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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. ELISA development.

Figure S2. eGFR stages versus plasma renalase.

Table S1. Listing of anti-RNLS antibodies evaluated.

Table S2. Selection of antibody pairs suitable for use in ELISA.

STROBE Statement.

Supplementary References.

Supplementary Methods.

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DNAJB9 Is Not Transcriptionally Upregulated in the Glomerulus in Fibrillary Glomerulonephritis

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Fibrillary glomerulonephritis (FGN) is an immune-complex–mediated GN with high rates of progression to end-stage kidney disease.[1,2] DnaJ homolog subfamily B member 9 (DNAJB9) is a sensitive and specific marker of FGN in kidney biopsies.[3–5] DNAJB9 is a heat-shock protein in the endoplasmic reticulum and is involved in the endoplasmic reticulum stress/unfolded response (UPR) pathway; it also binds aggregation-prone peptides.[6–9,S1–S3] Upregulation of other UPR proteins in FGN was not detected in mass spectrometry–based studies,[3] suggesting that the glomerular accumulation of DNAJB9 is not due to local upregulation of the UPR in glomeruli. In addition, serum levels of DNAJB9 were modestly increased in

patients with FGN, raising the possibility of local or systemic overexpression of this protein as a mechanism of disease.[S4,S5] In this study, we tested whether the mechanism of glomerular abundance of DNAJB9 was related to local upregulation of *DNAJB9* mRNA in glomeruli. Confocal microscopy and automated image analysis were performed and corroborated with DNAJB9 immunohistochemistry (IHC). To evaluate for the possibility of systemic manifestations, we assessed protein expression of DNAJB9 by IHC in extrarenal tissues from FGN and controls.

RESULTS

DNAJB9 RNA *In Situ* Hybridization and Immunohistochemistry

Kidney biopsies with FGN (n = 15 cases,171 glomeruli, median: 13) and non-FGN (n = 18 cases, 147 glomeruli, median: 9) including diabetic nephropathy (n = 6), AL amyloid (n = 4), cryoglobulinemic GN (n = 4), diffuse proliferative lupus nephritis (n = 2), and controls (allograft 3-month protocol biopsies, n = 2) were evaluated. By IHC, all FGN cases had glomerular reactivity with DNAJB9, and all non-FGN cases were negative. However, by RNA *in situ* hybridization using RNAscope, DNAJB9 mRNA signals were present in FGN, non-FGN, and controls (Figure 1). Signals were identified in podocyte and mesangial regions, in tubulointerstitial and vascular tissue. For FGN versus non-FGN, there were no significant differences in glomerular DNAJB9 mRNA signals, DNAJB9 per cell, number of nuclei, DNAJB9 signal intensity, or analyzed glomerular area (Table 1). There were no significant differences in DNAJB9/4′,6-diamidino-2-phenylindole signal ratios among non-FGN cases. When analyzed by combined total glomeruli rather than by case medians, FGN glomeruli had fewer DNAJB9 mRNA signals (334 vs. 421, $P = 0.001$), lower DNAJB9/4′,6-diamidino-

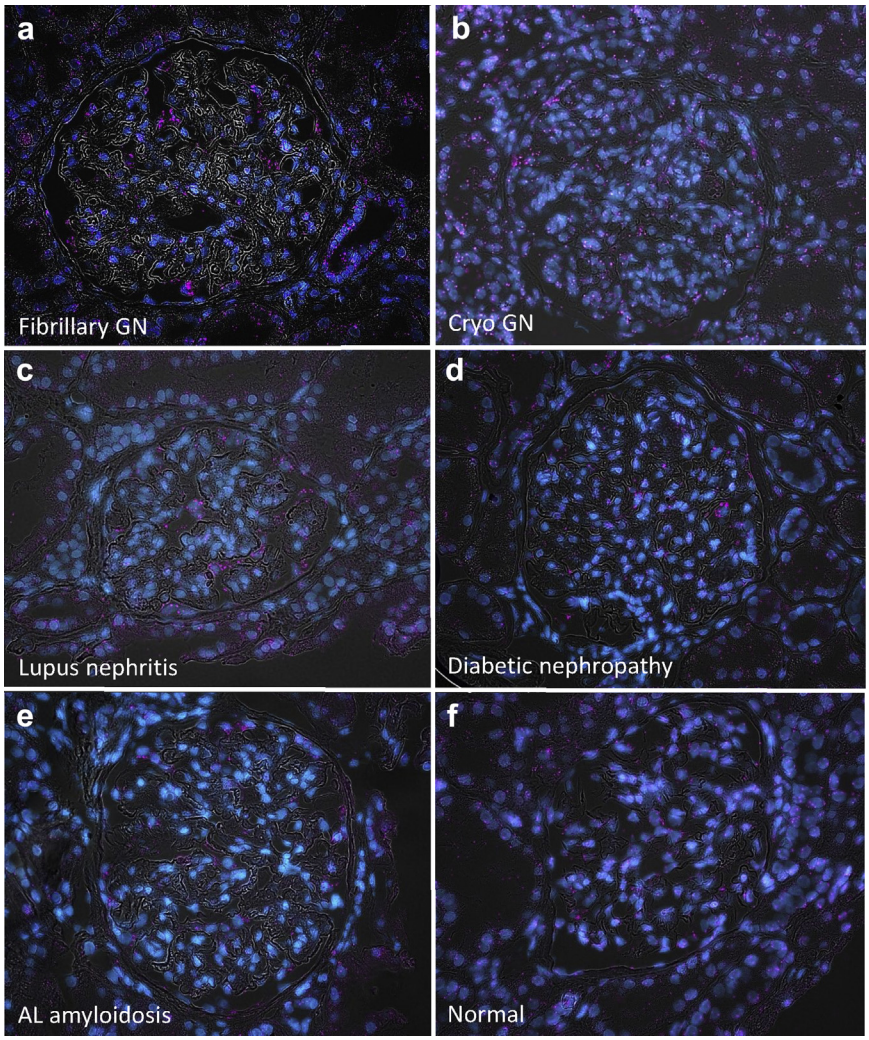

Figure 1. (a–f) DnaJ homolog subfamily B member 9 (DNAJB9) mRNA signals are seen in podocyte, mesangial, and endothelial cell regions as well as the tubulointerstitium. There is no significant difference in DNAJB9 mRNA signals or signal per cell ratios in glomeruli for fibrillary glomerulonephritis (GN) versus non-fibrillary GN controls (DNAJB9/ 4′,6-diamidino-2-phenylindole/transmitted light, at original magnification x200).

Table 1. DnaJ homolog subfamily B member 9 (DNAJB9) mRNA signals in fibrillary glomerulonephritis (FGN) and non-FGN patients

Diagnosis	No. cases	DNAJB9 IHC	Glomerular DNAJB9 mRNA signals	Nuclei (DAPI signals)	DNAJB9 mRNA signals per cell (DNAJB9/DAPI ratio)	DNAJB9 mRNA signal intensity	Analyzed glomerular area (μm^2)
FGN	15	Positive	332 (253–656)	163 (114–198)	2.4 (1.9–3.7)	562 (437–903)	20775 (15806–32363)
All non-FGN combined	18	Negative	366 (300–610)	137 (107–195)	3.1 (2.2–3.7)	718 (490–1086)	19081 (15211–23493)
Diabetic nephropathy	6	Negative	461 (288–810)	189 (124–196)	2.9 (2.1–4.3)	714 (602–1478)	17470 (11661–21891)
AL amyloid	4	Negative	298 (267–360)	99 (59–129)	3.6 (2.4–4.3)	487 (432–724)	17540 (12820–22260)
Cryo GN	4	Negative	681 (392–1205)	240 (138–319)	3.0 (2.3–3.5)	1101 (1004–1311)	27193 (16008–29670)
Lupus nephritis	2	Negative	278 (212–344)	142 (112–172)	2.0 (1.8–22)	576 (463–690)	22815 (15047–30583)
Control	2	Negative	399 (309–488)	113 (91–135)	3.1 (3.1–3.2)	782 (499–1064)	18946 (17201–20691)
P value		<0.001	0.60	0.74	0.46	0.24	0.63

DAPI, 4′,6-diamidino-2-phenylindole; IHC, immunohistochemistry.
Results are provided as median and interquartile range.

2-phenylindole ratios (2.5 vs. 3.2, $P = 0.0002$), and lower DNAJB9 signal intensity (595 vs. 758, $P = 0.004$) with similar 4′,6-diamidino-2-phenylindole signals (153 vs. 158, $P = 0.15$) and analyzed glomerular area (20,556 vs. 21,056 μm^2, $P = 0.79$) compared with non-FGN glomeruli. Overall, we found no correlation between glomerular DNAJB9 protein expression and DNAJB9 mRNA signals by *in situ* hybridization.

p53 Immunohistochemistry in Kidney Biopsies

DNAJB9 is a downstream target and negative feedback regulator of p53, a tumor suppressor; it has been shown to inhibit the pro-apoptotic function of p53 via interaction with its J domain.[8] Given that no significant differences were detected in DNAJB9 mRNA expression, we tested whether there were differences in this downstream target via IHC in a subset of the same biopsy cohort. p53 expression was infrequent in glomerular cells in all cases (0–2 cells positive), with no significant differences between FGN (n = 5) and non-FGN (n = 5 cases), providing evidence against dysregulation of p53.

DNAJB9 Immunohistochemistry in Non-Renal Tissue

To assess DNAJB9 expression in patients with other systemic conditions, we tested non-renal tissue from 5 FGN patients, including liver biopsy with cirrhosis due to hepatitis C virus (HCV), skin biopsy with fibrosing dermatitis concerning for early morphea, and skin biopsy with spongiotic dermatitis with eosinophils. These showed no significant DNAJB9 expression by IHC. One patient was pregnant at the time of FGN

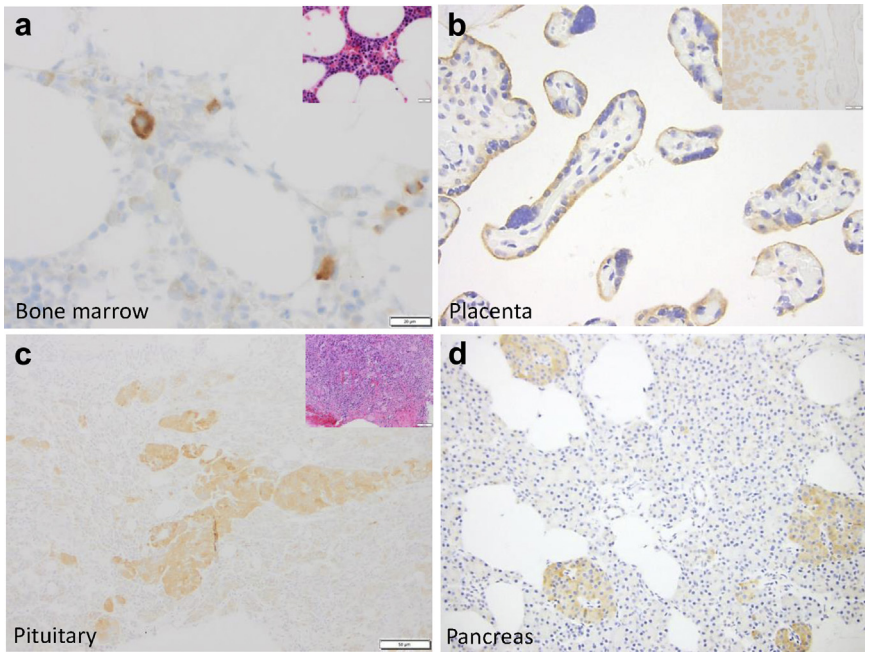

Figure 2. (a) Rare bone marrow cell from patients with and without fibrillary glomerulonephritis show focal staining for DnaJ homolog subfamily B member 9 (DNAJB9; with hematoxylin and eosin inset, original magnification x400). (b) Trophoblast and decidual cells (inset) from patients with and without FGN weakly express DNAJB9 (original magnification x200). (c) Scattered normal anterior pituitary cells (with hematoxylin and eosin inset, original magnification x200) and (d) pancreatic islet cells express DNAJB9 by immunohistochemistry (original magnification x200).

diagnosis; her placental tissue showed modest staining in trophoblast and decidual cells, similar to control placental tissue (n = 5; Figure 2). One patient with FGN had a concurrent bone marrow biopsy demonstrating normocellular marrow with no evidence of a lymphoproliferative disorder. Weak DNAJB9 cytoplasmic staining was present in scattered marrow cells (<5%) of uncertain type (Figure 2). A similar degree of DNAJB9 staining of bone marrow cells was present in 3 non-FGN patients (1 with 10% plasma cell neoplasm, 1 with myelodysplastic syndrome, 1 normal). DNAJB9 was negative in 2 marrow biopsies (1 with 10% plasma cell neoplasm and AL amyloidosis, 1 normal).

Given reported variable expression of DNAJB9 in other organs,[S6] we tested whether DNAJB9 is detected by IHC using an antibody and titer clinically specific for FGN, in patients without FGN. We identified patchy weak DNAJB9 staining in normal anterior pituitary (n = 6) without prominent expression in 5 common subtypes of pituitary adenomas (n = 15; Figure 2). Weak expression was seen in pancreatic islet cells (n = 1). Thus, no correlation was observed between extrarenal DNAJB9 staining and the presence of fibrillary GN.

DISCUSSION

In this study, we demonstrate that DNAJB9 protein expression in glomeruli of patients with fibrillary GN is not dependent on glomerular transcriptional upregulation of DNAJB9. Additionally, although FGN may be seen in the setting of systemic processes, we found no correlation between the presence of fibrillary GN and extrarenal DNAJB9 staining in a limited number of patients with autoimmune disease, hepatitis C virus, and pregnancy, nor in hematopoietic cells, providing evidence against systemic DNAJB9 upregulation in patients with FGN and these conditions. Splenic involvement has rarely been described in patients with FGN,[5,S7] supporting a more systemic mechanism that can include extrarenal deposits in some organs or settings.

Weaknesses in this study include limitations of RNA *in situ* hybridization studies: quantification is based on specific probes with supervised image analysis and is performed on 1 section rather than the entire glomerulus. Our study was performed at a single time point in disease, and it is possible that the UPR was upregulated prior to onset and subsequently normalized. Decreased local degradation of DNAJB9 protein in glomerular cells, rather than increased production, represents an additional potential mechanism of FGN not tested in this study. Like most cellular processes, the UPR is controlled by several mechanisms and it is possible that UPR alterations not directly related to DNAJB9 mRNA production—and thus not evaluated by the methodology in this study—contribute to the development of FGN.

In the context of increased serum levels of DNAJB9 in patients with FGN[S4] —and when compared with other immune-complex–mediated glomerular diseases—fibrillary GN may share mechanistic similarities with IgA nephropathy. Namely, an increased amount of circulating galactose-deficient IgA1 in IgA nephropathy (or DNAJB9 in FGN) is associated with disease in some patients, but an additional autoantibody response, glomerular, and host factors are required for disease development.[S8]

Thus, we demonstrate that DNAJB9 protein expression in FGN is not due to glomerular transcriptional upregulation of DNAJB9. Despite the role of DNAJB9 as a regulator of the UPR, our findings corroborate proteomic studies in which other components of the UPR were not upregulated in FGN,[3] suggesting that local activation of the UPR does not drive the pathogenesis of FGN. Taken together, the findings indirectly support alternate mechanisms—such as an auto-antibody, circulating source, and/or secondary DNAJB9 binding due to recognition of aggregation-prone motifs on misfolded IgG molecules[S9]—for the disease-defining DNAJB9 glomerular protein abundance in fibrillary GN.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplementary Methods.

Supplementary References.

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