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Glomerular endothelial expression of type I IFN-stimulated gene, DExD/H-Box helicase 60 via toll-like receptor 3 signaling: possible involvement in the pathogenesis of lupus nephritis

Takao Karasawa[a,b], Riko Sato[a,b], Tadaatsu Imaizumi[b], Shun Hashimoto[a], Masashi Fujita[a], Tomomi Aizawa[a], Koji Tsugawa[a], Shogo Kawaguchi[b], Kazuhiko Seya[b], Kiminori Terui[a] and Hiroshi Tanaka[a,c]

[a]Department of Pediatrics, Hirosaki University Hospital, Hirosaki, Japan; [b]Department of Vascular Biology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan; [c]Department of School Health Science, Hirosaki University Faculty of Education, Hirosaki, Japan

ABSTRACT

Background: Sustained type I interferon (IFN) activation *via* Toll-like receptor (TLR) 3, 7 and 9 signaling has been reported to play a pivotal role in the development of lupus nephritis (LN). Although type I IFN activation has been shown to induce interferon-stimulated genes (ISGs) expression in systemic lupus erythematosus, the implication of ISGs expression in intrinsic glomerular cells remains largely unknown.

Methods: We treated cultured human glomerular endothelial cells (GECs) with polyinosinic-polycytidylic acid (poly IC), R848, and CpG (TLR3, TLR7, and TLR9 agonists, respectively) and analyzed the expression of DExD/H-Box Helicase 60 (DDX60), a representative ISG, using quantitative reverse transcription-polymerase chain reaction and western blotting. Additionally, RNA interference against IFN-β or DDX60 was performed. Furthermore, cleavage of caspase 9 and poly (ADP-ribose) polymerase (PARP), markers of cells undergoing apoptosis, was examined using western blotting. We conducted an immunofluorescence study to examine endothelial DDX60 expression in biopsy specimens from patients with LN.

Results: We observed that endothelial expression of DDX60 was induced by poly IC but not by R848 or CpG, and RNA interference against IFN-β inhibited poly IC-induced DDX60 expression. DDX60 knockdown induced cleavage of caspase 9 and PARP. Intense endothelial DDX60 expression was observed in biopsy specimens from patients with diffuse proliferative LN.

Conclusion: Glomerular endothelial DDX60 expression may prevent apoptosis, which is involved in the pathogenesis of LN. Modulating the upregulation of the regional innate immune system *via* TLR3 signaling may be a promising treatment target for LN.

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Introduction

Sustained activation of type I interferon (IFN) has been reported to play a pivotal role in the pathogenesis of systemic lupus erythematosus (SLE) and the development of lupus nephritis (LN) [1,2]. Therefore, the involvement of innate immune system upregulation *via* regional Toll-like receptor (TLR) signaling, such as TLR3, TLR7, and TLR9, is believed to be involved in LN pathogenesis [3–7]. Considering that viral infections may trigger the development of inflammatory renal disease, upregulation of regional TLR3 signaling reportedly plays a role, at least partly, in the pathogenesis of glomerular diseases (GN), including LN [3,6,7]. Interestingly, the activation of TLR3 and downstream immune responses can be induced by both infectious organisms and endogenous ligands, leading to the development of "pseudo" antiviral immunity-related inflammations in the kidney [6]. Therefore, this theory is probably involved in the pathophysiology of LN [6,7].

Type I IFN induces DExD/H-Box helicase 60 (DDX60), as demonstrated by microarray analysis of genes induced by a viral infection in human dendritic cells [8]. DDX60 has been reported to degrade hepatitis C virus RNA and suppress viral replication [9]. In the clinical setting, DDX60 is reportedly a novel and unfavorable biomarker for tumorigenesis and prognosis of oral squamous cell carcinoma in a subsite-specific manner [10]. Furthermore, DDX60 expression in CD14+ monocytes has been observed to increase in patients with

CONTACT Hiroshi Tanaka hirotana@hirosaki-u.ac.jp Department of School Health Science, Hirosaki University Faculty of Education, Hirosaki, 036-8560, Aomori, Japan

childhood-onset SLE; this effect was believed to be a signature of type I IFN activation [11]. However, the expression and the role of DDX60 in intrinsic glomerular cells remain largely unknown.

Considering that the interferon-stimulated gene (ISG) family plays a pivotal role in innate immune reactions in various cell types [8–11], we examined the implication of ISGs in glomerular intrinsic cells [12–15]. Interestingly, we found intense immunostaining of these ISGs primarily in biopsy specimens of patients with LN, which suggested that the expression of ISGs is involved in LN pathogenesis [13,15]. It is known that the network of numerous ISGs regulates innate immune reactions in a complex manner. However, to our knowledge, the expression and roles of DDX60 in the inflammatory signaling pathways activated by TLR3 in human glomerular endothelial cells (GECs) have not been reported. In this study, we examined the expression of DDX60 and its postulated role in human GECs treated with TLR3, TLR7 and TLR9 agonists. Furthermore, we conducted an immunofluorescent study of DDX60 in biopsy specimens from patients with LN. Against our expectations, we observed that knockdown of DDX60 promoted apoptosis in poly IC-treated GECs. Thus, we further examined the glomerular endothelial expression of poly (ADP-ribose) polymerase (PARP), a marker of cells undergoing apoptosis [16,17].

Materials and methods

Reagents

Poly IC and rabbit anti-actin IgG were purchased from Sigma (St Louis, Mo, USA). R848, a TLR7 agonist, was obtained from InVivoGen (San Diego, CA, USA). CpG, a TLR9 agonist, was purchased from Novus Biologicals (Centennial, CO, USA). An anti-DDX60 rabbit antibody (GTX32082) was purchased from GeneTex (Irvine, CA, USA). An anti-caspase 9 rabbit antibody (9502) was from Cell Signaling Technologies (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was purchased from Medical & Biological Laboratories (Nagoya, Japan). Polyvinylidene difluoride (PVDF) membranes and Luminata Crescendo Westen HRP Substrate were purchased from Merk Millpore (Darmstadt, Germany). An illustrated RNA spin kit was obtained from GE Healthcare (Buckinghamshire, UK). SsoAdvanced Universal SYBR Green Supermix was obtained from Bio-Rad (Hercules, CA, USA). Small interfering RNAs (siRNAs) against IFN-β, dNTP mix, and Moloney murine leukemia virus (MMLV) reverse transcriptase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human (r(h)) IFN-β was purchased from ProSpec (Rehovot, Israel). Non-silencing negative control siRNA and siRNA against DDX60 were purchased from Qiagen (Hilden, Germany). An anti-PARP rabbit antibody (9542) was purchased from Cell Signaling Technologies (Danvers, MA, USA).

Cells

Normal human glomerular endothelial cells (GECs) were purchased from ScienCell (Carlsbad, CA, USA). The cells were cultured in endothelial growth mudium-2 (EGM-2) from Lonza (Walkersville, MD, USA) using gelatin-coated plates, as previously reported [15,18]. We treated the cells with 0.1–3 μg/mL poly IC to examine the concentration-dependent effect of poly IC. In subsequent experiments, the cells were treated with 3 μg/mL poly IC for up to 24 h. The cells were additionally treated with 5 μg/mL R848, 100 μg/mL CpG, or 1 ng/mL r(h) IFN-β for up to 24 h [19]. For RNA interference experiments, the cells were cultured in a medium without antibiotics for 24 h before transfection and then transfected with specific siRNA against IFN-β, DDX60, or a non-silencing negative control siRNA using the Lipofectamine RNAiMAX reagent, according to the manufacturer's protocol. After 48 h incubation, the cells were treated with 3 μg/mL poly IC.

qRT-PCR

Total RNA was extracted from cells using the illustra RNAspin kit and used for reverse transcription in accordance with the manufacturer's instruction. Single-stranded complementary DNA (cDNA) was synthesized from RNA using oligo(dT)$_{18}$ and MMLV reverse transcriptase. DDX60 cDNA, IFN-β cDNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were amplified using specific primers and SsoAdvanced Universal SYBR Green Supermix. GAPDH was used as an internal control.

The sequences of the primers used are shown as follows:

DDX60-F: 5′-AAGGTGTTCCTTGATGATCTCC-3′,
DDX60-R: 5′-TGACAATGGGAGTTGATATTCC-3′,
IFN-β-F: 5′-CCTGTGGCAATTGAATGGGAGGC-3′,
IFN-β-R: 5′-CAAGGCACAGTGACTGTACTCCTT-3′,
GAPDH-F: 5′-GCACCGTCAAGGCTGAGAAC-3′,
GAPDH-R: 5′-ATGGTGGTGAAGACGCCAGT-3′.

Western blotting

After incubation, as indicated the cells were lysed using Laemmli reducing sample buffer and detached from the bottom of the culture plate. The lysates were subjected to 5–20% polyacrylamide gel electrophoresis. The proteins were transferred to PVDF membranes, which were subsequently blocked with nonfat dry milk for 2 h at room temperature and probed with an antibody against DDX60 (1:1,000), caspase 9 (1:1,000), PARP (1:1,000), or actin (1:4,000) for 18 h at 4 °C. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG antibodies. Immobilon Crescendo western HRP chemiluminescence substrate was used for the detection. The optical density of bands was analyzed using Image J software.

Immunofluorescence staining of DDX60

Biopsy specimens were obtained during routine diagnostic procedures in clinical practice. In this study, we chose snapped frozen sections, stored in good condition. We performed DDX60 staining for specimens from patients with diffuse proliferative LN [$n = 3$, Class IV-G (A) in accordance with the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification for LN], non-proliferative LN ($n = 3$, Class II), proteinuric IgA nephropathy (IgAN, $n = 3$; defined as urinary protein/creatinine ratio greater than 1.0), and nutcracker syndrome ($n = 1$, as a non-inflammatory control) were stained for DDX60. These biopsy specimens were obtained from the patients before treatment initiation. Because nutcracker syndrome usually presents no inflammation in the kidney, we used this specimen as non-inflammatory control. The OCT-embedded specimens were cut into 5-μm-thick sections using a cryostat, briefly fixed in cold acetone, and air-dried. Sections were transferred to slides, and the slides were washed in phosphate-buffered saline (PBS) (pH 7.4) immediately before the immunohistochemical procedure. Rabbit anti-DDX60 antibody was added at a dilution of 1:1000. After incubation for 60 min at room temperature, the slides were washed in PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody at a dilution of 1:200 for 60 min at room temperature. To examine the localization of the DDX60 expressing cell, we performed a dual-labeling immunofluorescence study with anti-rabbit DDX60 antibody (1:1000) and anti-mouse CD34 antibody (Nichirei Bioscience, Tokyo, Japan, undiluted) as a marker of endothelial cells. The secondary antibodies used for the double-staining study were Alexa fluor 594 conjugated anti-mouse IgG (1:200) and FITC conjugated anti-rabbit IgG (1:200).

Statistical analysis

All qRT-PCR data are presented as mean ± standard deviation (SD). The student's *t*-test was used to assess the significance of the independent experiments. Statistically, significance was set as $p < 0.05$.

Results

Poly IC induces the expression of DDX60 in cultured human GECs

Normal human GECs cultured without stimulation displayed a low expression of DDX60 mRNA and protein. When the cells were treated with 0.1–3 μg/mL poly IC, the expression levels of DDX60 mRNA and protein increased in a concentration-dependent manner (Figure 1(A,B)). Additionally, the expression of DDX60 mRNA was increased after poly IC treatment in a time-dependent manner; the maximal level of DDX60 mRNA was observed at 4–8 h, and the level almost plateaued up to 24 h (Figure 2(A)). In contrast, no significant change was observed in the expression of DDX60 mRNA when the cells were treated with R848 or CpG ODN (Figure 2(A)). Furthermore, the expression of IFN-β mRNA after poly IC treatment showed a maximal level at 2 h (Figure 2(A)). The expression of DDX60 protein influenced mRNA expression and reached a maximum level of 16–24 h after treatment with poly IC (Figure 2(B)).

IFN-β is involved in DDX60 induction by poly IC

To examine whether IFN-β was involved in the expression of DDX60, RNA interference experiments were performed. When the cells were transfected with a siRNA against IFN-β, the poly IC-induced expression of DDX60 mRNA and protein decreased (Figure 3(A,B)). Knockdown of IFN-β mRNA was confirmed (lower part of Figure 3(A)). r(h)IFN-β treatment, as well as poly IC treatment, in the cells induced the expression of DDX60 mRNA (Figure 3(C)) and protein (Figure 3(D)).

Knockdown of DDX60 followed by poly IC treatment induces PARP cleavage

To examine whether DDX60 is involved in pro- or anti-apoptotic reaction, the protein levels of caspase 9, cleaved caspase 9, PARP and cleaved PARP were examined by western blotting. Cleaved caspase 9 (35 kDa) was increased by knockdown of DDX60 followed by

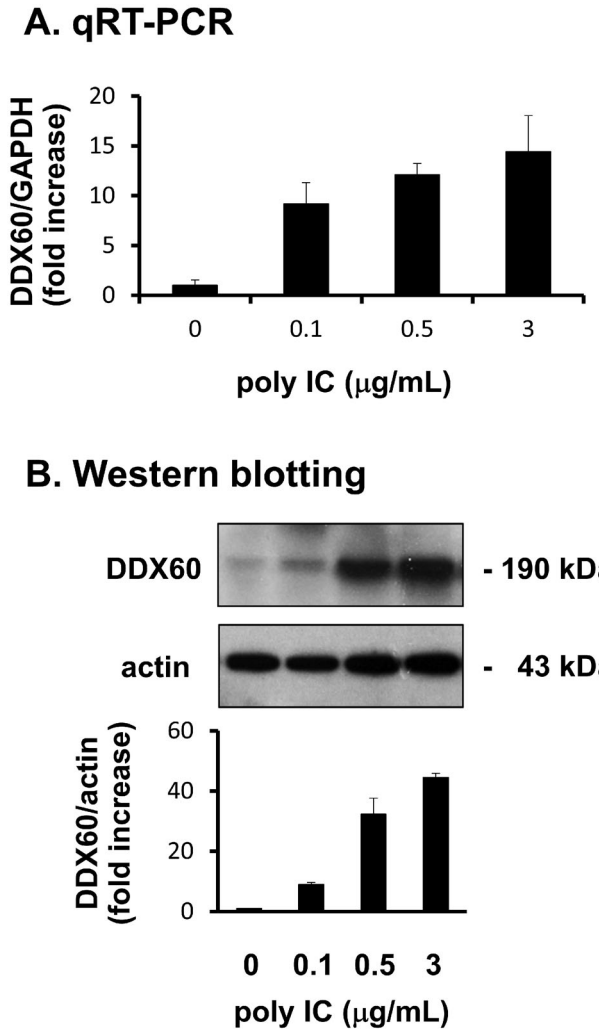

Figure 1. Poly IC induced DDX60 expression in cultured human glomerular endothelial cells (GECs) in a concentration-dependent manner. (A) GECs were cultured and treated with 0.1–3 μg/mL poly IC for 24 h. RNA was extracted from cells after incubation, and reverse-transcribed to cDNA using oligo $(dT)_{18}$ and M-MLV reverse transcriptase. cDNA was used as a template for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis for the expression of DDX60 and GAPDH mRNA. Data are presented as the mean ± standard deviation (SD) ($n = 3$). (B) After treatment of the cells with 0.1–3 μg/mL poly IC for 24 h, the cells were lysed using Laemmli sample buffer. The lysates were subjected to western blotting for DDX60 and the actin protein. The integrated optical density (OD) of the protein band in (B) was quantified using Image J and was normalized to that of actin (B, lower panel).

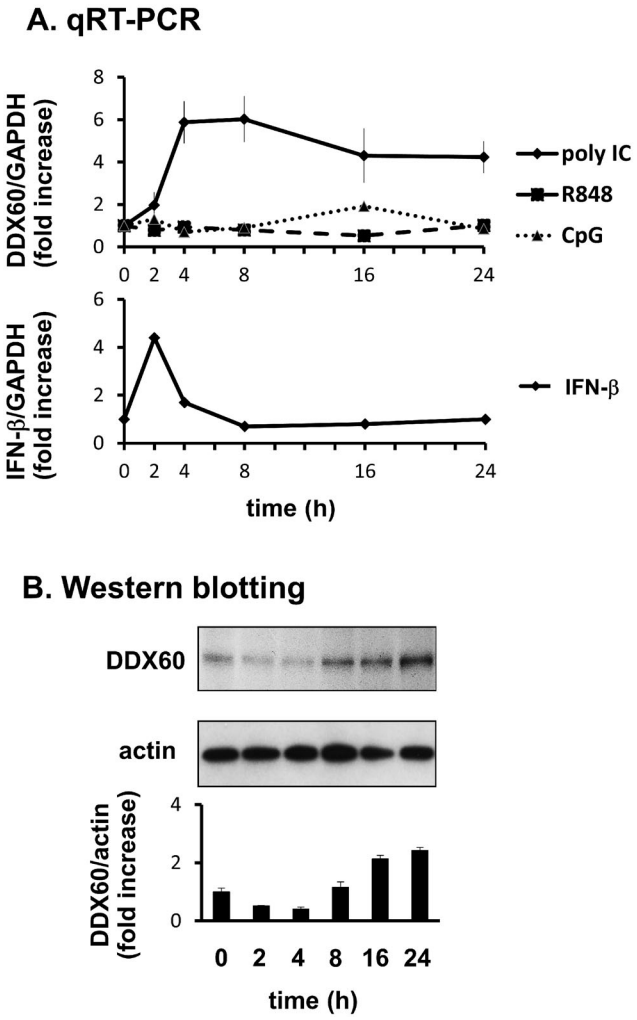

Figure 2. Poly IC induced the expression of DDX60 in a time-dependent manner. (A) GECs were treated with 3 μg/mL poly IC, 5 μg/mL R848, or 100 μg/mL CpG for up to 24 h. The mRNA expression of DDX60 was examined, as presented in the upper part of the Figure. IFN-β mRNA expression was shown in the lower part of the Figure. The data are shown as the mean ± SD ($n = 3$). (B) The cells were treated with 3 μg/mL poly IC as described in (a) and were lysed. Western blotting analysis was performed for DDX60 and actin. The integrated OD of the protein band was quantified using Image J and was normalized to that of actin (B, lower panel).

poly IC treatment (Figure 4(A)), and cleaved PARP (89 kDa) was increased by knockdown of DDX60 followed by poly IC treatment (Figure 4(A)). Additionally, the protein levels of caspase 9 and cleaved caspase 9 were examined by western blotting. On the other hand, knockdown of DDX60 inhibited the expression of IFN-β mRNA (Figure 4(B)). Effective knockdown of DDX60 protein was confirmed by western blotting (Figure 4(A)).

DDX60 is expressed in GECs in the biopsy specimen of proliferative LN

The immunoreactivity of DDX60 in the glomerulus was intense in the biopsy specimen of class IV LN, whereas DDX60 expression in the specimen of class II LN was traced. Dual immunostaining showed that DDX60 expression is located at GECs. Meanwhile, DDX60

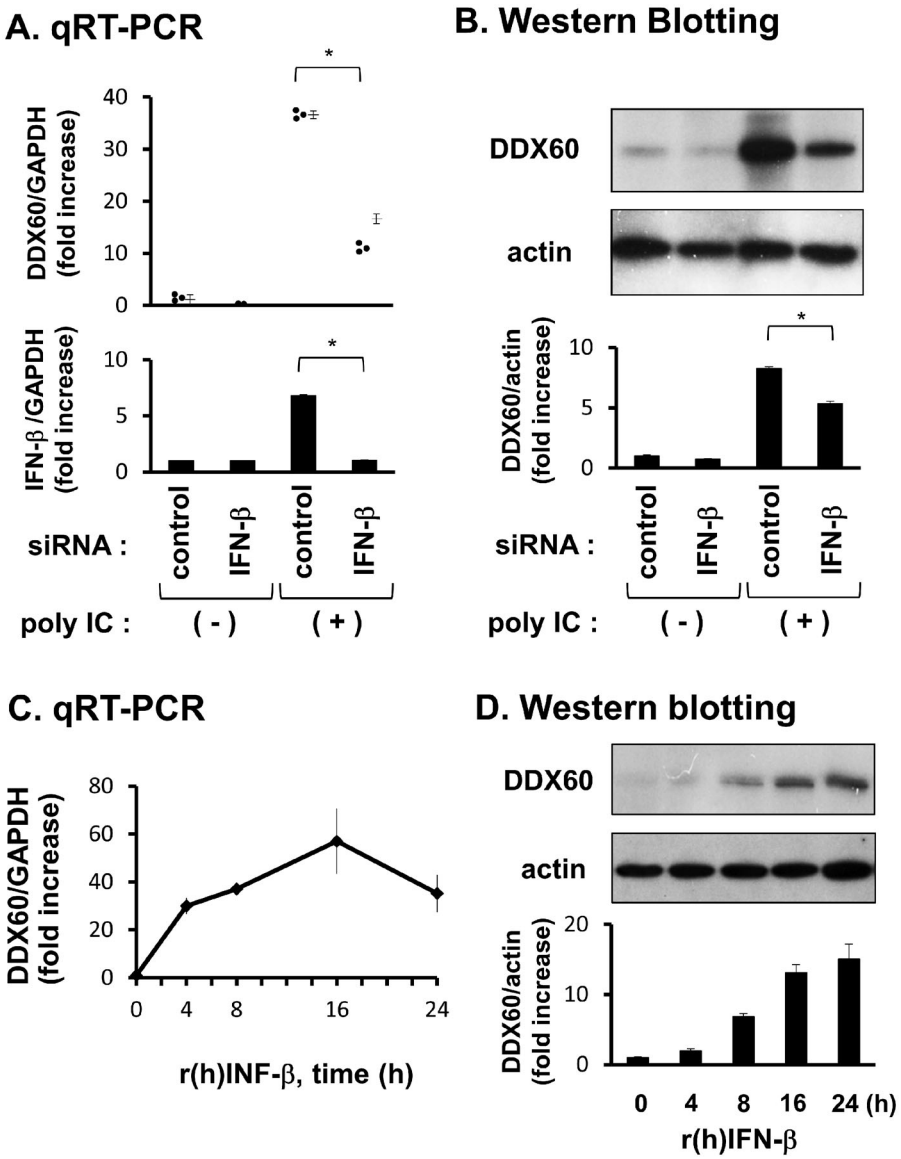

Figure 3. IFN-β was involved in poly IC-induced DDX60 expression. (A,B) Knockdown of IFN-β decreased the expression of DDX60 induced by poly IC. The cells were transfected with small interfering RNA (siRNA) against IFN-β or a non-silencing negative control siRNA and were incubated for 48 h. Subsequently, 3 μg/mL poly IC was added to the cultures. ($n = 3$, $^*p < 0.01$, *t*-test). (A) RNA was extracted from the cells after 24 h incubation, and qRT-PCR for DDX60 (A, upper panel) and IFN-β (A, lower panel) was performed. (B) The cells were lysed after 24 h incubation, and western blotting was performed for DDX60 and actin. The integrated OD of the protein band was quantified using Image J and was normalized to that of actin (B, lower panel). (C,D) The cells were treated with 1 ng/mL r(h) IFN-β for up to 24 h. The expression of DDX60 mRNA (C) and protein (D) was examined using qRT-PCR and western blotting, respectively. The integrated OD of the protein band was quantified using Image J and was normalized to that of actin (D, lower panel). Treatment of cells with r(h)IFN-β increased the expression of DDX60 mRNA and protein in GECs. The data in (A,C) are shown as the mean ± SD ($n = 3$, $^*p < 0.01$, *t*-test).

expression in the other specimens from patients with IgAN or nutcracker syndrome was negligible (Figure 5(A,B)). Representative dual-labeling staining with anti-DDX60 and anti-CD34 for biopsy specimens of proliferative LN showed that DDX60 was mainly expressed in the endothelial area (Figure 5(B)).

Discussion

Since GECs reportedly contribute to the glomerular filtration barrier *via* hemodynamic and physiologic cross-talk to podocytes [20], damages to GECs are believed to lead to the development of certain types of GN [15,18,19]. In clinical practice, viral infections are known

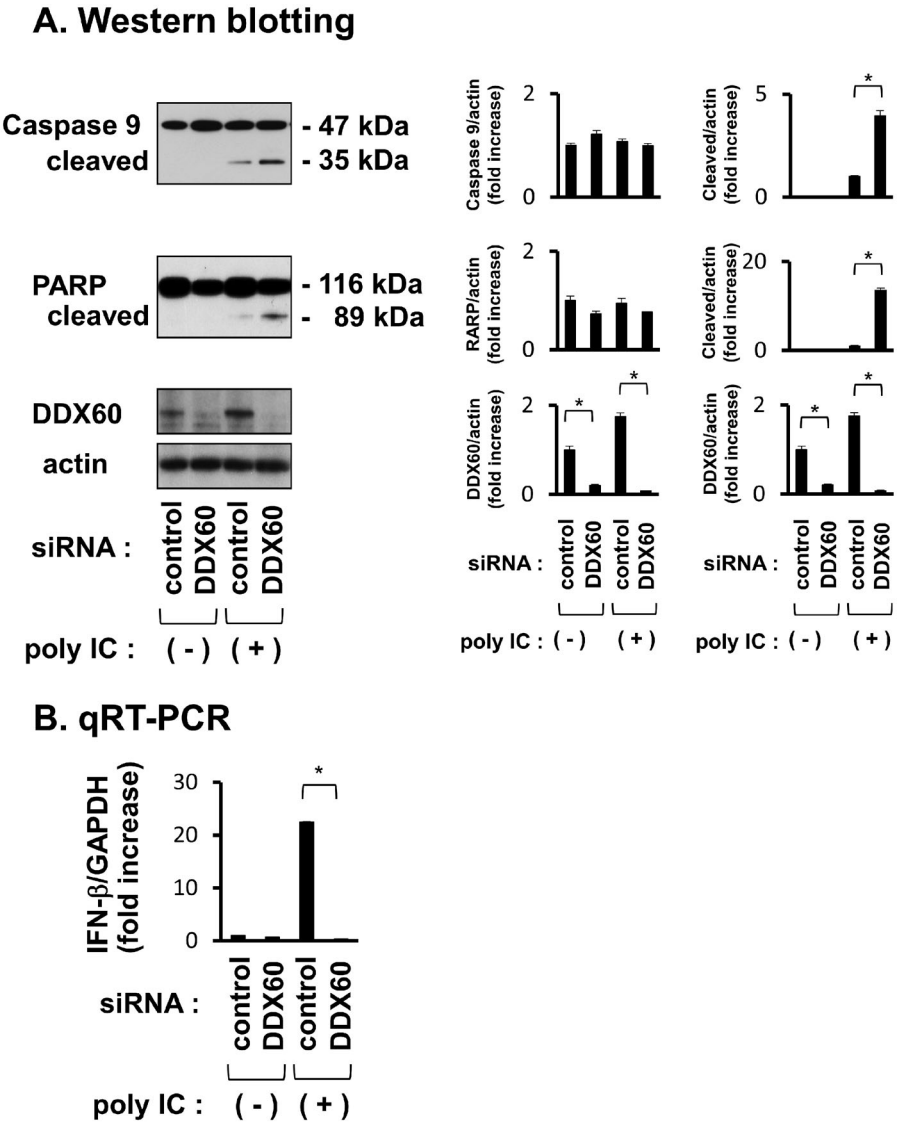

Figure 4. RNA interference against DDX60 followed by poly IC treatment induced the cleavage of caspase 9 and PARP. The cells were transfected with siRNA against DDX60 or a non-silencing negative control siRNA and incubated for 48 h. Subsequently, they were stimulated with 3 μg/mL poly IC. (A) After incubating for 6 h, following which they were lysed, and western blotting was performed for caspase 9, poly-ADP ribose polymerase (PARP), DDX60, and actin. The 47 kDa and 35 kDa bands of caspase 9 correspond to full length and cleaved form. The 116 kDa and 89 kDa bands correspond to full length and cleaved PARP, respectively. The integrated OD of the protein band of each molecule was quantified using Image J and was normalized to that of actin (B, right panel, $n = 3$, $^*p < 0.01$, *t*-test). (B) After incubating for 2 h, the cells were subjected to qRT-PCR analysis for IFN-β mRNA. ($n = 3$, $^*p < 0.01$, *t*-test).

to trigger the development of inflammatory GN or the worsening of preexisting GN [21]. Recognition of the molecular pattern of viral pathogens by TLR3 in intracellular endosomes and subsequent immunoreactions are important in host antiviral defenses [22]. Since GECs are directly exposed to circulating viral particles in the glomerulus, endothelial viral and noninfectious stimuli, such as endogenous ligands, can activate regional TLR3 signaling cascade [23]: the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways *via* transcriptional factors, leading to the subsequent release of type I IFN [22]. Thus, regional viral and "pseudo" viral immunoreactions, including proinflammatory cytokines/chemokines production *via* the activation of TLR3 signaling in the intrinsic glomerular cells have been postulated to be involved in the pathogenesis of LN [3,6,7,24]. Furthermore, sustained activation of type I IFN is believed to participate in LN pathogenesis [1,2].

In this study, considering the implications of TLR3 signaling in the pathogenesis of LN, we treated human GECs with poly IC and examined the expressions and

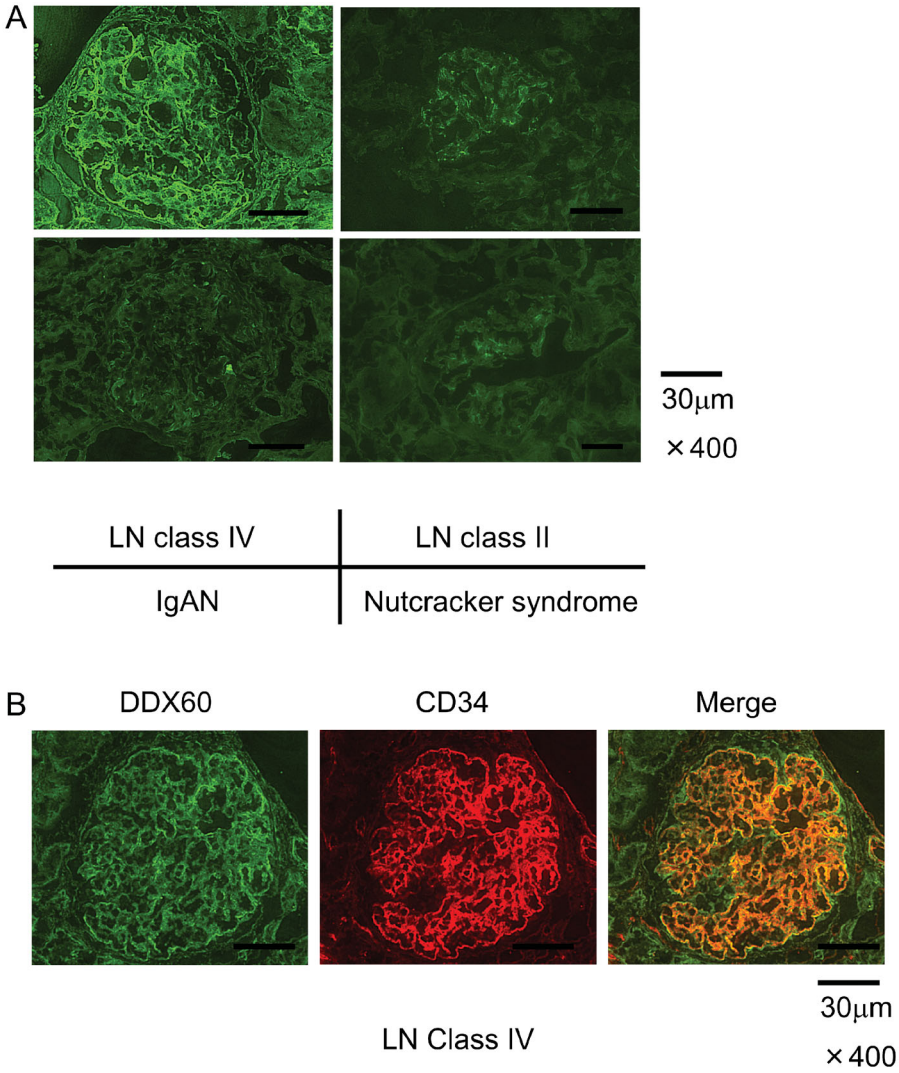

Figure 5. Immunofluorescence staining of DDX60 in renal biopsy specimens obtained from patients with proliferative LN [Class IV-G (A) in accordance with the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification for LN], proteinuric IgAN, and nutcracker syndrome (served as a non-inflammatory control), respectively. A significant increase in DDX60 immunoreactivity was observed in proliferative LN specimens, whereas immunoreactivity was negligible (A) in the other specimens (x400 magnification). Dual immunostaining of DDX60 and CD34 in renal biopsy specimens from a patient with proliferative LN (ISN/RPS 2003 classification class IV (A)). Positive staining of DDX60 (green) is detected mainly in the endothelial area (B, x400 magnification; Scale bar = 30 μm).

the role of an antiviral ISG, DDX60 which contributes to an inhibitor of viral replication [25]. We observed that DDX60 mRNA expression was induced by poly IC but not by R848 or CpG. This suggests that the expression of DDX60 in GECs is mainly regulated by TLR3 signaling. Knockdown of IFN-β decreased poly IC-induced DDX60 expression at both mRNA and protein level, whereas treatment of cells with r(h)IFN-β induced the expression of DDX60. The maximal level of IFN-β mRNA was observed at 2 h after treatment with poly IC, and DDX60 mRNA was observed to increase after 4 h. These results indicate that newly synthesized IFN-β, at least in part, mediates poly IC-induced expression of DDX60 in an autocrine and/or paracrine manner: a newly identified glomerular endothelial TLR3/IFN-β/DDX60 axis. Furthermore, we observed intense endothelial DDX60 expression in biopsy specimens from patients with diffuse proliferative LN but only trace or negligible DDX60 expression in specimens from patients with non-proliferative LN, IgAN or nutcracker syndrome; this result suggests that activated TLR3 signaling is involved in the glomerular lesions of patients with diffuse proliferative LN.

To examine the functional role of DDX60 in poly IC-treated GECs, we evaluated the effect of siRNA against DDX60. Because we found that poly IC treatment resulted in cell detachment from culture plate in DDX60-knockdown cells, whereas cells without

knockdown were intact, we speculated that DDX60 knockdown promoted apoptosis in GECs when TLR3 signaling was activated. Thus, we evaluated the effect of DDX60 knockdown on the expression of PARP, as a sensitive parameter for studying cell death [16,17]. PARP is also known as a post-translational modification of proteins with a multifunctional role in basic processes, such as DNA repair, transcription, cell proliferation, and death [16]. We found that DDX60 knockdown followed by poly IC treatment induced the proteolytic cleavage of 116 kDa PARP to its 89 kDa fragment [17], and of 47 kDa caspase to its 35 kDa fragment, which suggested that glomerular endothelial DDX60 expression might act as an anti-apoptotic molecule downstream of poly IC treatment; however, this theory remains speculative. DDX60 knockdown decreased IFN-β expression, and positive feedback between DDX60 and IFN-β may be involved in preventing excessive apoptosis. Dysregulated balance between pro-apoptosis and anti-apoptosis in GECs may be in the pathogenesis of LN.

Regarding the implication of apoptotic glomerular cells in the pathogenesis of LN, the availability of extracellular chromatin released from apoptotic glomerular cells has been reported to be a prerequisite for the binding of anti-dsDNA antibodies in the glomeruli, and this may be involved in nephritic processes in LN [26]. The binding of anti-dsDNA antibodies to instinct renal cells further triggers downstream inflammatory pathways, indicating that increased glomerular cell apoptosis is involved in LN progression [27]. Thus, the balance between apoptosis and anti-apoptosis is an important issue in the pathogenesis of inflammatory lesions in LN. To date, we observed that ISG15, a member of the ISG family, acts as a negative feedback molecule against excessive inflammation *via* TLR3 signaling activation in glomerular intrinsic cells [13]. Overall, glomerular endothelial DDX60 expression observed in biopsy specimens of patients with proliferative LN may act as a negative feedback molecule in the inflammatory reaction *via* TLR3 signaling, which supports the theory that it prevents excessive apoptosis; however, this theory needs to be explored in a future study. DDX60 is reportedly to degrade viral RNA [9]. Therefore, if poly IC-induced DDX60 could degrade extrinsic poly IC in GECs, such dsRNA decomposition activity may contribute to anti-apoptosis, although this theory remains speculative. Regarding this issue, further studies are needed. We believe that modulation of certain ISGs, such as DDX60 and ISG15, is a possible treatment option in patients with LN [13]. However, further detailed studies are needed to confirm this hypothesis.

This study has certain limitations. The molecular mechanism by which DDX60 functions as an anti-apoptotic molecule remains to be clarified. In addition, the number of biopsy specimens examined for DDX60 immunostaining was small. However, this is the first study to demonstrate TLR3 activation-induced expression of DDX60 in cultured human GECs and intense DDX60 expression in GECs in the biopsy specimen of patients with proliferative LN. We believe that modulating the upregulation of the regional innate immune system *via* TLR3 signaling may be a promising treatment target for LN.

Ethics statement

This article does not contain any studies with human participants or animals except for immunohistologic study using renal biopsy specimens, which was approved by the ethics committee of Hirosaki University Graduate School of Medicine (2018-098).

Author contributions

T.I. and H.T. designed and supervised the study. T.K., R.S., T.I., M.F., T.A., and K.T. performed experiments. T.K. and S.H. performed immunostaining. T.K. and H.T. wrote the manuscript. S.K., K.S. and K.T. gave technical support and conceptual advice. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data generated or analyzed during this study are included in this article.

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