Title page

Supplemental materials

Renal Injuries in Primary Aldosteronism (PA)

~Quantitative Histopathological Analysis of 19 PA patients~

Corresponding author:

Hironobu Sasano, MD, PhD

Department of Pathology,
Tohoku University Graduate School of Medicine

2-1 Seiryo-machi, Aoba-ku, Sendai, 980-8575, JAPAN.

Tel: +81-22-717-7450
Fax: +81-22-717-8634

E-mail: hsasano@patholo2.med.tohoku.ac.jp

Contents

Supplemental text

Materials and Methods

Clinical features of the patients studied

Estimated glomerular filtration rate (eGFR) was calculated by the following formula,

male; $eGFR=194 \times Age(y)^{-0.287} \times sCr^{-1.094}$,

female; $eGFR=194 \times Age(y)^{-0.287} \times sCr^{-1.094} \times 0.739$.

Histopathological analysis

All specimens were fixed with 10% buffered formalin and embedded into paraffins. The thickness of all tissue sections was set at 3μm.

Arterioles

Afferent arterioles were identified as the larger diameter and thicker wall than efferent arterioles and being located adjacent to the juxtaglomerular apparatus at the glomerular vascular pole.

Adrenals

a: the maximal diameter of the tumor, b: the diameter orthogonal to 'a'

$$\text{APA H-score} = \frac{4}{3}\pi \times \frac{a \times b \times (\frac{a+b}{2})}{2^3} \times \text{CYP11B2 H-score}$$

Ultrastructural analysis

The pieces of renal specimens were harvested among the renal cortex from the biopsy specimens under magnifying glass and fixed with glutaraldehyde, following post-fixation in osmium tetroxide, embedding epoxy, sliced at 90 nm, and staining with uranylacetate and lead citrate.

Figure legend

Fig. 2. The clinical information of the left picture was 59-year-old female with eGFR 27.94 mL/min/1.73m^2 (adrenalectomy not done yet), PRA 0.5ng/ml/h, PAC 54.6ng/dl, one antihypertensive agent, including eplerenone, and 20-year of past history of hypertension. Histopathologically, the median diameter of glomerulus was 261.9μm, GGS 11.1%, SGS 0%. The middle was 62-year-old male with post-operative eGFR 68.30 mL/min/1.73m^2, PRA 0.7ng/ml/h, PAC 31.9 ng/dl, two antihypertensive agents, including spironolactone, and 15-year of past history of hypertension. Histopathologically, the median diameter of glomerulus was 176.3μm, GGS 14.0%, SGS 42.9%. The right was 58-year-old male with post-operative eGFR 60.49 mL/min/1.73m^2, PRA 0.1ng/ml/h, PAC 89.3 ng/dl, three antihypertensive agents, including spironolactone, and 18-year of past history of hypertension. Histopathologically, the median diameter of glomerulus was 168.8μm, GGS 33.3% and SGS 0%.

Major Resources Table

Antibodies for immunohistochemistry. Primary antibodies used for immunohistochemical analysis in this study were collagen type 3, 11βHSD1, 11βHSD2, Renin, MR, ICAM-1 and CYP11B2.

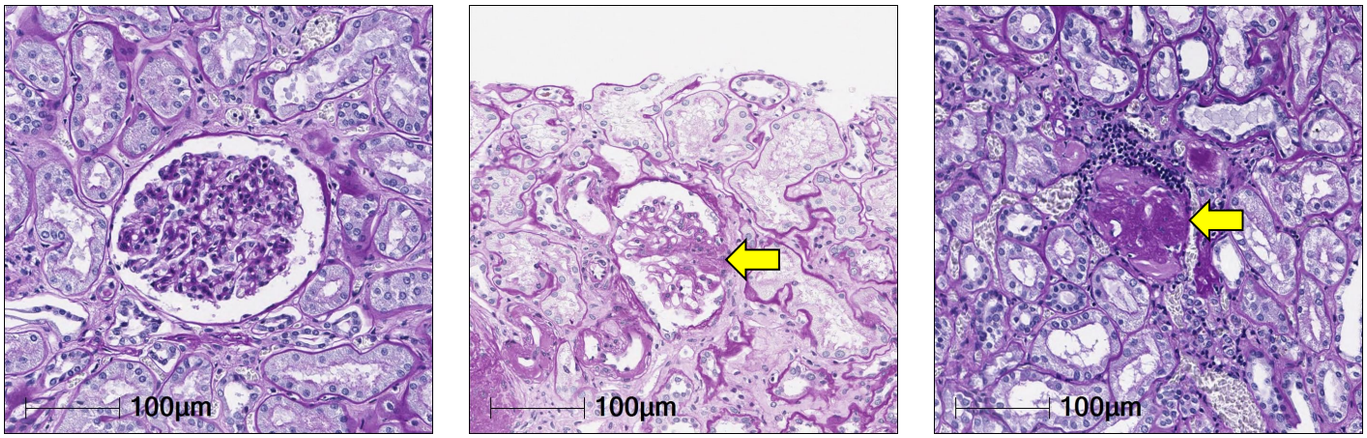
Antibody	origin / clone	Product	Localization	Evaluation
Collagen type 3	Mouse / FH-7A	ab6310 (abcam)*	Interstitium of tubules	area
11βHSD1	Rabbit / EP9406(2)	ab169785 (abcam)†	Cytoplasm of tubules	H-score
11βHSD2	Mouse / C-9	sc-365529 (Santa Cruz)‡	Cytoplasm of tubules	H-score
Renin	Sheep / polyclonal	LS-B144 (LifeSpan BioSciences) §	Cytoplasm of juxta glomerular cells	area (μm^2)/ glomerulus
MR	Mouse / 6G1	rMR1-18-6G1-3G3 (Gomez Sanchez)‖	Nuclear of tubules	Labeling Index (%)
ICAM-1	Rabbit / EP14424	ab43013 (abcam) ¶	Cell membrane of tubules or endothelium	NA
CYP11B2	Mouse / 41-17	AB_2650562 (Gomez Sanchez)#	Cytoplasm of adrenal cortical cells	H score

Pretreatment/dilution; * -/1:300, † autoclave/1:100, ‡ -/1:2000, § -/1:3600, ‖autoclave/1:100, ¶autocrave/1:1000, #autoclave/1:500

Table S1. Correlations between MR, 11βHSD2 and aldosterone concentration.
There were no correlations between MR, 11βHSD2, plasma aldosterone concentration (PAC) and urinary aldosterone concentration (UAC). However, we need further investigation to clarify their exact relationship because of the small number of this study.
11βHSD2: 11β-hydroxysteroid dehydrogenase type 2

Factors	MR	11βHSD2	PAC	UAC
MR		ρ=0.3768 p=0.25	ρ=0.2605 p=0.44	ρ=-0.0186 p=0.96
11βHSD2			ρ=0.4000 p=0.22	ρ=0.2727 p=0.42
PAC				ρ=0.7091 p=0.01
UAC				

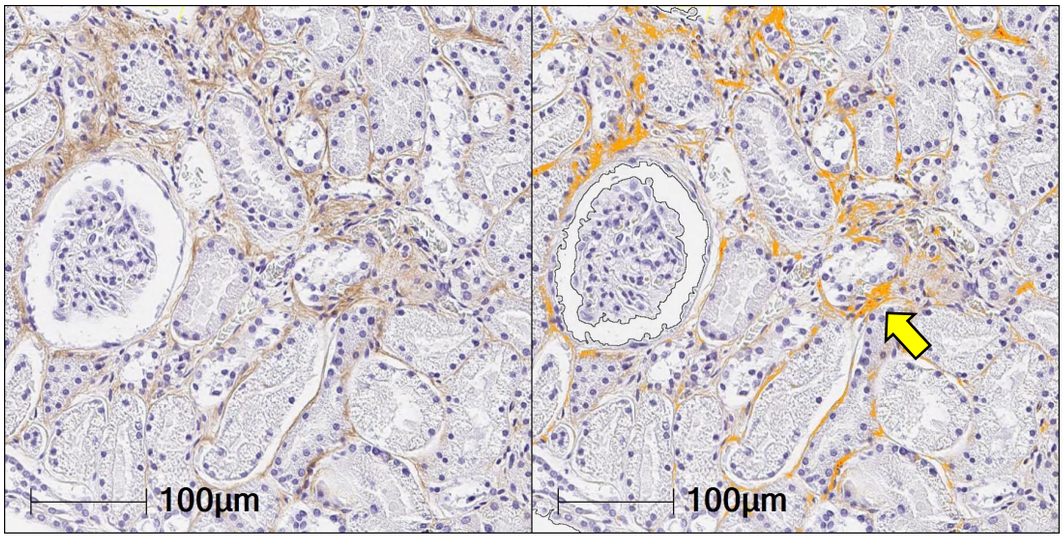
Figure S1. Representative pictures of PAS-stained glomeruli. The left had almost normal structure. Partial PAS-dense lesion meant SGS could be found in capillary lesion in the middle picture. GGS was demonstrated in the right.



control SGS GGS

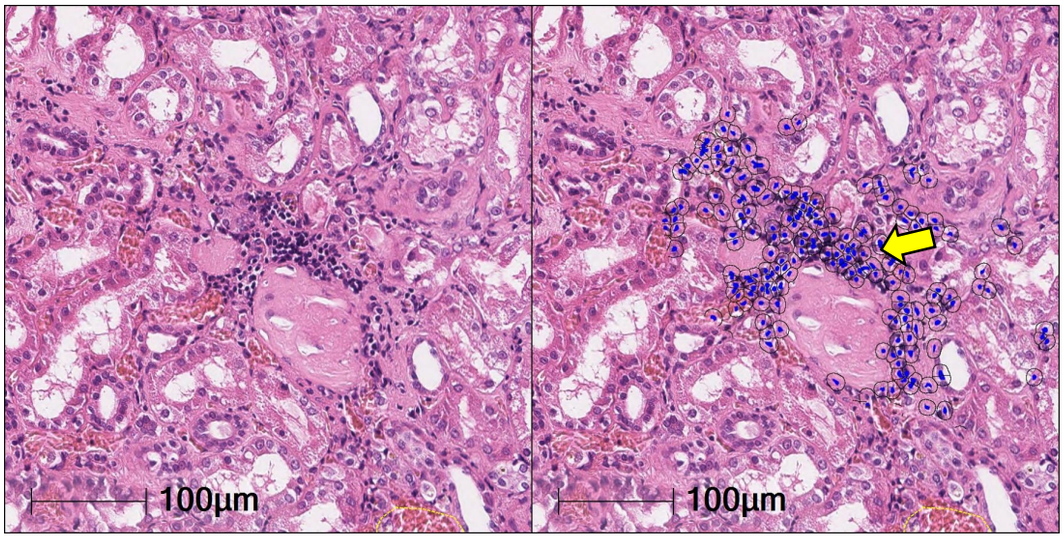
Figure S2. Procedures of image analysis for interstitial findings. A. Interstitial fibrosis. Fibrotic area was evaluated by Collagen type 3 IHC. Brown-colored area was detected by imaging software and marked up with orange colored area(Right). **B. Infiltration of inflammatory cells.** Lymphocytes were marked up with blue and automatically counted by imaging software. Lymphocytes were selected as cells which had small round nuculei and few cytoplasm.
IHC; immunohistochemistry, HE; hematoxylin and eosin

A



Original picture of collagen type 3 IHC Marked up with orange

B



HE-stained specimen Marked up with blue

Figure S3. Procedures of image analysis for immunohistochemistry. A. Renin. Renin immunoreactivity was evaluated by the ratio of the renin-positive area divided by the number of glomeruli. Renin-positive area was detected by the imaging software, recognizing the DAB strongly stained area (orange or red colored). The number of glomeruli was manually counted. **B. 11βHSDs.** They were immunolocalized in tubular cytoplasm. Immunointensity was divided into four degrees based on the DAB gradient and each number of positive cells was calculated by the imaging software [negative (blue), weak (yellow), moderate (orange) and intense (red)]. Then immunoreactivity was semi-quantified by the formula of H-score. H-score ranged from 0 to 300, which was obtained according to the gradients of intensity score (0: negative, 1: weak, 2: moderate, 3: intense) and proportion of positive cells (%). The representative image of analysis in 11βHSD2 IHC was shown below. 11βHSD1 was analyzed in the same fashion. DAB; 3,3'-diaminobenzidine, IHC; immunohistochemistry

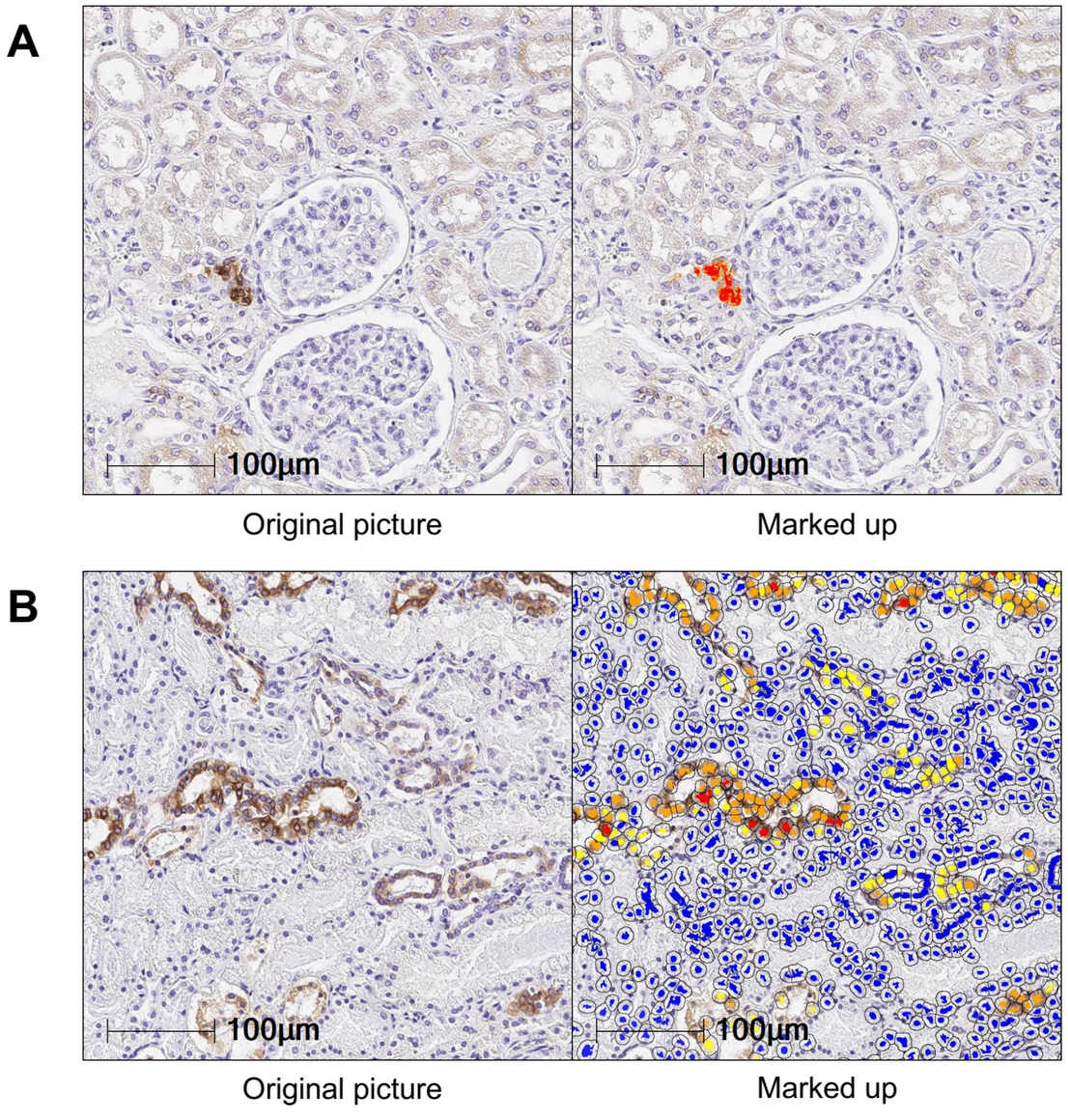
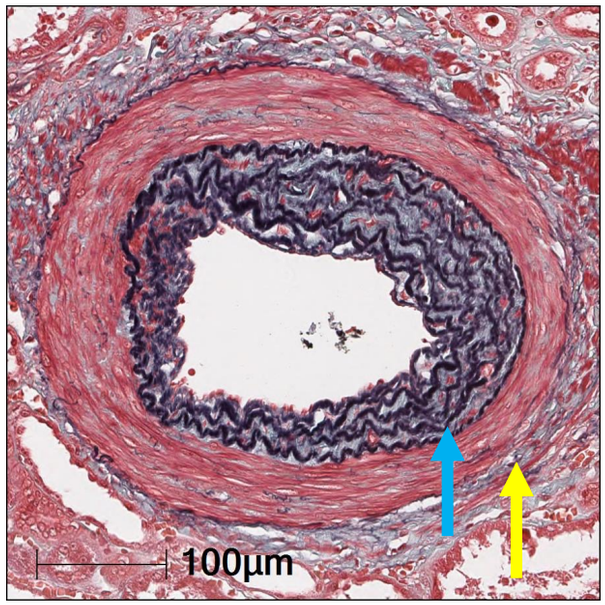
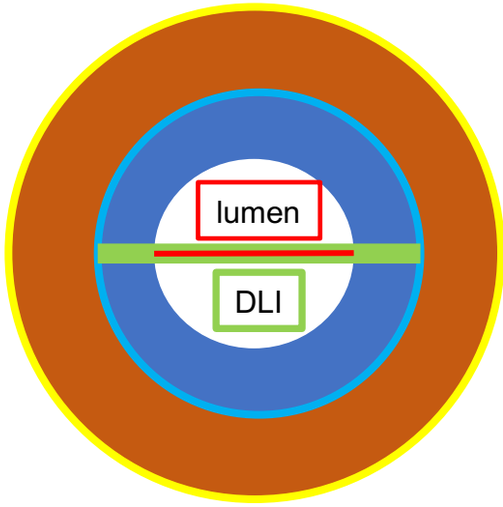


Figure S4. Analytical method of evaluating arteries. According to the previous report, we evaluated the most sclerosed or damaged artery in each specimen. **A.** Arcuate artery from EM-stained specimen. Blue arrow indicated internal elastic lamina (IEL) and yellow arrow did external elastic lamina (EEL). **B.** Schematic diagram of artery and the formula for intima-media-ratio (IMR) and luminal stenosis.

A



B



Width of intima (distance between intima and IEL)
Width of media (area between IEL and EEL)
Diameter internal to the media (Diameter of lumen plus intima; DLI)
Diameter of lumen/2 = r
DLI/2 = R

Lumen area (mm^2) = πr^2
IEL area (in mm2) = $\pi R2$
Intimal area (in mm2) = IEL area – Lumen area

Luminal stenosis = 100 X intimal area / IEL area
IMR = Width of intima at maximal intimal thickness / width of media at maximal intimal thickness

Figure S5. Example pictures of arteriole in PAS-stained sections. A. Arrow indicated hyalinization of arteriole, intensely PAS positive area. **B.** Afferent arteriole was surrounded by cells including cytoplasmic granules, juxtaglomerular cells (arrow).
PAS; Periodic acid-Schiff

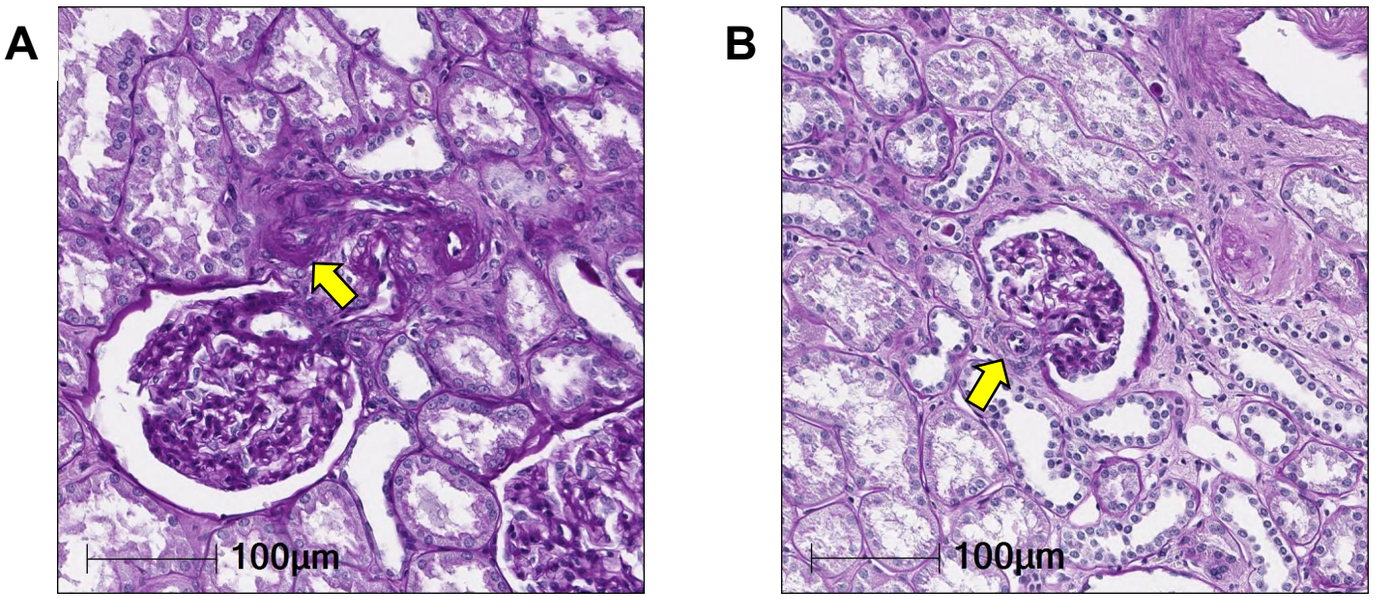
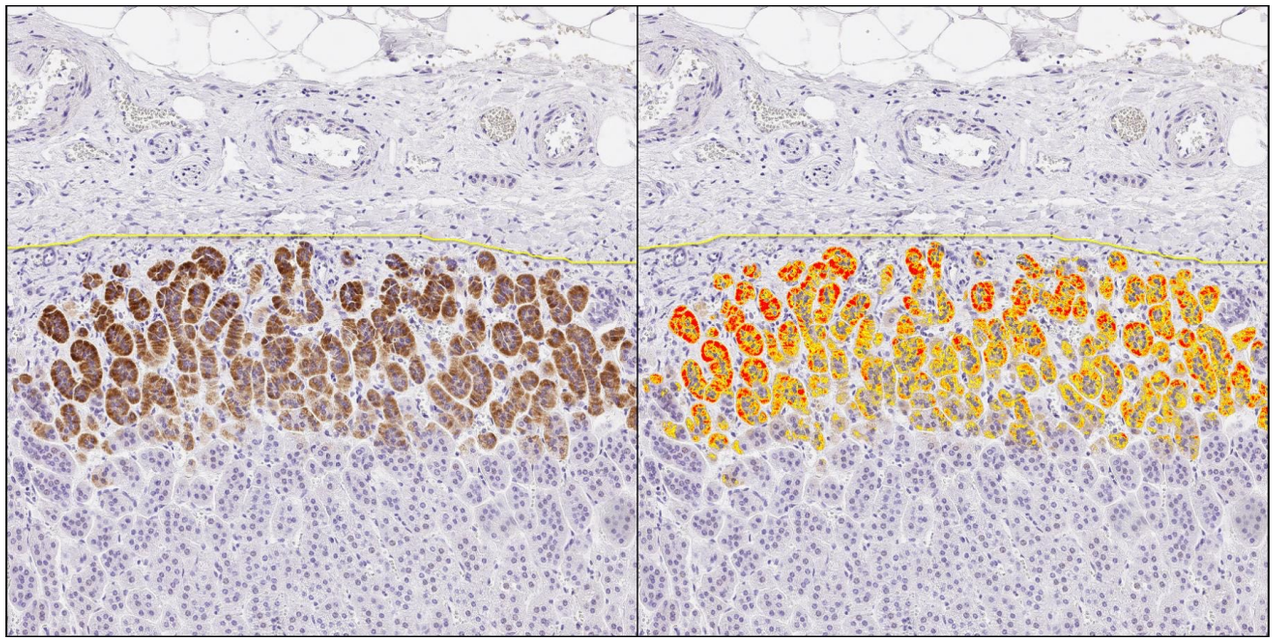


Figure S6. Example of analyzing CYP11B2 IHC. Representative picture of CYP11B2 positive lesion. Imaging software detected immunoreactive area and divided 3 colors according its intensity (weak: yellow, moderate: orange, strong: red), then we calculated H-score.
CYP11B2; Cytochrome P450 Family 11 Subfamily B Member 2, IHC; immunohistochemistry



Original picture Marked up

Figure S7. Representative illustration of MR immunohistochemistry of glomeruli. There were nuclear MR-translocated pericapillary cells (arrow), considered as podocytes.

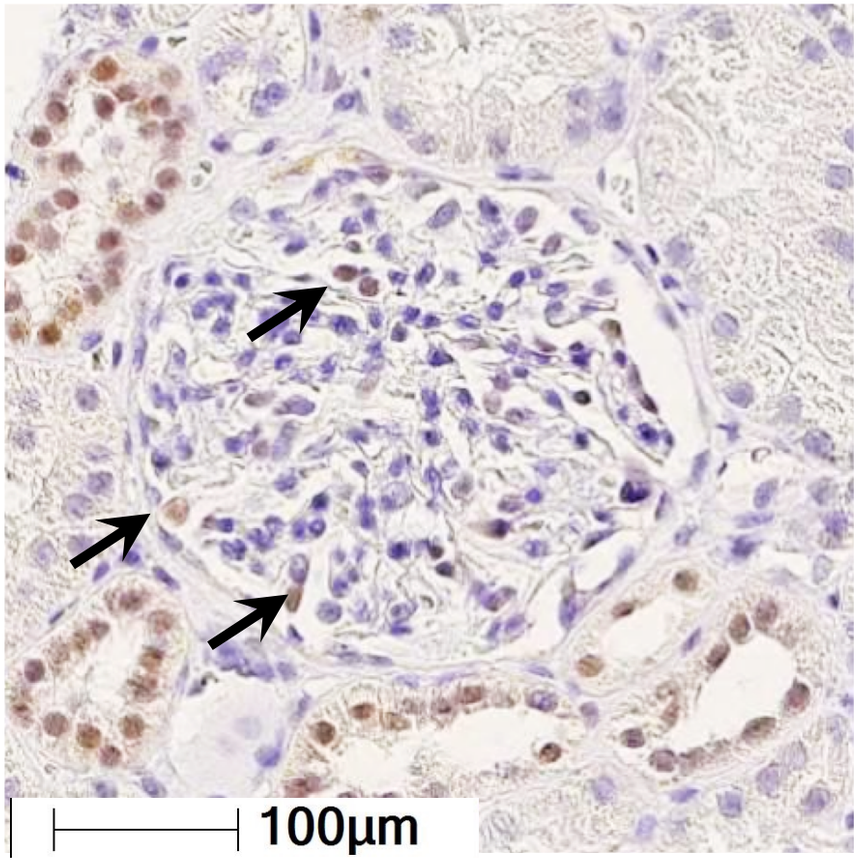


Figure S7. Aldosterone stimulated arterio-arteriolosclerosis and glomerulosclerosis. Under the status of autonomous aldosterone excess, aldosterone could reduce tubule-glomerular feedback and subsequently caused glomerular hypertension. These dysregulated status could also result in uncontrolled glomerular hyperfiltration or proteinuria. Proteinuria was known to exacerbate glomerulosclerosis and progressed from segmental- and developed into global glomerulosclerosis. Aldosterone could also directly damage podocytes by itself and/or signaling via mineralocorticoid receptor. Aldosterone itself could also cause endothelial swelling or endothelial dysfunction. Lower NO production could result in decreased vasodilation, resulting in peripheral resistance. Endothelial injury could also increase oxidative stress, resulting in fibrotic change. Renal topical ischemia could eventually occur, increasing oxidative stress. These were mostly due to non-genomic effect of aldosterone, but genomic effects could also occur. After endothelial damages, fibrotic changes and proliferation of vascular smooth muscle cells could occur, inducing luminal stenosis and increased IMR. Aldosterone also harmed arterioles via both pathways and predominance of non-genomic effect has been known in afferent arterioles.

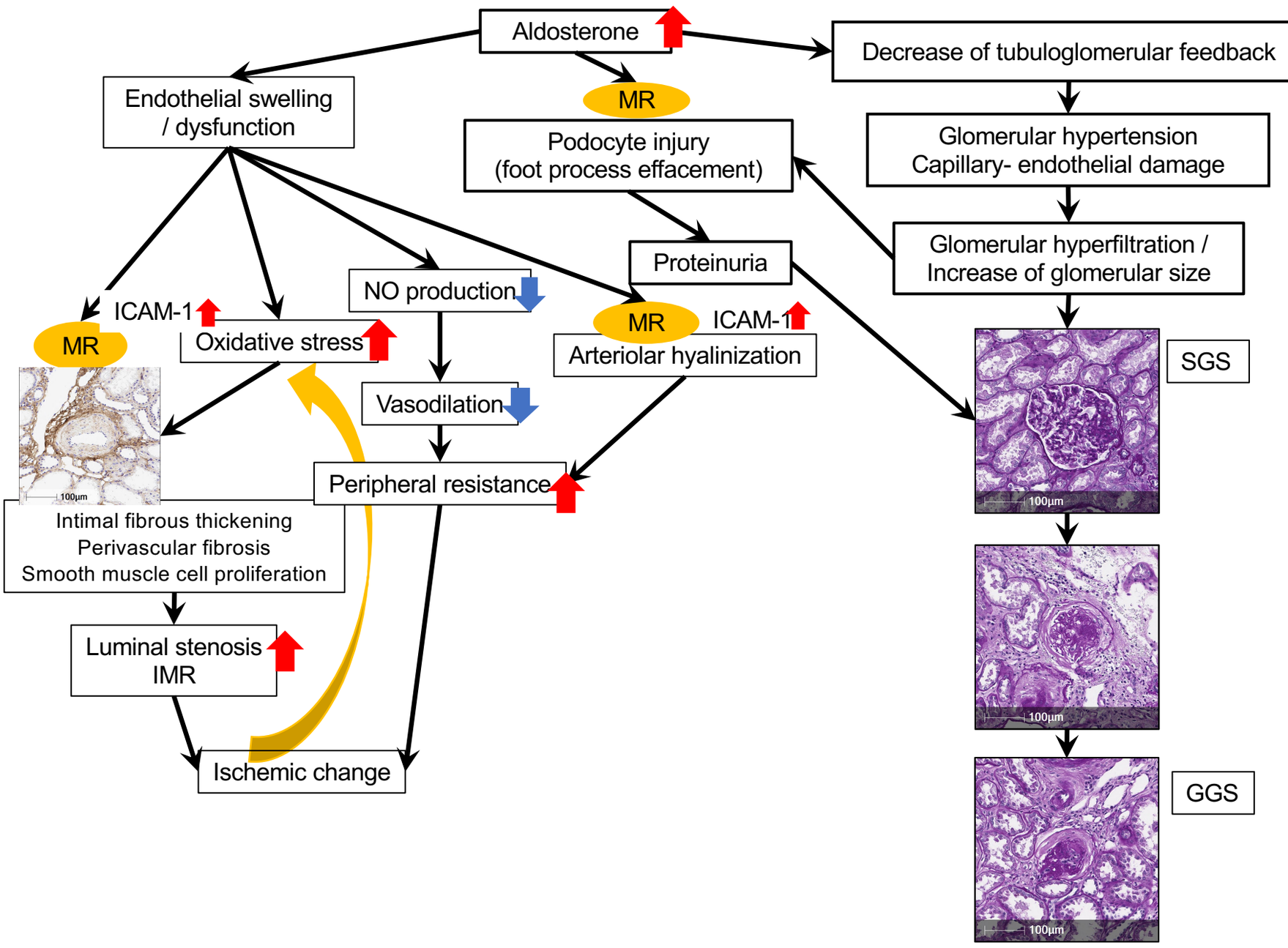


Figure S8. Representative pictures of ICAM-1 immunohistochemistry.
A, B and C demonstrated ICAM-1 in PA. Endothelial cells had immunoreactivity of interlobular arteries or veins, arterioles and glomerular capillary. **C.** Lymphocytes were also expressed ICAM-1. **D and E** demonstrated that in EH. ICAM-1 were less pronounced than that in PA. Bowman's capsule and few proximal tubules were reported to be positive for ICAM-1 in normal renal tissue [38].