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Original Article

Liver fatty acid binding protein protects renal function through down-regulation of oxidative stress in IgA nephropathy

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Abstract: IgA nephropathy (IgAN) is the most common type of primary glomerulonephritis worldwide and there is still no specific treatment for this disease. Increasing evidence has indicated that overload oxidative stress is closely related to IgAN. Liver fatty acid binding protein (L-FABP) has certain anti-oxidant properties. However, its role in IgAN has not been investigated. In this study, we evaluated the protective role of hL-FABP against IgAN on a transgene mouse model. hL-FABP was introduced in wild type (WT) and ddY (IgAN mice) mice and consequently transgene mice (Tg-WT and Tg-ddY) were generated. IgA measurement in kidney showed that Tg-ddY mice could effectively reduce IgA accumulation in the kidney. Blood urea nitrogen and creatinine were also decreased in Tg-ddY mice, indicating that renal function was protected. RT-PCR and western blot analysis of oxidative stress-related gene expression revealed that hL-FABP could decrease the expression of oxidative genes 4-HNE, HO-1, MCP-1, FN, TNF-alpha on both mRNA and protein levels. Taken together, our results here indicate that hL-FABP has a protective role in renal function against IgAN via reduction of oxidative stress by down-regulation of oxidative gene expression. The results herein could provide useful information for the understanding of IgAN pathogenesis as well as the development of IgAN specific treatments.

Keywords: IgA nephropathy, liver fatty acid binding protein, oxidative stress, renal function

Introduction

IgA nephropathy (IgAN), characterized by dominant deposition of IgA in the glomerular mesangium, is the most common type of primary glomerulonephritis worldwide [1, 2]. At the early-stage of IgAN, it is benign. However, within 20 years of diagnosis, about 40% of patients would progress into end-stage renal failure [3]. Up to date, there is still a lack of specific treatment for this disease.

The exact mechanism underlying IgAN pathogenesis remains elusive, however, there is increasing evidence indicates that overload oxidative stress in the kidney may be closely related to this disease [4, 5]. Previous studies have shown that reactive oxygen species (ROS) as well as ROS-related proteins, such as heme oxygenase-1 (HO-1) and 4-hydroxy-2-nonenal (4-HNE), were significantly elevated in the kidney of IgAN patients than that of normal population [6-8]. Moreover, anti-oxidants have demonstrated some value in IgAN treatment on both pre-clinical and clinical cases [6, 9].

Liver fatty acid binding protein (L-FABP), a type of cytoplasmic protein expressed in liver and human renal proximal tubule epithelium, binds to long-chain fatty acids and other hydrophobic ligands and contributes to fatty acid uptake, transport and metabolism [10, 11]. Other than its function in fatty acid metabolism, L-FABP has also been discovered to participate in the reduction of cellular oxidative stress through limiting the toxic effects of oxidative products of fatty acids [12, 13]. Previous studies have established that L-FABP has predictive value in both chronic kidney disease and acute kidney injury [14-16]. Even more, renal L-FABP expression is associated with prevention of tubulointerstitial damage caused by protein overload [17].

Table 1. PCR primers used in the study

Primer name	Sequence (5'→3')
M-β-actin-F	GTGACGTTGACATCCGTAAAGA
M-β-actin-R	GTAACAGTCCGCCTAGAAGCAC
hL-FABP-F	AAATCGTGCAGAATGGGAAG
hL-FABP-R	TCTCCCCTGTCATTGTCTCC
M-HO-1-F	GTGACAGAAGAGGCTAAGACCG
M-HO-1-R	ACAGGAAGCTGAGAGTGAGGAC
M-TNF-a-F	TACTGAACTTCGGGGTGATCG
M-TNF-a-R	GGGTCTGGGCCATAGAACTGA
M-4-HNE-F	AGGTGTCCCAAAGAAGCTGTAGT
M-4-HNE-R	TTTGGTTCCGATCCAGGTTTT
M-FN1-F	GCCTCAATCCAAATGCCTCTAC
M-FN1-R	CAGTCACAACCTCTTCCCGAAC
MCP1-F	TCCCAATGAGTAGGCTGGAG
MCP1-R	CCTCTCTCTTGAGCTTGGTGA

F: forward primer; R: reverse primer.

The effect of L-FABP in IgAN, however, has yet remained to be investigated.

In the current study, we aimed to investigate the protective effect of L-FABP in IgAN on transgenic ddY mouse model. The ddY mouse is known as a spontaneous IgAN prone mouse. By introduction of human L-FABP into wild type (WT) or ddY mouse through transgene technology, we studied the possible protective effect of L-FABP in IgAN.

Materials and methods

Ethics statement and animals

This study was approved by the Ethics Review Committee of Shanghai University of Traditional Chinese Medicine and performed in accordance with the provincial guidelines of Laboratory Animal Science Association. All animals were purchased from Shanghai slack laboratory animal co., LTD and kept in the Specific Pathogen Free (SPF) environment with water and food supplied. The human L-FABP gene was purchased from Origene and cloned into pBR322-piggybac vector (SBI) for generation of transgene animals as previously described [18].

Real-time PCR

Gene expression on RNA level was determined by RT-PCR as previously described [19]. Briefly, total RNA was extracted using the RNeasy mini kit (Qiagen, MD, USA) and subsequently reverse-transcribed into cDNA using reverse transcriptase M-MLV (Promega), both according to the manufacturer's instructions. Quantitative real-time PCR was then performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermal protocol was as follows: one cycle of 48°C 30 min and 95°C 10 min, and then followed by 40 cycles of 95°C 15 sec and 60°C 60 sec. Each sample was tested in triplicate. The comparative $2^{-\Delta\Delta Ct}$ calculation method was used to calculate the expression level of target genes using GAPDH as an internal control. The primers pairs used in the study were listed in **Table 1**.

Western blot

Western blot was performed as previously described with modifications [20]. In brief, tissue samples were first homogenized and centrifuged. Then supernatants were separated by 12% SDS-PAGE. Gel-separated proteins were subsequently transferred on to a PVDF membrane. Non-specific binding sites on the membrane were blocked with 5% non-fat milk. Subsequently, membrane was incubated with primary antibodies and corresponding HRP-conjugated secondary antibodies for 2 h and 1 h at room temperature, respectively. After extensive washes with PBS-T, immunobands on the membrane was visualized using ECL substrate (Biotime) under a CCD camera (Bio-Rad). The following antibodies were used: Anti-hL-FABP (ab78428), anti-Fibronectin (ab2413), anti-TNF alpha (ab6671), anti-4 Hydroxynonenal (ab48506), anti-Heme Oxygenase 1 (ab13248), anti-MCP1 (ab7202), anti-Collagen IV (ab6586) and anti-Actin (ab199406). All the antibodies were purchased from Abcam.

ELISA

The levels of urine proteins were tested by ELISA. Samples were collected and appropriately diluted and the levels of hL-FABP, IgA, Blood urea nitrogen (BUN) and creatinine (CRE) were determined using commercial ELISA kits according to the manufacturer's instructions. The concentrations of tested proteins were calculated according to corresponding standard curve generated in the tests. The kits for hL-FABP, IgA, BUN and CRE quantification were

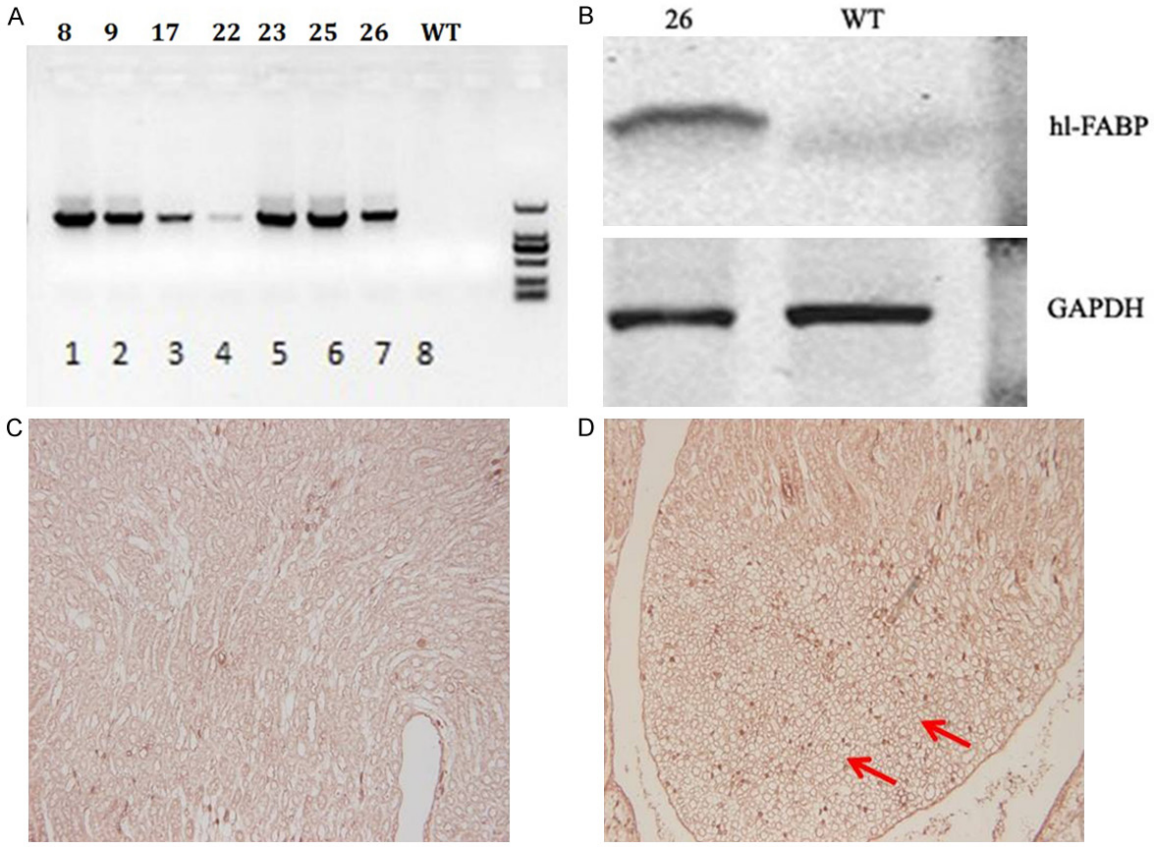

Figure 1. Detection of hL-FABP expression in transgene mice. Human (h)L-FABP was first introduced into WT or ddY mice and then the expression of hL-FABP on both mRNA and protein levels were determined by RT-PCR (A), Western blot (B) and IHC (C, D), respectively. Representative results are shown. (A) Lanes 1-7 (left to right) were positive hL-FABP transgene mice. Lane 8 was WT mice. Numbers indicated above the lanes were IDs of the mice. (C, D) were IHC detection of hL-FABP in WT and hL-FABP transgene mice, respectively.

purchased from R&D systems, eBiosciences, Biotime and Abcam, respectively.

Histology and electron microscopic analysis

Mice were euthanized at the specified ages and 5-micron sections of renal tissue were fixed in buffered formalin, stained with periodic acid-Schiff (PAS), and processed for light microscopic evaluation. Specimens that contained >30 glomeruli were used for histopathologic analysis and quantitation of glomeruli with segmental and global sclerosis and/or mesangial cell proliferation and/or an increase in mesangial matrix. Each specimen was assigned a score representative of the calculated percentages of affected glomeruli (0: 0%; 1: 1-24%; 2: 25-49%; 3: >50%). The total maximal score for each specimen was 9 using this scoring system. Specimens were evaluated in a triple-blinded manner by 3 nephrologists. Immunofluorescence was performed on four micron cryostat sections of the other cryopreserved kidney with the use of goat anti-mouse FITC-conjugated polyclonal antibodies to IgG, IgA, IgM (K&P Laboratories, Gaithersberg, MD), and rat anti-mouse FITC-conjugated monoclonal antibody to FABP (Cedarlane, Hornby, Ontario, Canada). For electron microscopic examination, the samples were fixed with glutaraldehyde and osmium tetraoxide, embedded in EPON™ resin. Sections (100 nm) were stained with uranyl acetate and lead citrate, and examined under the electron microscope.

Statistical analysis

All numeric data were expressed as mean ± SD. Statistical analysis was performed by Student's t-test using SPSS 17.0 (SPSS). *P*-values were less than 0.05 were considered as statistically significant.

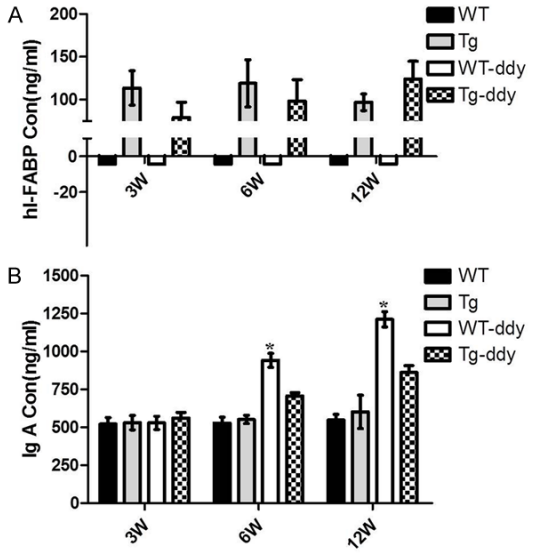

Figure 2. Detection of hL-FABP and IgA in urine samples. Urine samples from all mice were collected at week 3, 6 and 12 and the concentrations of hL-FABP and IgA were measured by ELISA. A. hL-FABP levels in urine samples (n=5). B. IgA levels in urine samples. Data shown are mean ± SD of three independent experiments.

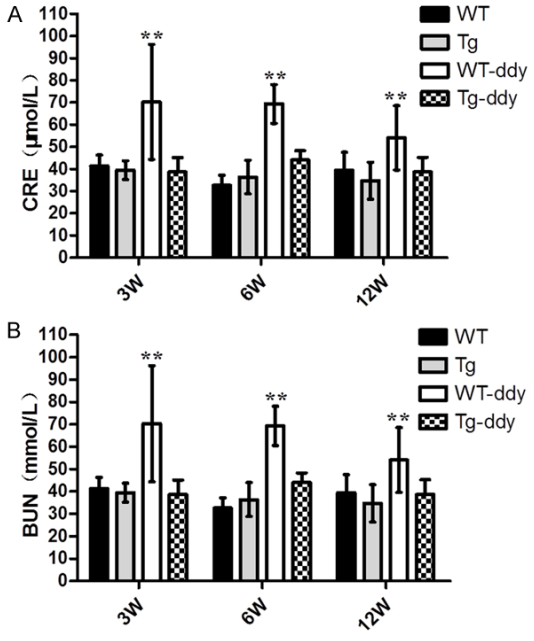

Figure 3. Detection of CRE and BUN in urine samples. Urine samples from all mice were collected at week 3, 6 and 12 and the concentrations of CRE and BUN were measured by ELISA. A. CRE levels in urine samples (n=5). B. BUN levels in urine samples. Data shown are mean ± SD of three independent experiments.

Results

Establishment of hL-FABP transgene animal model

First, hL-FABP gene was introduced into both WT and ddY mice by transgene technology and then the transgene efficiencies were determined by RT-PCR, Western blot and immunohistochemistry (IHC) analysis. HL-FABP expression on mRNA level was measured by RT-PCR. As shown in **Figure 1A**, hL-FABP expression was detected in all hL-FABP transgene mice (lanes 1-7) but not non-transgene mice (lane 8). Further analysis on protein level by Western blot and IHC showed that hL-FABP was highly expressed in the kidney of transgene mice, but not non-transgene mice (**Figure 1B-D**). Taken together, the results here indicated that hL-FABP transgene mice were successfully generated. Four groups of mice were used in this study and they were designated as WT (WT mice), Tg (WT mice with hL-FABP gene introduction) WT-ddY (WT-ddY mice) and Tg-ddY (ddY mice with hL-FABP gene introduction), respectively.

HL-FABP alleviates IgAN symptoms in Tg-ddY mice

The protective effect of hL-FABP on IgAN was then investigated. First, urine samples from WT or ddY mice with or without hL-FABP introduction at week 3, 6 and 12 and the levels of L-FABP, IgA, BUN and CRE were measured. Our results showed that hL-FABP expression was constantly detected in both Tg and Tg-ddY mice, but not in WT and WT-ddY mice, further confirming the success of transgene model (**Figure 2A**). Further determination of IgA concentration revealed that WT-ddY mice at week 6 and 12 exhibited significantly higher levels of urine IgA than WT and Tg mice. In Tg-ddY mice, urine IgA concentration at week 6 and 12 was also slightly higher than in WT and Tg mice; however, it was significantly lower than that in WT-ddY mice (**Figure 2B**). These data implied that hL-FABP might alleviate IgA accumulation in the kidney.

Renal function was further evaluated by determining the CRE and BUN levels in urine samples and similar results were observed. Namely, hL-FABP could decrease the levels of CRE and

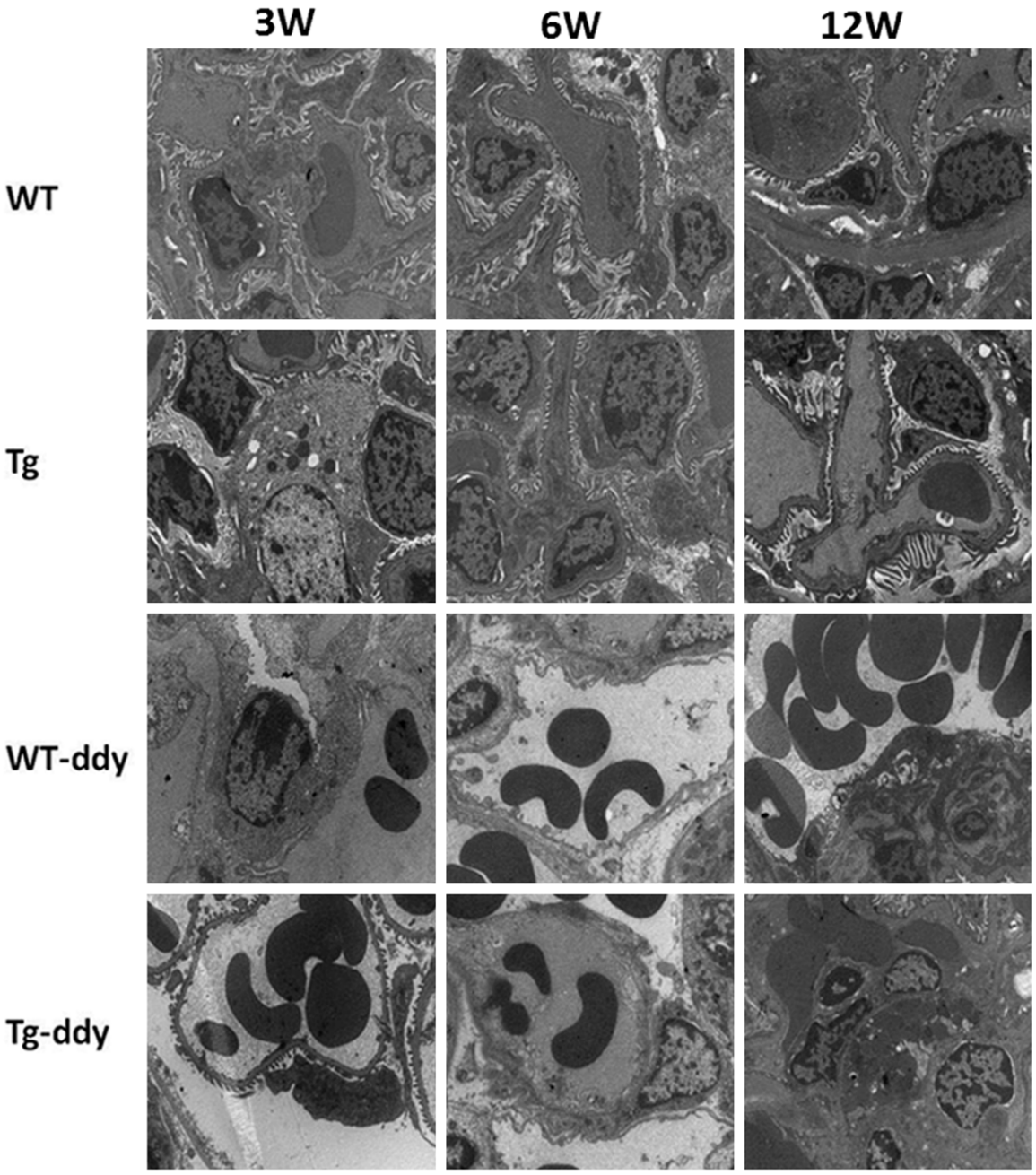

Figure 4. hL-FABP decreases IgA accumulation in the glomerular mesangium. At week 3, 6 and 12, kidney samples from all mice were harvested and tested IgA accumulation by electron microscopy. Representative result is shown.

BUN in urine samples of Tg-ddY mice, but not in Tg mice (**Figure 3**).

In order to obtain visualized results of hL-FABP on IgAN, We also detected the Ig A in glomerular mesangium via electron microscope. As shown in **Figure 4**, no apparent IgA accumulation was observed in WT and Tg mice throughout the observation time period. However, in WT-ddY mice, IgA precipitation was observed at week 3 and it was increasing at week 6 and 12. Of note, Tg-ddY mice also exhibited some IgA accumulation in the glomerular mesangium at week 3, but unlike WT-ddY mice, IgA precipitate

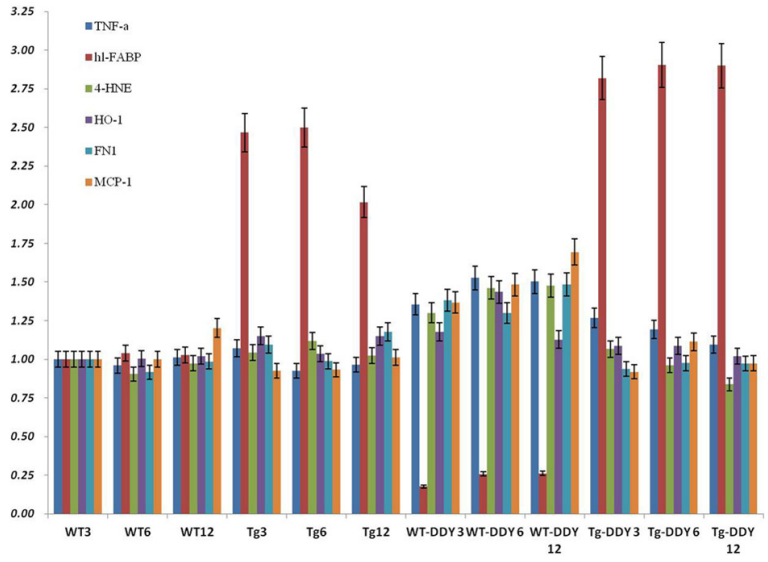

Figure 5. RT-PCR analysis of oxidative stress-related gene expression in kidney. At week 3, 6 and 12, kidney samples from all mice were harvested and TNF-α, hL-FABP, 4-HNE, HO-1, FN1 and MCP-1 expression was determined by RT-PCR. Data shown are mean ± SD of three independent experiments.

slowly decreased as time pass-by. At week 12, no apparent IgA precipitate was observed in Tg-ddY mice. Taken together, the results here indicate that hL-FABP could protect renal function against IgAN via decrease of IgA accumulation in the glomerular mesangium.

L-FABP protects renal function by down-regulating oxidative stress

Next we explored possible mechanism underlying hL-FABP protection of renal function against IgAN. Since hL-FABP could reduce cellular oxidative stress which has been believed to be one of the causes for IgAN, we hypothesized that hL-FABP might exert its renal protection function by the reduction of oxidative stress in the kidney. Consequently, we tested the expression of oxidative stress-related genes on both mRNA and protein levels. As shown in **Figure 5**, in the control groups (WT and Tg mice), the mRNA levels of 4-HNE, HO-1, FN-1 and MCP-1 remained at basal level from week 3 to 12. On the contrary, the levels of these genes were all significantly elevated in WT-ddY mice throughout week 3 to 12. Interestingly, in Tg-ddY mice, the mRNA of the tested oxidative stress-related genes was at the same levels as in the control WT and Tg mice. The determination of the expression of these genes on protein levels demonstrated similar results (**Figure 6**). These results indicate that hL-FABP could reduce oxidative stress in the kidney of IgAN mice, and consequently protect renal function from IgAN.

Discussion

IgAN, the most common form of primary glomerulonephritis, exhibits IgA deposition in the glomerular mesangium [1, 21]. However, up to now, specific treatment is still in lack [22-24]. In the current study, we investigated the protective role of hL-FABP in renal function against IgAN on the hL-FABP transgene mouse model. Our results demonstrated that hL-FABP could reduce the IgA accumulation in the glomerular mesangium. Moreover, the renal function of IgAN mice with hL-FABP introduction (Tg-ddY) was significantly improved comparing to WT-ddY mice. Further mechanism study revealed that hL-FABP could down-regulate the expression of oxidative stress-related genes on both mRNA and protein levels. Taken together, our study herein implies that hL-FABP has a renoprotective role against IgAN and this is achieved by reduction of oxidative stress through down-regulation of oxidative stress-related genes.

L-FABP is initially identified in liver and plays essential roles in fatty acid uptake, metabolism and transportation. There are also studies showing its function in antioxidation. However, its pathophysiological roles in in kidney diseases, especially IgAN, have not been fully investigated. Recent studies have described the function of L-FABP in renal protection in tubulointerstitial diseases on animal models [17, 18]. In the current study, hL-FABP expression, comparing to Tg-WT mice, was significantly elevated in the glomerular mesangium as well as urine in Tg-ddY mice, indicating that the expression of hL-FABP might be enhanced by certain stimuli and consequently demonstrated renoprotection in Tg-ddY mice.

The role of L-FABP in fatty acid metabolism has been well-studied. However, its function in antioxidation has been described only recently and

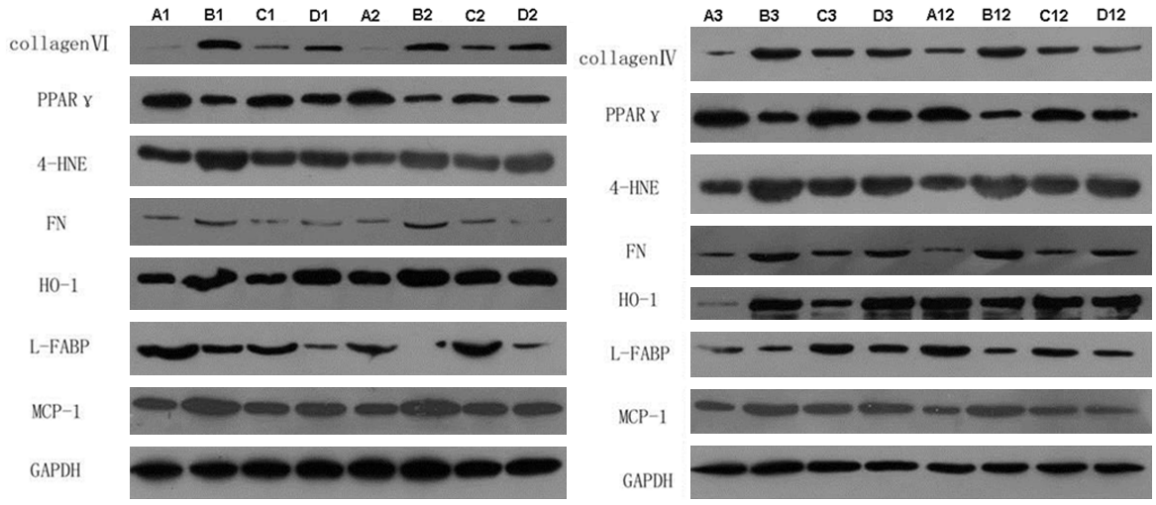

Figure 6. Western blot analysis of oxidative stress-related gene expression in kidney. At week 3, 6 and 12, kidney samples from all mice were harvested and TNF-α, hL-FABP, 4-HNE, HO-1, FN1 and MCP-1 expression was determined by western blot. Representative result is shown. The samples were indicated above each lane and designated as follows: A-D represent WT, Tg, WT-ddY and Tg-ddY, respectively. Numbers 3, 6 and 12 indicate samples were taken on week 3, 6 and 12, respectively.

its exact mechanisms still remain controversial. Some claimed that L-FABP reduces cellular oxidative stress by promotion on fatty acid metabolism, while others believe that the oxidative stress reduction was due to hypoxia-reoxygenation by L-FABP [12, 25, 26]. Our study here has also confirmed that L-FABP could reduce oxidative stress in the glomerular mesangium of IgAN mice. However, our results indicated that L-FABP reduced oxidative stress by down-regulating the expression of oxidative stress-related genes including 4-HNE, HO-1, FN-1 and MCP-1. Given the complicity of gene regulation network, how the down-regulation of the oxidative stress-related gene impact the oxidative stress in the glomerular mesangium requires further investigation.

Our results has determined the impact of L-FABP on the expression of many oxidative stress-related genes, however, which one or more genes exert predominant roles in reducing oxidative stress remains to be further explored. Moreover, if oxidative stress-related gene was responsible for the overload oxidative stress in the glomerular mesangium of IgAN patients, then whether direct regulation of these genes instead of adopting L-FABP could provide better renoprotection could be also investigated.

Of note, we and others have also discovered that traditional Chinese medicine "GubenTongluo Formula" has also demonstrated promising results in IgAN, probably through mediating both humoral and cellular immune responses [27, 28]. Although out of the scope of the current study, it would be an interesting topic to compare the efficacy between L-FABP and GubenTongluo Formula in IgAN.

In conclusion, our study showed that L-FABP could protect renal function in IgAN by reducing oxidative stress in the glomerular mesangium through down-regulation on oxidative stress-related genes. The results of this study could provide useful information for the understanding of IgAN pathogenesis as well as the development of specific treatment.

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Disclosure of conflict of interest

None.

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