Supplementary material to article by H. Sueki et al. "A Case of Subepidermal Blistering Disease with Autoantibodies to Multiple Laminin Subunits who Developed Later Autoantibodies to Alpha-5 Chain of Type IV Collagen Associated with Membranous Glomerulonephropathy"

**Appendix S1.**

**MATERIALS AND METHODS**

**Chemicals, recombinant proteins (RPs), antibodies and antigen sources**

All chemicals used for biochemical analyses were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) or nacalai tesque (Kyoto, Japan).

Commercially available RPs of NC1 domain of human COL4A5 (commercial COL4A5 RP) and N-terminal region of human COL4A6 (commercial COL4A6 RP), which were prepared by E. coli and wheat germ in vitro expression system, respectively, were obtained from MyBioSource (San Diego, CA, USA) and Abnova (Taipei, Taiwan), respectively. Monoclonal antibody (mAb) against human COL4A5 NC1 domain (COL4A5 mAb) was provided by Dr. Borza, Texas, USA. Anti-His-tag HRP-DirecT mAb and Anti-GST-tag HRP-DirecT polyclonal antibody (pAb) were obtained from MBL (Nagoya, Japan).

**Routine immunoblot (IB) analyses of various antigen sources**

We performed IB analyses of various antigen sources, including normal human epidermal extract (S2), RPs of BP180 NC16a domain (S3) and C-terminal domain (S4), concentrated culture supernatant of HaCaT cells (S5), normal human dermal extract (S6), and purified human laminin (LM)-332 (S7).

**Novel IB using hemidesmosome-rich fraction**

We have established a novel IB using hemidesmosome-rich fraction. The method was reported briefly in our previous reports (S8, S9).

**Novel IB of RPs of LM-521**

We have established a novel IB using commercially available LM-521 trimer RP (Biolamina, Sundbyberg, Sweden) (Li et al. in preparation). Briefly, RP was added with 2× sample buffer, boiled for 2 min, and then applied to SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide e-PAGEL, ATTO, Tokyo, Japan). Separated proteins were transferred electrophoretically onto nitrocellulose membrane by iBlot Transfer Stacks, nitrocellulose, regular size (Invitrogen, Carlsbad, CA, USA). Following blocking with 3% skim milk in Tris-buffered saline containing 0.05% Tween 20, and then reacted with HRP-conjugated goat antibodies to human IgG or mouse IgG (Dakocytomation, Glostrup, Denmark) for 3 hrs at room temperature. Blot was developed with ECL Primer Western Blotting Reagent (GE healthcare, Buckinghamshire, UK).

**Preparation of RPs of NC1 domains of human COL4A3 and COL4A5**

RPs of NC1 domains of COL4A3 and COL4A5 were prepared in mammalian cell expression system, as described previously (S10, S11).

**ELISAs of RPs of NC1 domain of human COL4A3 and COL4A5**

For ELISAs, Maxi-sorp strip plates (Nunc A/S, Roskilde, Denmark) were coated with 100 µl of 1.0 µg/ml COL4A3 or COL4A5 RPs in TBS at 4°C overnight. After removing the coating buffer, the plates were blocked with 320 µl of 10% Adult bovine serum (ABS), 5% Sucrose and 0.05% sodium azide in TBS at room temperature for 3 h and they were dried and stored at 4°C. One hundred times diluted patient or normal sera or 200 times diluted COL4A5 mAb with sample buffer (0.01% Tween 20, 2% ABS in TBS) were added into the plates and incubated for 1 h on RT. After washing with TBS containing 0.05% Tween 20, the bound autoantibodies were detected by peroxidase detection using anti-human or anti-mouse IgG-HRP (1:8000) mAb and TMB (Moss inc, Pasadena, MD). One hundred µl of 0.5 M HCl were added to stop the reaction. Then, the optical density (OD) was determined on a microplate reader (Infinite 50, Tecan, Austria) at the wavelength of 450 nm. We set the cut-off value as mean +3 SD of OD_{450} values of normal control sera.

**IB analyses of RPs of NC1 domain of human COL4A3 and COL4A5**

For IB analyses, the RPs were separated by electrophoresis on SDS-polyacrylamide gel (5–20% polyacrylamide e-PAGEL, ATTO) and transferred onto Immobilon polyvinylidene difluoride membrane (Invitrogen). Membranes were incubated in 3% skim milk in TBS for 1 h at room temperature. Ten µl patient or normal control sera were diluted with 190 µl 3% skim milk in TBS. One µl COL4A5 mAb was diluted with 199 µl 3% skim milk in TBS. One µl COL4A5 mAb was diluted with 199 µl 3% skim milk in TBS. Membrane was incubated with diluted sera or COL4A5 mAb at 4°C overnight, washed thoroughly with TBS with 0.05% Tween 20, and then reacted with HRP-conjugated goat antibodies to human IgG or mouse IgG (Dako, Glostrup, Denmark) for 3 hrs.
at room temperature. The reaction with Anti-His-tag HRP-DirecT mAb and Anti-GST-tag HRP-DirecT pAb were performed according to manufacturer’s instruction. Proteins were visualised using EzWestBlue (ATTO).

**WB analyses of commercial COL4A5 and commercial COL4A6**

In WB analyses of commercial COL4A5 and commercial COL4A6, Anti-His-tag HRP-DirecT mAb and Anti-GST-tag HRP-DirecT pAb strongly reacted with commercial COL4A5 and commercial COL4A6 RPs, respectively.

**SUPPLEMENTARY RESULTS**

**ELISAs of RPs of NC1 domain of human COL4A3 and COL4A5**

In ELISA analyses, COL4A5 mAb specifically reacted with COL4A5 RP, but not with COL4A3 RP (Fig. S1). Patient and normal control sera did not react with the both COL4A3 and COL4A5 RPs (Fig. S1).

**IB analyses of RPs of NC1 domain of human COL4A3 and COL4A5**

In WB analyses of COL4A3 and COL4A5 RPs, COL4A5 mAb showed a strong reactivity with the COL4A5 RP (Fig. S2). Patient and normal control sera did not react with the both COL4A3 and COL4A5 RPs (Fig. S2).

In IB of commercially available RPs of COL4A5 and COL4A6, anti-His-tag HRP-DirecT mAb and Anti-GST-tag HRP-DirecT pAb strongly reacted with commercial COL4A5 RP and commercial COL4A6 RP, respectively. Our patient sera, which were taken at both skin and kidney disease stages, reacted with commercial COL4A5 RP, but not with commercial COL4A6 RP (Fig. S3). Normal control sera did not react with both commercial RPs (Fig. S3). These results suggest that our patient sera contained significant amount of autoantibodies to COL4A5. Absence of reactivity with mammalian RP of COL4A5 might be explained by the speculation that higher conformation in mammalian RP hampered the reactivity of autoantibodies, which could react with linear epitope on bacterial commercial COL4A5 RP.

**LITERATURE**


