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Increased urinary exosomal SYT17 levels in chronic active antibody-mediated rejection after kidney transplantation via the IL-6 amplifier

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Abstract

Chronic active antibody-mediated rejection (CAAMR) is a particular problem in kidney transplantation, and about 25% of grafts are lost by CAAMR. Further, the pathogenesis remains unclear, and there is no effective cure or marker. We previously found that a hyper NFκB-activating mechanism in non-immune cells, called the IL-6 amplifier, is induced by the co-activation of NFκB and STAT3, and that this activation can develop various chronic inflammatory diseases. Here we show that synaptotagmin-17 (SYT17) is increased in an exosomal fraction of the urine from CAAMR patients, and that this increase is associated with activation of the IL-6 amplifier. Immunohistochemistry showed that SYT17 protein expression was increased in renal tubule cells of the CAAMR group. While SYT17 protein was not detectable in whole urine samples by western blotting, urinary exosomal SYT17 levels were significantly elevated in the CAAMR group compared to three other histology groups (normal, interstitial fibrosis and tubular atrophy, and calcineurin inhibitors toxicity) after kidney transplantation. On the other hand, current clinical laboratory data could not differentiate the CAAMR group from these groups. These data suggest that urinary exosomal SYT17 is a potential diagnostic marker for CAAMR.

Introduction

Kidney transplantation (KTx) is the most effective replacement therapy for end-stage renal diseases. Data from the U.S. Renal Data System (2013) (https://www.usrds.org/) indicate that survival after KTx is significantly better than that of patients treated with dialysis. Furthermore, recent improvements of immunosuppressants have significantly reduced the risk of acute rejection (1). However, chronic allograft rejection, which represents relatively slow and progressive diseases such as chronic active antibody-mediated rejection (CAAMR), has proven a more difficult problem (1,2). Pathological changes during chronic rejection occur gradually even without clinical manifestations, followed by dysregulation of the function of the kidney transplant. Allograft biopsies are considered the gold standard for the diagnosis of chronic allograft rejection (3), but the outcome after diagnosis, especially by episode biopsies, is still poor. A reliable clinical index to predict chronic rejection and determine the timing of biopsy is therefore awaited. Liquid biopsy is a technology that potentially predicts outcomes of diagnosis and treatment by analyzing body fluid samples (4), and urine may be one type of liquid biopsy useful as a non-invasive diagnostic marker for kidney dysfunction due to its close relationship with the renal system (5).

Recent studies have suggested that chronic inflammation is critical for the development of various diseases (6,7). We have reported a molecular mechanism of chronic inflammation, the IL-6 amplifier

(8-10). The IL-6 amplifier is triggered by the simultaneous activation of two transcriptional factors, NFκB and STAT3, in non-immune cells such as synovial cells, fibroblasts, and endothelial cells (8-10). The co-activation of NFκB and STAT3 enhances NFκB signaling to synergistically promote the production of various NFκB-driven inflammatory mediators including IL-6, chemokines and growth factors, and induce chronic inflammation in the affected tissues (8-10). Inhibition of the IL-6 amplifier significantly improves pathological outcomes in mouse disease models including allogenic transplantation rejection, multiple sclerosis, dermatitis, uveoretinitis and rheumatoid arthritis (7,8,11-19). Consistently, its activation is observed in human allogeneic lung transplantation with chronic rejection phenotypes, and target molecules of the IL-6 amplifier are elevated in patients with inflammatory diseases such as atherosclerosis, multiple sclerosis, lung transplant rejection, keloid, and rheumatoid arthritis (7,13,20). Genome-wide screenings have identified over 1,000 positive regulators and 500 target genes of the IL-6 amplifier, and human disease–associated genes are highly enriched in the gene lists (7). Additionally, it has been demonstrated that some disease-associated genes positively regulate its activation (18).

Synaptotagmin-17 (SYT17), also known as Brain/kidney protein, is selectively expressed in the brain and kidney, and contains two C2-like domains structurally homologous to those seen in other synaptotagmins (21). Although SYT17 has no transmembrane domain, it can bind to the plasma

77 membrane via its N-terminus (22). It is reported that the protein expression of SYT17 is increased in
78 the hippocampus in a kainate-induced rat seizure model (23) and in proximal tubules in the kidney
79 after ischemia-reperfusion injury (24), indicating that the expression level of SYT17 changes in
80 pathological conditions. However, the functional role of SYT17 in a pathological and/or
81 inflammatory condition in the kidney remains unknown.
82
83 A recent study suggested that blockade of the IL-6 signaling pathway improved the outcomes of
84 CAAMR (25), suggesting the functional involvement of IL-6-producing mechanisms including the
85 IL-6 amplifier in CAAMR. We here aimed to explore novel urinary diagnostic markers for CAAMR
86 by analyzing genes associated with the IL-6 amplifier and identified urinary exosomal SYT17 as a
87 potential candidate.
88

Materials and Methods

Patients and urine samples

Urine samples were obtained from healthy volunteers, chronic kidney disease (CKD) patients, and KTx patients between 2016 and 2019. Samples were collected at Hokkaido University Hospital and Sapporo City General Hospital under the approval of clinical research No. 014-0391 and No. 015-0220. KTx patient samples were collected at the time of the protocol biopsy, and samples at the episode biopsy were excluded. All KTx patients received standard immunosuppressive treatment with prednisone, mycophenolate, and tacrolimus or cyclosporine. Samples were centrifuged at 1,500 rpm for 5 minutes, and the supernatant was stored at -80℃. Based on the histological results of the biopsy, KTx patients were divided into 4 groups (NED, normal histology; IF/TA, interstitial fibrosis and tubular atrophy; CNI-T, calcineurin inhibitors toxicity; and CAAMR). Patient information is shown in Table 1.

Immunohistochemistry

To confirm the expression of SYT17 protein and proteins related to the IL-6 amplifier in kidney tissue, we performed immunohistochemistry using frozen or paraffin sections of transplanted kidney biopsies. All samples were surplus specimens of the protocol biopsy. The frozen samples were embedded in compound and stored at -80℃. The cut surface was covered with an adhesive film

(Cryofilm type IIC9, SECTION-LAB, Japan), and the frozen sections (10 μm) were prepared with a microtome (Leica CM3050S, Germany) according to a method described previously(26). The resulting sections were fixed with formaldehyde after dehydration with ethanol. As a first antibody reaction, anti-SYT17 antibody (1/200, Proteintech) and Rabbit IgG Isotype Control (1/5,000, Cell Signaling Technology) were reacted overnight at 4℃. The second antibody reaction was carried out with donkey anti-rabbit IgG (H+L), Alexa Fluor 546 (1/1000, Life Technologies) and Hoechst 33342 trihydrochloride trihydrate (1/3,000, Life Technologies) at room temperature for 1 hour. The resulting sections were embedded with 25% glycerol. Paraffin sections were prepared after formalin fixation of the specimen. Immunohistochemistry of serial sections was performed using rabbit anti-STAT3 pY705 (1/200, Cell Signaling Technology, Danvers, MA), rabbit anti-phospho NFκB p65 Ser276 (1/400, Sigma Aldrich), or control antibody (Cell Signaling Technology). The rabbit IgG Elite ABC Kit and ImmPACT DAB (Vector Laboratories, Burlingame, CA) were used for signal enhancement and visualization, respectively. For double staining of SYT17 with STAT3 pY705 or with phospho NFκB p65 Ser276, the Polink DS-RR-Hu/Ms A Kit (GBI Labs, Bothell, WA) was used with a slight modification. Sections were analyzed with a BZ-9000 microscope (KEYENCE).

Isolation of urinary exosome

We used 1 ml of urine to extract exosomes using the MagCapture Exosome Isolation Kit PS

(Fujifilm Wako Pure Chemical Industries, Ltd.) according to the instruction protocol. A protease inhibitor (Z-Leu-Leu-Leu-al®, Sigma Aldrich) was added to the extracted urinary exosome samples, which were then stored at -20°C until analysis.

Western blotting

Urinary exosome samples were lysed with SDS sample buffer (10% SDS, 10% glycerol, 0.04% bromophenol blue, 0.5 M Tris HCl [pH 6.8], and 10% 2-mercaptoethanol) and boiled at 60°C for 10 minutes. The exosome lysates were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). Immunoblotting was performed using Can Get Signal Immunoreaction Enhancer Solution (Toyobo) according to the manufacturer's protocol. The antibodies used were anti-SYT17(1/2,500, Atlas antibody), anti-CD9 (1/1,000, System Bioscience), goat anti-rabbit lgG (H+L) HRP secondary antibody (1/10,000, Thermo Fisher Scientific), and goat anti-rabbit HRP secondary antibody (1/20,000, System Bioscience). Signal enhancement solution was used for the detection of SYT17 (Pierce® Western Blot Signal Enhancer, Thermo Fisher Scientific). Proteins were visualized by Pierce™ ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions. The films were developed by using an automatic X-ray film developing apparatus (FPM 100, Fuji Medical Film). Western blot results were quantified using ImageJ software.

143

144 **Luciferase assay**

145 Either the pGL4.20 [luc2/Puro] vector with IL-6-promoter sequence (Promega, Tokyo) or the
146 pGL4.32 [luc2P/NFκB-RE/Hygro] vector (Promega, Tokyo), pGL4.74 [hRluc/TK] vector (Promega),
147 and SYT17 expressing vector (pEF-BOS vector (27)) were transiently cotransfected into 293T cells
148 by using polyethylenimine. Cells were harvested 24 hours after the transfection and stimulated with
149 TNFα (50 ng/ml) for 6 hours. The luciferase activities of the total cell lysates were measured using
150 the Dual-Luciferase Reporter Assay System (Promega).

151

152 **siRNA-mediated knockdown**

153 Small interfering RNAs (siRNAs) were transfected into human renal proximal tubular epithelial cells
154 (RPTEC) or human renal glomerular endothelial cells (HRGEC) by using Lipofectamine
155 RNAiMAX (Thermo Fisher Scientific). The cells were incubated for 48 hours, and after 2 hours of
156 starvation, they were stimulated with human IL-6 (100 ng/ml) plus human soluble IL-6Rα (100
157 ng/ml), and human IL-17 (50 ng/ml) or human TNFα (50 ng/ml) for 3 hours to activate STAT3 and
158 NFκB. Total RNA samples were prepared from the cells using an RNA Extraction Kit (Toyobo,
159 Tokyo, Japan) and then subjected to reverse transcription and quantitative PCR. For confocal
160 microscopy, siRNA-transfected RPTEC were seeded on channel microscopy slides (μ-Slide VI, Ibidi

GmbH, Germany) and cultured for 48 hours. After 3 hours of starvation, they were stimulated with human IL-6 (100 ng/ml) plus human soluble IL-6Rα (100 ng/ml), and human IL-17 (50 ng/ml) for 30 min at 37°C. The cells were then fixed in 4% formaldehyde/PBS and permeabilized in ice-cold 100% methanol. After blocking non-specific binding using 10% FCS in the Perm/Wash buffer (BD Biosciences), the cells were stained using rabbit anti-SYT17 (1/200, Proteintech), rabbit anti-STAT3 pY705 (1/100, Cell Signaling Technology), or rabbit anti-NFκB p65 (1/50, Santa Cruz Biotechnology) overnight at 4°C. Alexa Flour 488-conjugated goat anti-rabbit IgG (H+L) (Invitrogen) was used as a secondary antibody to visualize the signals. Hoechst33342 (Invitrogen) was used for nuclear staining. Fluorescence signals were observed using a confocal microscope LSM980 (Zeiss). The signal intensity of SYT17 was calculated using ImageJ software.

Statistical analysis

The sample size in each KTx group was set to 20 for feasibility reasons. Assuming an effect size of about 1.4 and two-sided significance level of 0.05, the sample sizes provide 80% power using Tukey’s test. The chi-square test or Student’s t test was used for two-item test. Tukey's test or Dunnett’s test was used for multi-item tests. The receiver operating characteristic (ROC) curve in figure 4 shows the performance of a binary classifying system (rejection versus nonrejection). We used JMP® 14 (SAS Institute Inc., Cary, NC, USA) or PRISM 8 for the statistical analysis. P values

179 less than 0.05 were considered significant differences between groups (*P <0.05, **P <0.01, and

180 ***P < 0.001). Error bars reflect the standard deviation (SD) or standard error of the mean (SEM)

181 for each test group.

182

Results

SYT17 is highly expressed in tubular cells of CAAMR patients.

The genes required for activation of the IL-6 amplifier have been identified and include the synaptotagmin (SYT) family. In this study**,** we focused on SYT17 because it is selectively expressed in the brain and kidney (21). Tubular cells are an interface of urine excretion and a potential cell type to affect urinary content. Immunohistochemistry analysis revealed that SYT17 protein was expressed strongly in the CAAMR group compared to other KTx groups including normal histology (NED), interstitial fibrosis and tubular atrophy (IF/TA), and calcineurin inhibitors toxicity (CNI-T) (Figs. 1A and B). We found that SYT17 was mainly expressed in the tubular cells of the kidney but not other cell populations including endothelial cells (glomeruli) and epithelial cells from the CAAMR group (Fig. 1C and Supplementary Fig. 1). We also found that the localization of SYT17 was mainly in the cytoplasm (Supplementary Fig. 2). Thus, SYT17 is highly expressed in the tubular cells of CAAMR patients.

The IL-6 amplifier is activated in tubular cells of CAAMR patients.

We have reported that the IL-6 amplifier is triggered by the simultaneous activation of two transcriptional factors, NFκB and STAT3, in non-immune cells, and that this activation is associated with various inflammatory pathologies (7,8,11-19). The co-activation of NFκB and STAT3

synergistically promotes the production of various NFκB-target genes including IL-6 (7,8,11-19). We hypothesized that the IL-6 amplifier is induced in the tubular cells of CAAMR. Consistent with our idea, activated (phosphorylated) NFκB p65 and STAT3 were more evident in the tubular region of the CAAMR group compared to the other KTx groups (Figs. 2A and B). In addition, a transcriptome analysis using human tubular cells showed that the IL-6 amplifier was activated by co-stimulation with TNFα and IL-6, which also activate NFκB and STAT3, respectively, and that this activation induced SYT17 expression (Supplementary Fig. 3A). Interestingly, a deficiency of SYT17 suppressed IL-6 expression and activation of the IL-6 amplifier, while forced expression of SYT17 enhanced NFκB promoter activity (Supplementary Figs. 3B and 3C). Moreover, SYT17 knockdown cells showed reduced nuclear localization of NFκB p65 after stimulation with IL-6 and TNFα, whereas no difference was observed for the nuclear localization of phospho STAT3 (Supplementary Figs. 4A and 4B). SYT17 siRNA significantly decreased the expression of SYT17 protein in knockdown cells (Supplementary Fig. 4C). These results suggest that SYT17 is positively involved in NFκB signaling but not STAT3 signaling. They also suggest that the expression of SYT17 is induced in CAAMR tubular cells through activation of the IL-6 amplifier to promote an inflammatory status in the grafts, most likely via positive feedback.

SYT17 is highly present in the urinary exosomal fraction of CAAMR patients.

SYT proteins are a family of membrane trafficking proteins, and it is reported that SYT4 is released extracellularly via exosomes (28). We therefore hypothesized that the SYT17 induction in CAAMR tubular cells may correlate with an elevated level of exosomal SYT17 secreted in urine. A western blotting analysis was performed to evaluate SYT17 protein levels in urine, but no SYT17 protein was detected using whole urine samples (Fig. 3A). On the other hand, SYT17 proteins were detectable in urinary exosomal fractions, particularly in the urine of CAAMR patients (Fig. 3B). We investigated the SYT17 protein level in urinary exosomal fractions normalized to the expression level of an exosomal marker, CD9 (29), and found a high enrichment of SYT17 in exosomes from the urine of CAAMR patients compared to healthy volunteers and individuals in the KTx NED group (Fig. 3C). These results suggest that SYT17 in urinary exosomes can be a potential diagnostic marker for CAAMR.

Exosomal SYT17 can potentially be a novel diagnostic marker for CAAMR.

To assess the possibility of exosomal SYT17 as a marker for CAAMR, we examined SYT17 levels in urinary exosome samples from other KTx groups with increasing patient numbers. CAAMR patients had significantly higher urinary exosomal SYT17 protein levels than the other groups (Fig. 4A; healthy: 0.12±0.09, NED: 0.13±0.17, IF/TA: 0.22±0.35, CNI-T: 0.40±0.35, CAAMR: 1.34±1.05,

236 $p<0.01$ by Tukey's test). Importantly, current clinical markers including
237 N-acetyl-β-D-glucosaminidase (NAG), estimated glomerular filtration rate (eGFR), and proteinuria
238 were unable to differentiate the CAAMR group from the other KTx groups (Fig. 4B-4D), and a
239 significant correlation of the SYT17 levels with these clinical parameters was not observed
240 (Supplementary Fig. 5). The ROC curve of SYT17/CD9 showed an area under the curve (AUC) of
241 0.82 with intermediate accuracy results (Fig. 4E). These results suggest that urinary exosomal
242 SYT17 levels may be a novel, non-invasive diagnostic marker candidate for CAAMR.
243

Discussion

In this study, we investigated SYT17 as a potential diagnostic marker for CAAMR. Despite the several reports of markers for acute rejection (30-36), non-invasive markers for CAAMR are currently not available (37). The NIH Biomarkers Definitions Working Group defined a marker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (38). Current clinical markers for kidney functions including NAG, proteinuria, and eGFR have low disease specificity, and often show outliers after CAAMR has progressed (30,39,40). Moreover, there are patients with "subclinical rejection", who are diagnosed histopathologically by chance even without clinical dysfunction of the transplanted kidneys (35). Allograft biopsy is the current gold standard for the diagnosis of transplant rejection. However, needle biopsy analysis not only has complications, but a tendency to underestimate the grade of the rejection or missing the diseased area (41,42). Therefore, novel, non-invasive markers for CAAMR that accurately reflect graft function are highly desired for clinical use. In this context, urine is an ideal sample that can be collected non-invasively.

Exosomes are extracellular vesicles smaller than 150 nm in diameter and are actively secreted from cells (43,44). Exosomes play a role in cell-to-cell communications and contain information

molecules such as RNA or proteins. The molecules included in/on exosomes vary with the type of cell. Therefore, in the field of liquid biopsies, researchers have considered exosomes secreted from disease-specific cells, such as immune cells or cancer cells, as diagnostic markers (45,46). Previous reports have shown that exosomes are contained in all body fluids tested such as urine and blood, and are released from epithelial cells that are in contact with these fluids (45,46). It is reported that about 70% of urinary proteins are derived from the kidney, and urinary exosomes are delivered from tissues that contact urine, including the kidney, ureter, bladder and prostate (47-50). Hence, we hypothesized that some urinary exosomal proteins from KTx patients are derived from transplanted kidney, which might inform the functional status of the allografts. Here we showed that SYT17 was highly enriched in urinary exosomes from patients with CAAMR. Our immunohistochemistry data suggested that SYT17 protein is expressed in the distal tubular cells of CAAMR patients. In agreement with this observation, it is reported that many urinary exosomal proteins are secreted from renal distal tubular cells (46,51). On the other hand, it is generally considered that donor-specific anti-HLA antibody (DSA) is a trigger for CAAMR, and the deposition of DSA is mainly observed in glomerular epithelial cells (52). In the present study, however, SYT17 was mainly expressed in tubular epithelial cells from CAAMR patients. Tubular epithelial cells might have high sensitivity for inflammatory cytokines such as IL-6, IL-17a, and TNFα. Additionally, even small amounts of soluble inflammatory factors such as IL-6, TNFα, and growth factors released from the glomerulus

may induce the production of SYT17. This idea is supported by the upregulation of SYT17 by IL-6 and TNFα stimulation in tubular epithelial cells (Supplementary Fig. 3). The concept of the IL-6 amplifier is a hyper activation of NFκB in nonimmune cells caused by the simultaneous activation of STAT3 and NFκB. Co-stimulation with TNFα and IL-6, which are stimulators of NFκB and STAT3, respectively, is a typical way to activate the IL-6 amplifier. SYT17 expression is mainly observed in tubular cells expressing p-STAT3 and p-p65 in clinical samples of CAAMR (Fig. 2B). In addition, an open-access database of transcription factor binding (53) detected several putative NFκB-binding sites within the 3-kb promoter region of the SYT17 gene. These lines of evidence strongly suggested that SYT17 induced by the amplifier activation enhances inflammatory responses in the tubular regions of CAAMR.

Moreover, we found evidence that SYT17 itself positively regulated activation of the IL-6 amplifier (Supplementary Figs. 3 and 4), suggesting that SYT17 might also be not only a target but also a regulator of this pro-inflammatory mechanism via the IL-6 amplifier. Consistently, a human protein interactome study suggested that SYT17 binds with a TNFα signaling component, TRAF2 (54). On the other hand, it is reported that the overexpression of another member of the synaptotagmin family, SYT1, binds to NEMO to suppress NFκB-promoter activity (55). Therefore, synaptotagmin family members may have reciprocal roles in NFκB activation, although the binding capacity of SYT17 to

TRAF2, NEMO or other components of NFκB signaling was not examined in this study. The precise mechanism of action of SYT17 for NFκB activation is a matter of future study.

It is also reported that anti–IL-6 receptor monoclonal antibody provides good long-term outcomes for CAAMR patients (25), further suggesting the involvement of the IL-6 amplifier in CAAMR. Moreover, in addition to higher SYT17 levels in urine exosomes of CAAMR patients, we found evidence that the same is true for chronic kidney disease patients (Supplementary Fig. 6). Thus, SYT17 could be a biomarker candidate and therapeutic target for kidney inflammatory diseases and conditions including CAAMR and chronic kidney diseases.

To conclude, the absence of an effective biomarker for CAAMR prediction led us to discovering urinary exosomal SYT17 levels as a candidate. Three stages are needed for markers to be used in clinical practice: discovery, validation, and implementation (56). Our current study is only the first stage. A long-term prospective observation is necessary to assess if SYT17 satisfies the other two.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

325 **Table 1. Patient characteristics**

	NED (n=20)	IF/TA (n=19)	CNI-T (n=17)	CAAMR (n=22)	p value
Gender, Male	60%	47%	76%	68%	NS[*1]
Age at transplantation	43±22	29±20	**48**±16	26±19	<0.05[*2]
Years after transplantation	**3.1**±4.1	9.3±6.3	6.9±4.7	8.9±4.2	<0.01[*2]
ABO incompatible, yes	**45**%	16%	29%	5%	<0.05[*1]
Living donor, yes	85%	74%	76%	77%	NS[*1]
Kidney age# (year)	56±11	56±15	58±12	56±11	NS[*2]
Dialysis period (year)	3.4±5.8	4.1±6.8	6.0±6.9	4.7±8.7	NS[*2]
HLA mismatch	3.2±1.8	2.6±1.5	2.8±1.5	2.8±0.6	NS[*2]

326

327 Significant differences were observed in age at transplantation, observation period, and ABO

328 incompatible transplantation. Numbers underlined have significant differences from the other

groups.

Kidney age# = Donor age at transplant + Observe period.

*1: chi squared test, *2: Tukey's test. NS = no significant difference.

Figure legends

Figure 1. SYT17 is highly expressed in tubular cells from CAAMR patients

A: Immunohistochemistry of biopsy specimens from KTx patients with normal histology (NED), interstitial fibrosis and tubular atrophy (IF/TA), calcineurin inhibitors toxicity (CNI-T), and chronic active antibody-mediated rejection (CAAMR) using anti-SYT17. Representative images are shown. Scale bars are 100 μm.

B: Fluorescence intensity of the SYT17 signals in (A) were quantified using ImageJ. The vertical axis represents pixels. *** $P < 0.001$ by Tukey's test. (NED: 0.08±0.07, IF/TA: 0.83±0.39, CNI-T: 0.80±0.93, CAAMR: 8.85±2.09; n = 6 each).

C: Immunohistochemistry of SYT17 in CAAMR and NED samples at higher magnification. Yellow and blue arrows indicate tubular cells and glomeruli, respectively. Representative images of a single patient sample are shown for each group. Scale bars are 100 μm.

Figure 2. NFκB and STAT3 activation in tubular cells of CAAMR patients

A: Immunohistochemistry of phosphorylated NFκB p65 (p-p65) and phosphorylated STAT3

(p-STAT3) using biopsy specimens from KTx patients with NED, IF/TA, CNI-T, or CAAMR. Representative images of a single patient sample are shown for each group. Scale bars are 100 μm. Red arrows are examples of p-p65 and pSTAT3 double positive cells.

B: Immunohistochemistry of co-staining with p-p65 (brown) and SYT17 (red), or p-STAT3 (brown) and SYT17 (red) in the tubular and glomeruli regions of specimens from a CAAMR patient. Examples of SYT17-expressing cells with phosphorylated p65 and STAT3 are indicated with red arrows. Representative images are shown. Scale bars are 100 μm.

Figure 3. SYT17 protein in urinary exosomes

A: Western blotting analysis of whole urine samples using anti-SYT17. PC (positive control) is cell lysate overexpressing SYT17 protein.

B: Western blotting analysis of urinary exosomal fraction samples using anti-SYT17. CD9 was used as a marker of exosomes.

C: The graph shows the SYT17/CD9 ratio from the results of (B). Image J was used to digitize the bands for comparisons between samples.

Figure 4. Urinary exosomal SYT17 protein can distinguish CAAMR patients from other groups

A-D: The graphs show data of SYT17/CD9 (A) and current clinical markers (B-D; NAG (N-acetyl-β-D-glucosaminidase), Proteinuria, and eGFR (estimated glomerular filtration rate)) between KTx groups (NED, IF/TA, CNI-T, and CAAMR) and healthy volunteers. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ by Tukey's test.

E: The ROC curve of SYT17/CD9 is shown.

Supplementary Figure 1. Localization of SYT17 in kidney segment

A: Hematoxylin-eosin (HE) and SYT17 staining of the CAAMR sample shown in Fig. 1C.

B: SYT17-positive areas in each kidney segment are shown. *** $P < 0.001$ by Student's t test. G, glomeruli; PT, proximal tubule; DT, distal tubule. Scale bars are 100 μm.

Supplementary Figure 2. Localization of SYT17 protein in cells

Immunohistochemistry of CAAMR at high magnification. Scale bars are 100 μm in upper panels and 20 μm in lower panels.

Supplementary Figure 3. Inflammatory amplifier of kidney is present in tubular cells

A: The levels of IL-6 amplifier signature genes (IL-6 and CCL20) and SYT17 in tubular epithelial cells by transcriptome analysis. Results are relative to no stimulation.

B: IL-6 mRNA levels in siRNA-mediated knockdown cells following activation of the IL-6 amplifier with IL-6 and IL-17. Control siRNA (C), three different siRNAs for SYT17 (S1, S2 and S3), and siRNA for NFκB p65 (P, positive control) were used. IL-6 levels relative to GAPDH levels are shown. *: $P < 0.05$ by Dunnett's test.

C: Luciferase assay using tandem NFκB-binding elements (left) or IL-6 promoter (right) was performed with (SYT17) or without (Mock) overexpression of SYT17 in the presence or absence of TNFα stimulation. *: $P < 0.05$ by Student's t test.

Supplementary Figure 4. SYT17 positively regulates NFκB pathway

A: SYT17-knockdown or control RPTEC were stimulated with or without IL-6 and TNFα for 30 min, and the localization of NFκB p65 (green) was observed by confocal microscopy. Nuclei were stained by Hoechst33342 (blue). Quantification of the localization of NFκB p65 is shown in the right graph. C > N indicates cells with more NFκB p65 localized in the cytoplasm than nucleus; C = N indicates cells with equal amounts of NFκB p65 localized in the cytoplasm and nucleus; and C < N indicates cells with more NFκB p65 localized in the nucleus than cytoplasm. NTC, non-target control siRNA. Each data point represents the mean + SEM. *** $P < 0.001$, Student's t test.

B: SYT17-knockdown or control RPTEC were stimulated with or without IL-6 and TNFα for 30 min, and the localization of phospho-STAT3 (green) was observed by confocal microscopy. Nuclei were stained by Hoechst33342 (blue). Quantification of the localization of phospho-STAT3 is shown in the right graph. NTC, non-target control siRNA. Each data point represents the mean + SEM.

C: SYT17 expression levels (green) were evaluated in SYT17-knockdown or control RPTEC. Nuclei were stained by Hoechst33342 (blue). Quantification of the SYT17 expression levels is shown in the right graph. The relative signal intensity was obtained by dividing the mean SYT17 signal intensity by the area of a cell. Red bars indicate the mean. *** $P < 0.001$, Student's t test.

Supplementary Figure 5. No significant correlation between SYT17/CD9 and current clinical markers

A-C: The correlation between SYT17/CD9 and current clinical markers is shown. The current clinical markers are NAG (N-acetyl-β-D-glucosaminidase, A), Proteinuria (B), and eGFR (estimated glomerular filtration rate, C).

Supplementary Figure 6. SYT17 is also a biomarker for CKD patients

The graphs show data of SYT17/CD9 in urinary exosomes from healthy volunteers (Healthy) or patients with chronic kidney diseases (CKD) including idiopathic membranous nephropathy,

membranous nephropathy, primary nephrotic syndrome, and lupus nephritis. *: $P < 0.05$ by Student's t test.

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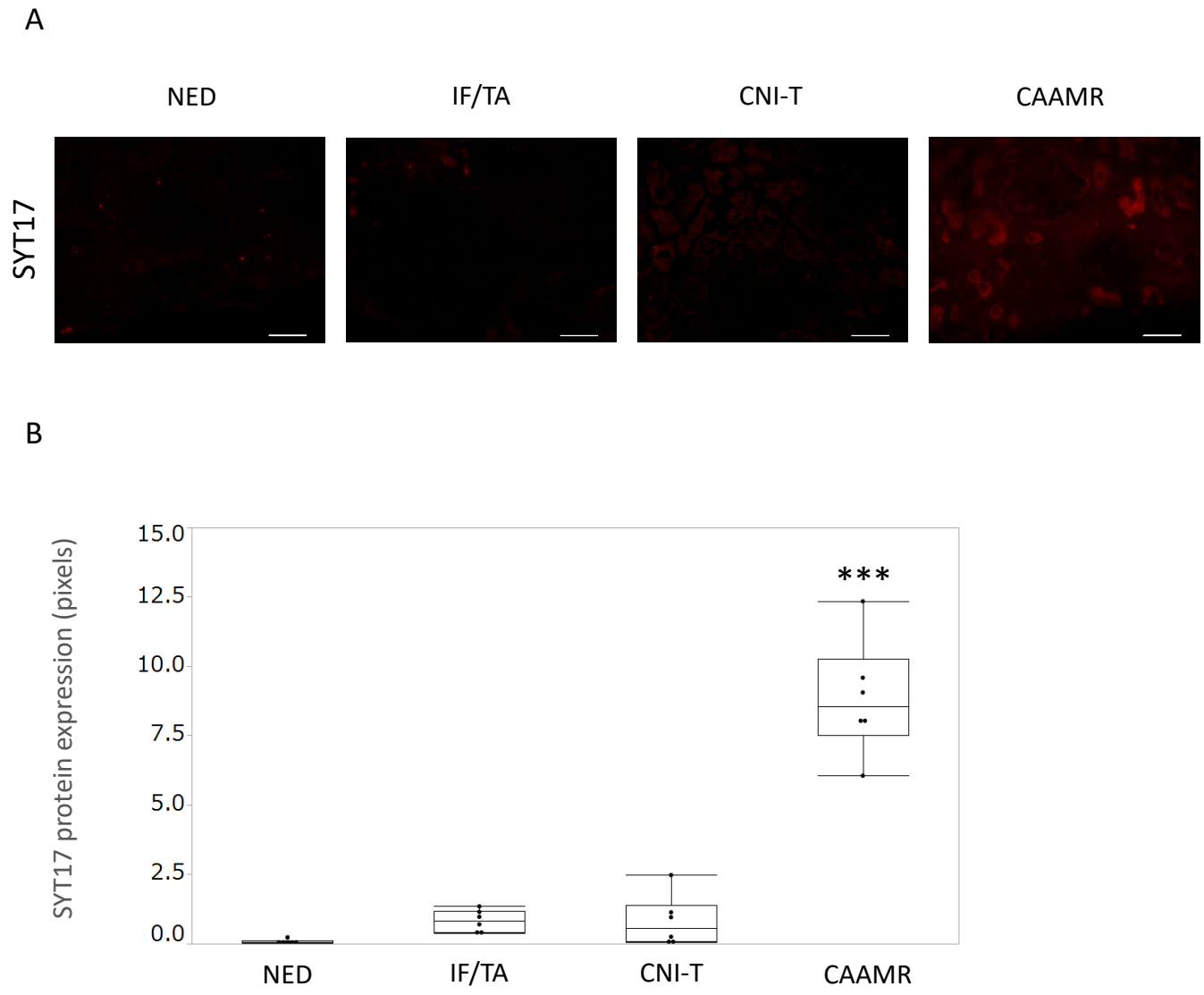
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Fig 1. SYT17 is highly expressed in tubular cells from CAAMR patients



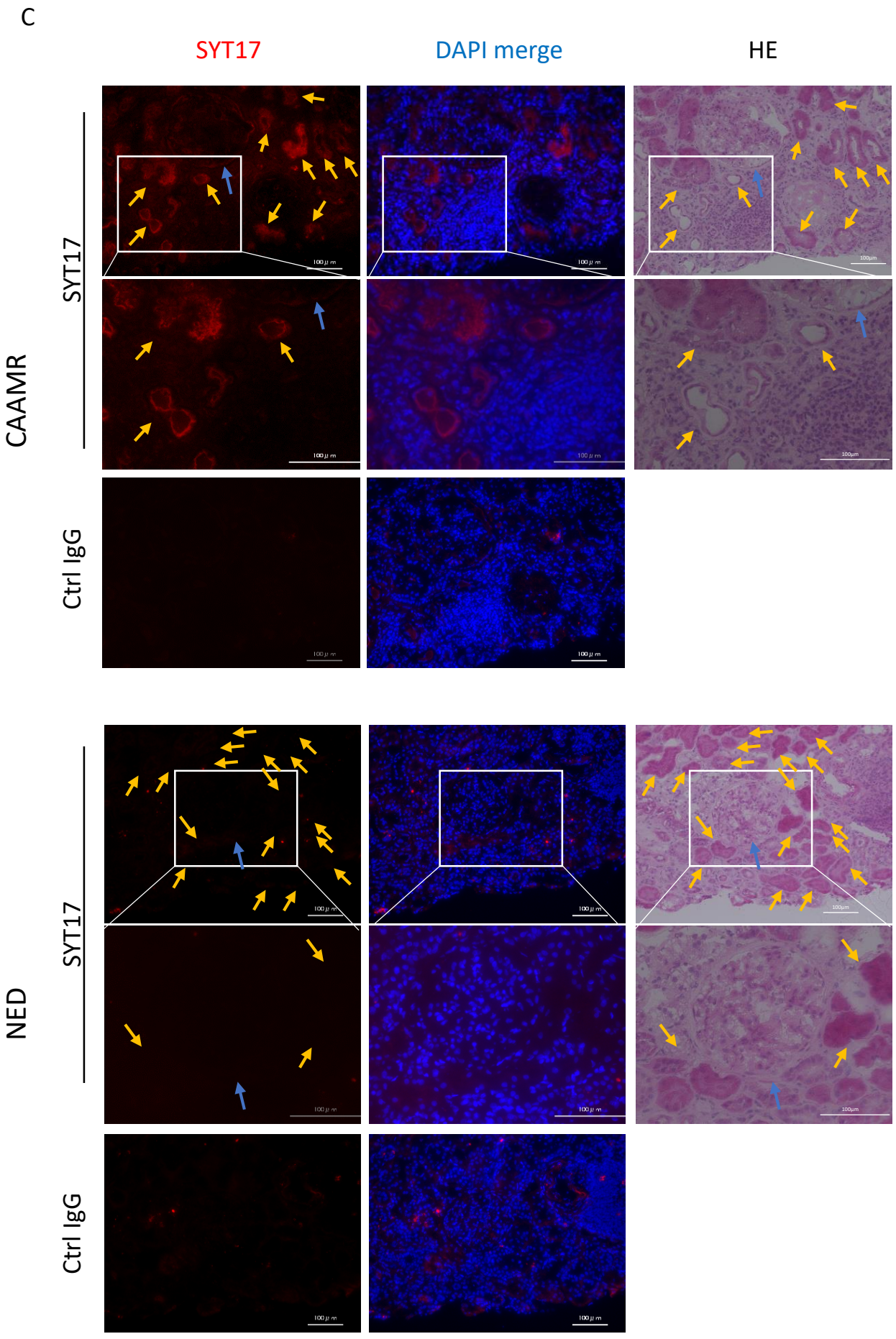
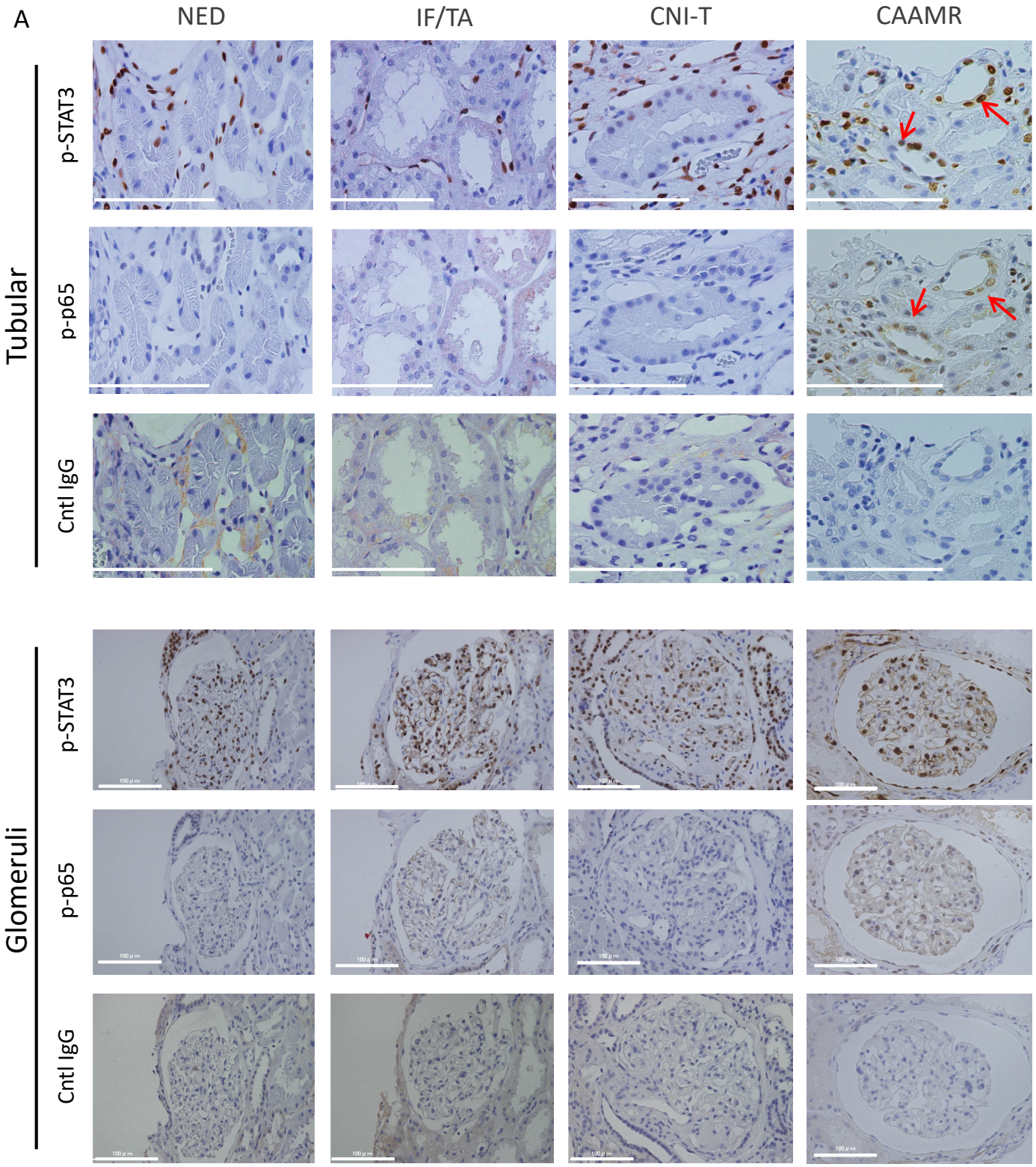

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Fig 2. NFκB and STAT3 double positive cells are existed in tubular cells of CAAMR patients



B

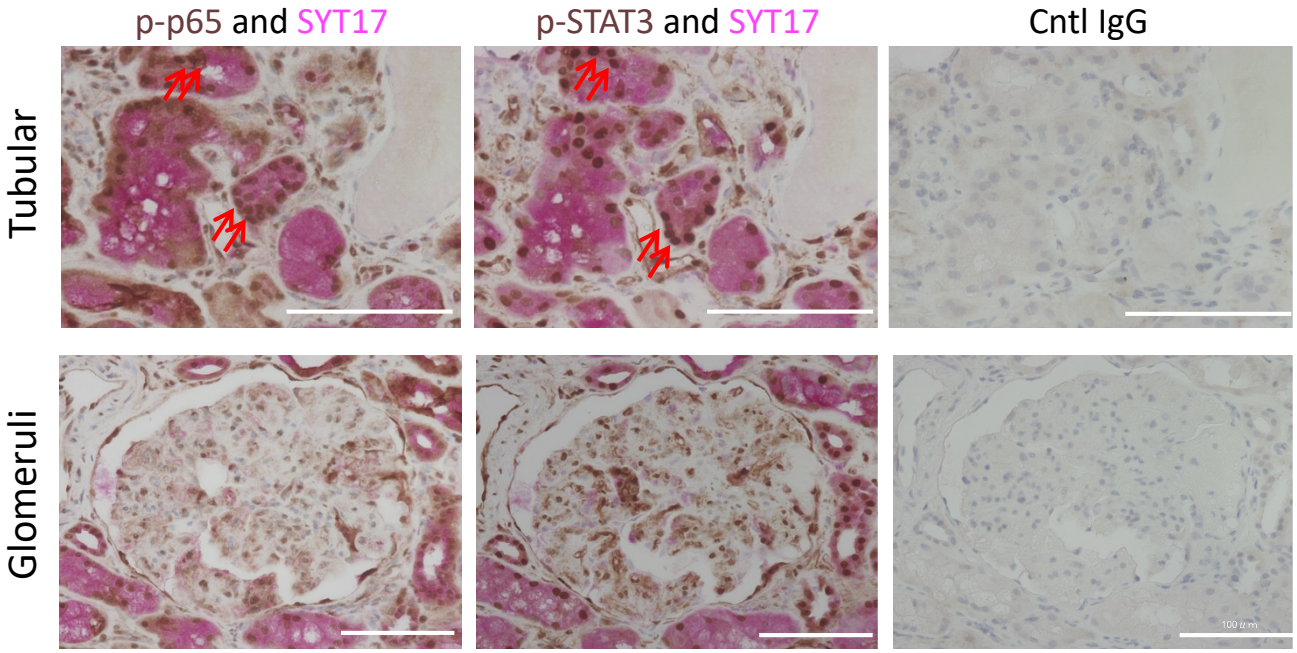
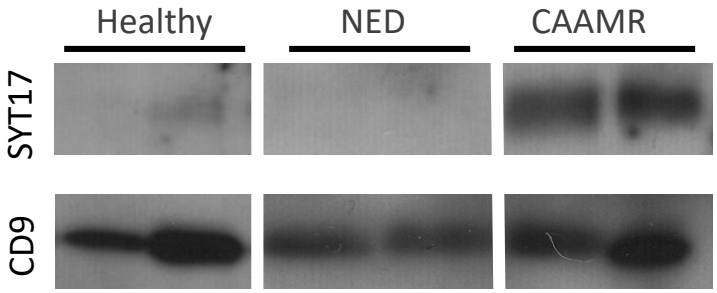


Fig 3. Urinary exosomes enable detection of SYT17 protein

A



B



C

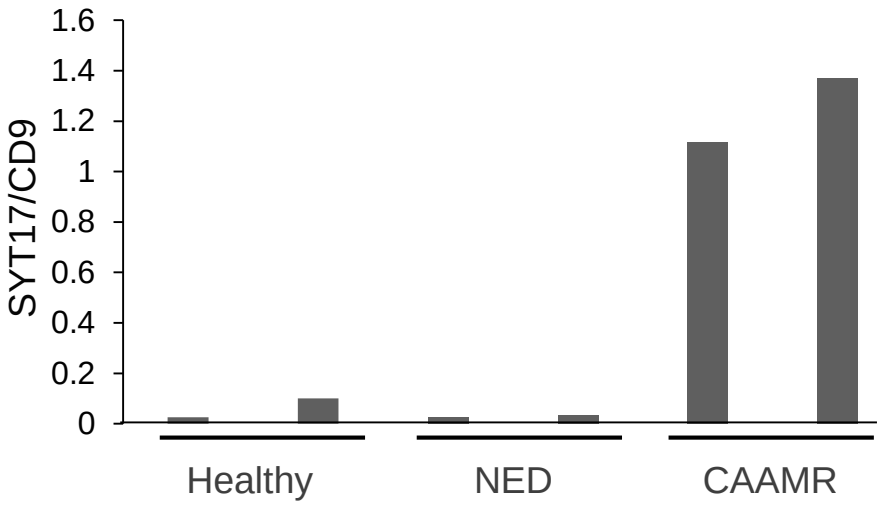


Fig 4. Urinary exosomal SYT17 protein can distinguish CAAMR patients from other groups

