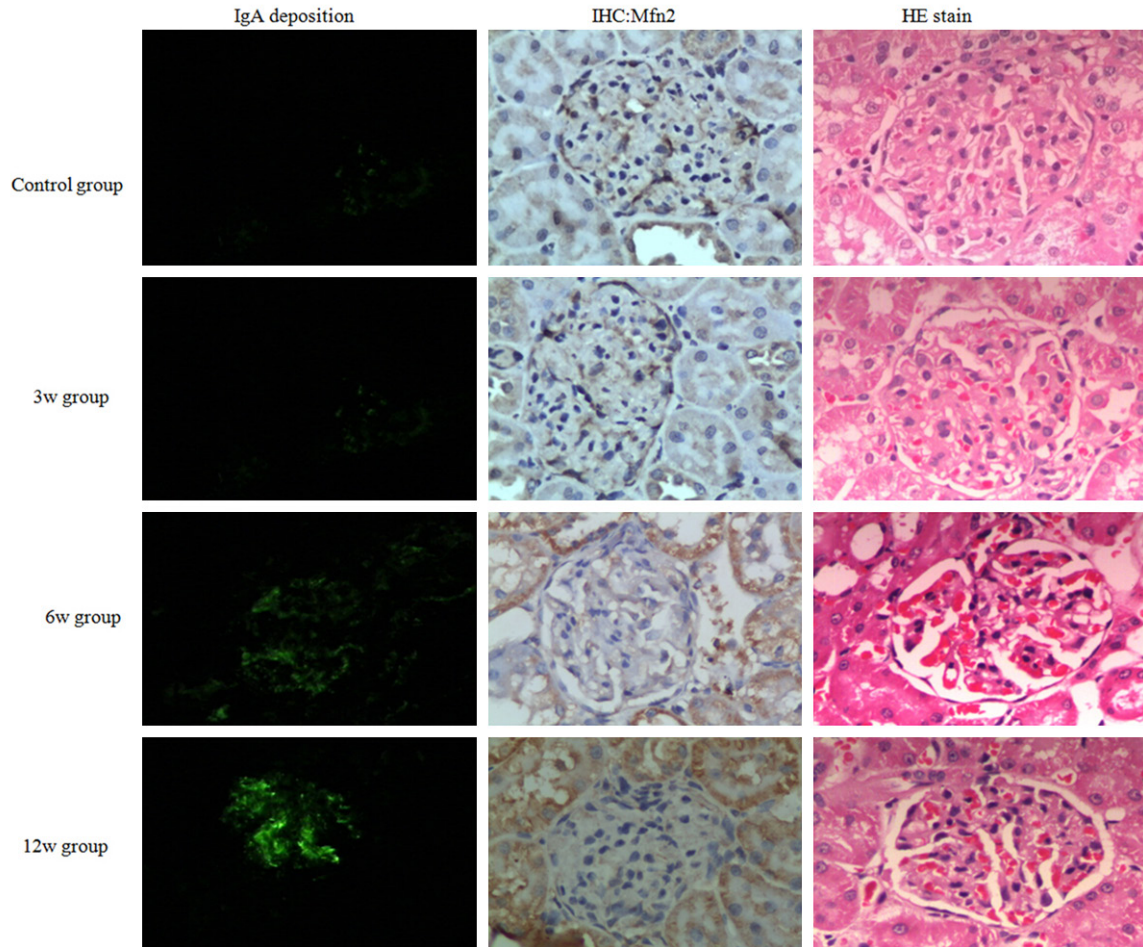*The relationship between Mfn2 expression and mesangial cell proliferation in IgAN animal model*

In order to define the relationship between Mfn2 expression and mesangial cell proliferation in vivo. According to the sacrifice time, IgAN animal model were divided into 3 groups, 3 w group, 6 w group and 12 w group. As time extension, IgA deposition and mesangial cell proliferation gradually increased. At the same time, Mfn2 expression gradually decreased (**Figure 4**). As the expression of Mfn2 gradually

reduce, hyperplasia of GMCs increased significantly.

Discussion

Mfn2 is a recently discovered multifunctional protein; as a transmembrane GTPase, Mfn2 is embedded in the mitochondrial outer membrane and mediates mitochondrial fusion [10]. Its deficiency or dysfunction is associated with many diseases, such as human neurodegenerative diseases, Charcot-Marie-Tooth type 2A, Parkinson's disease, and Alzheimer's disease [11-13]. while Mfn2 also plays an important role in regulating the proliferation and apoptosis of VSMCs through the MAPK and Ras-PI3K-Aktpathways; notably, this is independent of its mitochondrial fusion function [7, 9]. Mounting evidence suggests that Mfn2 plays a role in a variety of proliferative disorders, such as cardiovascular proliferative diseases and certain tumors (urinary bladder carcinoma, hepatocellular carcinoma, and breast carcinoma) [14, 15]. Brook et al. studied the effects of Mfn2 in cisplatin-induced acute tubular necrosis and

Figure 4. The relationship between Mfn2 expression and mesangial cell proliferation in IgAN animal model.

found that Mfn2 had a protective influence on the kidney by inhibiting excessive apoptosis of renal tubular epithelial cells [16]. Tang et al. found that overexpressed Mfn2 could alleviate high glucose-induced GMC proliferation and increase apoptosis, which may contribute to reversing early diabetic nephropathy pathological changes [11]. However, there are few studies connecting IgAN and Mfn2.

In our recent research, compared with a control group, the expression of Mfn2 was markedly inhibited inIgA1-induced GMC proliferation. The same result was published in 2012; Tang et al. found that high glucose-induced Mfn2 gene expression decreased with time, and was lowest after 48 h [17]. In our research, we found there was concentration-dependent manner in IgA1 induced GMCs proliferation and Mfn2 expression. The higher stimulated concentration, the higher proliferate of GMCs and lower

expression of Mfn2. We further through the animal experiment confirmed the above conclusion. In IgAN animal model group, Mfn2 expression obviously decreased comparing with control group. The more mesangial cell proliferation, the lower expression of Mfn2. These findings indicate that no matter in vivo or in vitro, GMCs proliferation is accompanied by decreased Mnf2 expression. In other word, Mfn2 take part in the regulation proliferation and apoptosis of GMCs in IgAN.

Increasing evidence indicates that overexpression of Mfn-2 suppresses cell proliferation and triggers apoptosis in cultured VSMCs by inhibiting the Ras-Raf-ERK/MAPK signaling pathway and the Ras-PI3K-Akt cell survival pathway [9, 17]. Tang demonstrated that Mfn2 inhibited the antiapoptosis effects of p-Akt and Bcl-2 in diabetic nephropathy [11]. In our research, we found similar results; no matter in vivo or in

vitro, reduced expression of Mfn2 was accompanied by higher expression of Bcl-2 and p-Akt and lower expression of caspase 3. This finding provides the first evidence that in IgAN, GMC proliferation is associated with an activated Akt signaling pathway.

There were some limitations to our study. We also require Mfn2 transfer *in vitro* or *in vivo* to produce an Mfn2 overexpression model for further characterization of the causal relationship between Mfn2 and GMCs proliferation in IgAN.

In summary, our research is the first aimed at Mfn2 and IgAN; we found a marked change of Mfn2 in test group, indicating that Mfn2 is associated with GMC proliferation in IgAN, and the connection apparently involves activates Ras-PI3K-Akt pathway. This well-conserved gene might open new a pathogenesis and therapeutic target for IgAN.

Acknowledgements

Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

None.

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