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Paper-Based ELISA for the Detection of Autoimmune Antibodies in Body Fluid—The Case of Bullous Pemphigoid

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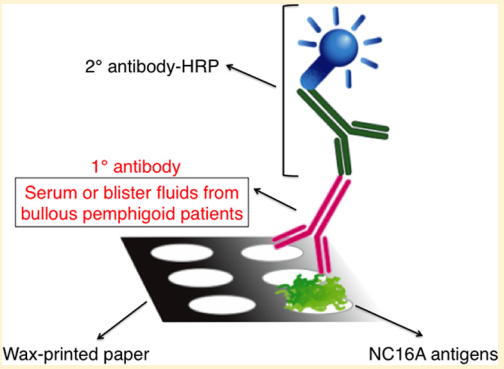
Supporting Information

ABSTRACT: Bullous pemphigoid (BP), a common autoimmune blistering disease, is increasing in incidence and conveys a high mortality. Detection of autoantibodies targeting the noncollagenous 16A (NC16A) domain of type XVII collagen using enzyme-linked immunosorbent assay (ELISA) has demonstrated high sensitivity and specificity for diagnosing BP. We have developed a rapid, low-cost, and widely applicable ELISA-based system to detect the NC16A autoimmune antibody and then diagnose and monitor BP disease activity using a piece of filter paper, a wax-printer, and NC16A antigens. Both sera and/or blister fluids from 14 untreated BP patients were analyzed. The control group included healthy volunteers and patients with other blistering disorders such as pemphigus vulgaris. In our established paper-based ELISA (P-ELISA) system, only 2 μL of serum or blister fluid and 70 min were required to detect anti-NC16A autoimmune antibodies. The relative color intensity was significantly higher in the BP group than in the control groups when using either serum ($P < 0.05$) or blister fluid ($P < 0.001$) specimens from BP patients. The results of P-ELISA were moderately correlated with the titer of the commercial ELISA kit (MBL, Japan) (rho = 0.5680, $P = 0.0011$). This newly developed system allows for rapid and convenient diagnosis and/or monitoring of BP disease activity.



Bullous pemphigoid (BP), a common autoimmune blistering disease, is noted for its high mortality and increasing incidence.[1−3] The one-year mortalities after diagnosis of BP in various nations were 37.8% (France; 502 patients), 29% (Germany; 369 patients), and 19% (the United Kingdom; 869 patients), respectively.[1,2,4] Clinically, patients with BP show multiple features, such as tense blisters, erosions, and crusts with itchy urticarial plaques, and erythema developed on the entire body.

Type XVII collagen (BP180 or BPAG2) of the dermoepidermal junction is thought to be the main target recognized by autoantibodies in patients with BP, and the noncollagenous 16A domain (NC16A) is identified as the major pathogenic epitope.[5,6] The diagnosis of BP requires clinical features, histopathological findings, and immunofluorescent studies.[7] Direct immunofluorescence (DIF) analysis of peri-lesional skin shows linear in vivo depositions of immunoglobulin G (IgG) and complement C3 at the dermal−epidermal junction. Indirect immunofluorescence (IIF) analysis of patient sera also reveals linear depositions of IgG at the dermal-epidermal junction.[7] Recently, the enzyme-linked immunosorbent assay (ELISA) has gained status for diagnosing BP because of its high sensitivity (~92%) and specificity (~98%) for detecting circulating autoantibodies, primarily those to recombinant BP180.[8−13] In addition to the diagnosis of BP, ELISA has proven useful for evaluating disease activity and assessing BP treatment effectiveness.[14] However, commercial ELISA kits (e.g., MBL and Euroimmun) are expensive, time-consuming to

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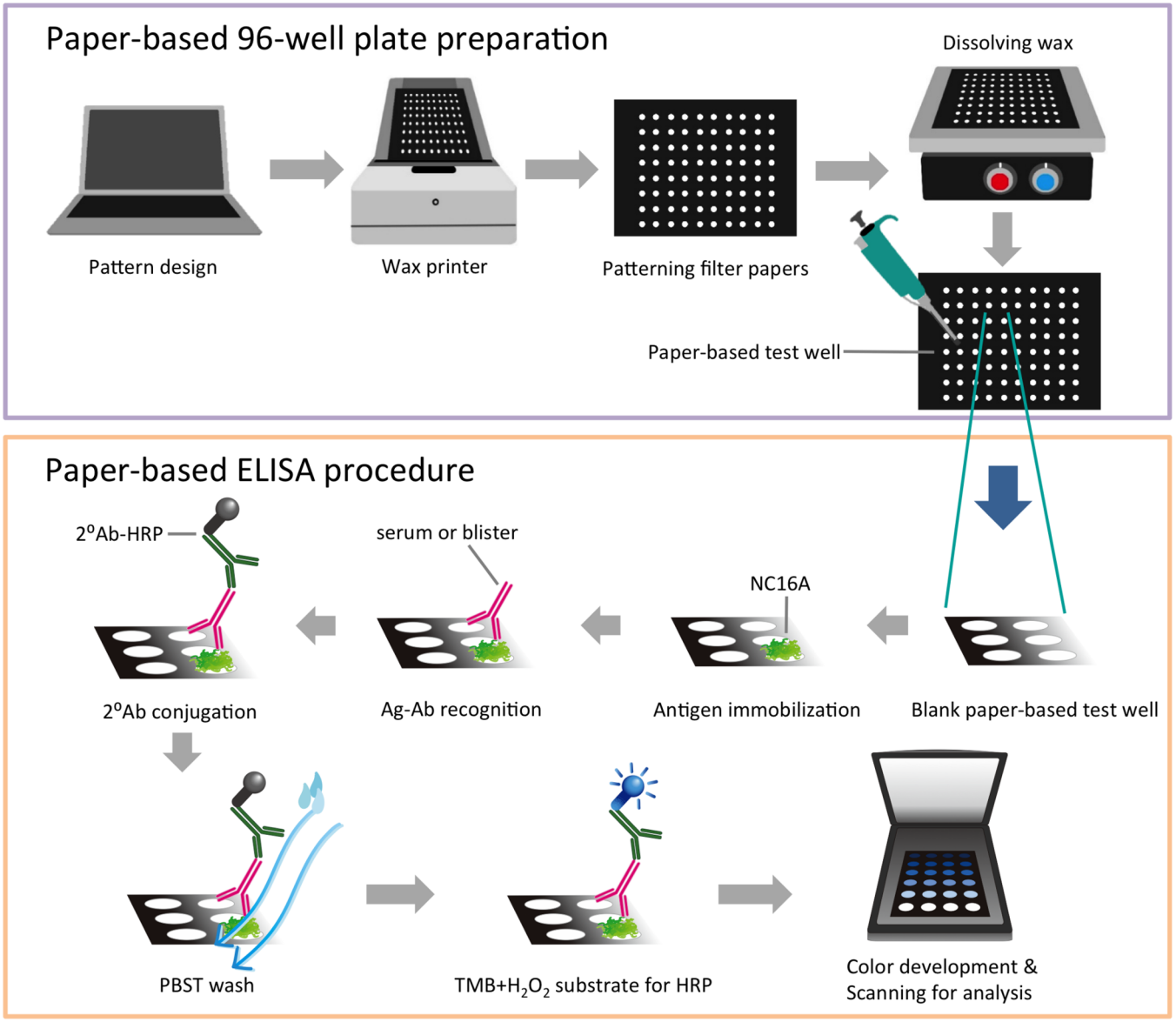

Figure 1. Schematic of the detailed procedure for paper-based ELISA (P-ELISA) detection of anti-NC16a autoimmune antibody. We first designed a 96-well plate pattern using a personal computer. Wax was printed out onto filter paper (Whatman grade no. 1) via a commercial wax printer. The wax-printed paper was remelted via a hot plate (temperature at 135 °C) to generate hydrophobic−hydrophilic boundaries. We then immobilized 2 μL (0.1 μg) of recombinant NC16a antigen to the test wells for 20 min through physical adsorption. Thereafter, the steps of ordinary ELISA were followed, including the use of 2 μL of patient serum or blister fluid as the primary antibody, 2 μL (160 ng/mL) of the horseradish peroxidase-conjugated goat-antimouse IgG as the secondary antibody, washing with PBST, and an enzyme-induced color reaction using a 2 μL mixture of 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2. The output color signal was analyzed by a commercial desktop scanner.

use (at least 150 min), and are only executable in some medical institutes equipped with the ELISA reader. In order to increase the feasibility of ELISA test and help the clinicians to modify the treatment of BP patients efficiently, the development of a low-cost and rapid diagnostic tool for detecting autoimmune antibodies in patients with BP is clinically mandatory.

Dot immunoassays on nitrocellular and filter papers has been an established diagnostic tool for decades.[15−17] Paper-based ELISA (P-ELISA)−first developed by the Whitesides Research Group at Harvard University while carrying out the "Zero-Cost Diagnostics" project sponsored by the Bill and Melinda Gates Foundation−has provided a useful procedure for performing immunoassays using a piece of filter paper (Whatman grade no.1 in this study) for antibody−antigen recognition.[18] We have extended the potential clinically relevant applications of P-ELISA from the diagnosis of infectious diseases (e.g., HIV and dengue fever) to ophthalmological diseases (e.g., proliferative diabetic retinopathy, age-related macular degeneration).[18−20] P-ELISA has proven to be faster and less costly than conventional ELISA, and it conveys a similar level of sensitivity and specificity, in particular, while using tiny amounts of clinical sample (few microliter sampling). Here, we have developed a P-ELISA approach for diagnosing BP by testing serum or blister fluid of individual BP patients. Our focus is twofold: (1) to expand ELISA-based BP diagnostics, which are not routinely offered either in the hospital or at clinical setting; and 2) to provide a point-of-care diagnostic tool (or in vitro diagnostic tool) with reduced cost (potentially for resource-limited settings such as small laboratories or local clinics in rural regions). To our knowledge, this study is the first applying P-

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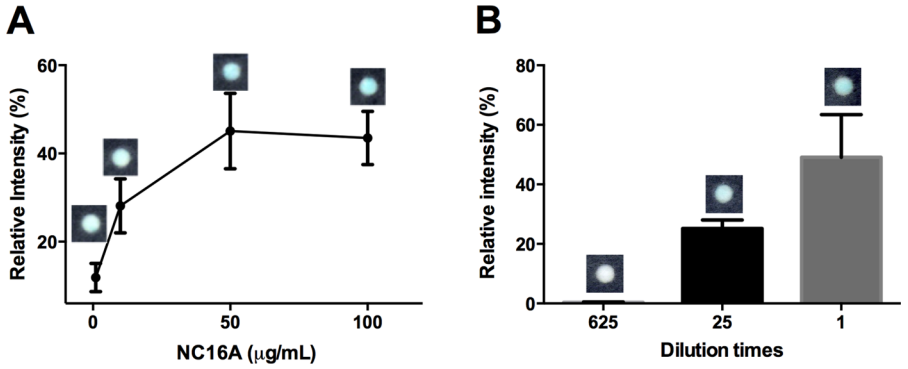

Figure 2. Calibration plots of P-ELISA by placing (A) different concentrations of NC16A antigen (N = 4) and (B) different dilution of blister fluid (N = 4).

ELISA to detect autoimmune antibodies in a human specimen sample (e.g., serum and blister fluid).

EXPERIMENTAL SECTION

Materials and Reagents. To make our paper-based diagnostic tool, we used a wax printer (no. Phaser 8560, Xerox, Norwalk, CT, U.S.A.) to manufacture hydrophobic barriers, in 96-well plate fashion, on filter paper (Whatman grade no. 1). Recombinant NC16A antigen, which was prepared as reported previously,[21] was immobilized in filter paper. Horseradish peroxidase (HRP)-conjugated polyclonal rabbit antihuman IgG (DakoCytomation, Denmark) was used as the secondary antibody to label the primary antibody. Phosphate-buffered saline containing 0.1% Tween-20 (PBST) was used for our washing step. A mixture of 3,3′,5,5′-tetramethylbenzidine and H_2O_2 (Komabiotech, Korea) was used as a coloring reagent to measure HRP oxidization. The output color signal was recorded using a commercial desktop scanner (no. GT-10000, EPSON, Japan).

Patients. BP patients recruited in our study fulfilled the following criteria: (1) clinical features of BP; (2) pathological features with subepidermal blister with a superficial perivascular inflammatory cell infiltrate that was predominantly eosinophilic; and (3) linear depositions of IgG and/or C3 along the basement membrane zone detected by DIF.[22,23] In total, there were 14 patients with BP recruited for this study. We collected sera from eight BP patients, blister fluids from three BP patients, and both sera and blister fluids from another three BP patients. The control group for our serum experiment included four patients with pemphigus vulgaris (PV) and four healthy subjects, while the control group for the blister fluid experiment included six patients with burn injury, one patient with blister induced by cryotherapy, and one patient with blister induced by long-term compression (bedsore). The detailed information on BP patients and control subjects is listed in Table S1 of the Supporting Information. We aspirated blister fluid using a 30G needle. Both serum and blister fluid were stored at −20 °C until use. Informed consent was obtained from all patients, and the procedures were approved by the Ethical Committee of National Cheng Kung University Hospital (no. B-ER-101-038).

Clinical severity was evaluated using the BP Disease Area Index (BPDAI).[24] The BPDAI provides a total score (maximum 360) by combining the scores from three distinct parameters (scored 0 to 120 each), which included the following: (1) number and size of erosions or blisters; (2) number and size of erythematous nonbullous lesions; and (3) number and size of mucosal lesions. A high score is associated with high clinical severity of BP.[24] In our group of patients, there were no mucosal lesions.

Serologic Examinations. Serologic examinations included indirect immunofluorescence (IIF) analysis and a commercially available ELISA test (Medical and Biological Laboratories, Nagoya, Japan) to assess anti-BP180 autoantibody reactivity. The substrate for IIF analysis was prepared from anonymously donated human neonate prepuce skin tissue. Both serum and blister fluid were incubated in serial dilutions between 1:10 and 1:1280, and bound IgG autoantibodies were visualized by subsequent incubation with fluorescein isothiocyanate-labeled secondary antibodies (Dako, Kyoto, Japan) directed against total human IgG. We followed the manufacturer's instructions for the ELISA test, which defined positivity above the cutoff value of 9 U/mL. The upper limit of the assay result was 150 U/mL.

Preparation of Paper-Based ELISA. P-ELISA is basically the same as conventional ELISA, but the method differs in the fact that it employs paper-based 96-well plates prepared via a wax printing method (in this study), rather than plastic microtiter plates or tubes as the reaction medium (Figure 1).[25,26] To detect the presence of autoimmune antibodies in the serum or blister fluid of BP patients, we developed a multiple-step procedure to carry out P-ELISA, as follows: (1) Recombinant NC16A antigen, which was prepared as reported previously,[21] was immobilized in filter paper (Whatman grade no. 1) for 20 min. (2) Two microliters of either patient serum or blister fluid was spotted onto the paper-based test zones and dried for 30 min at room temperature. (3) Two microliters of HRP-conjugated goat-antimouse IgG (160 ng/mL, or 320 pg/test zone) was used (20 min) as our secondary antibody to label the primary antibody. (4) After carrying out the washing step with PBST, we placed 2 μL of a solution of the enzyme substrate (a mixture of 3,3′,5,5′-tetramethylbenzidine and H_2O_2) onto our paper-based test wells to induce a measurable color change (from colorless to blue). The output color signal was recorded using a commercial desktop scanner that cost approximately $100.00 (U.S.). The color intensity was analyzed using Adobe Photoshop software. P-ELISA results were expressed by relative intensity, which was defined as (intensity of [experiment group] − intensity of [water])/intensity of [water] (%). The P-values were obtained using Student's t test. Pearson's correlation coefficient was used to explore relationships between the titer from the ELISA kit and the relative intensity of P-ELISA.

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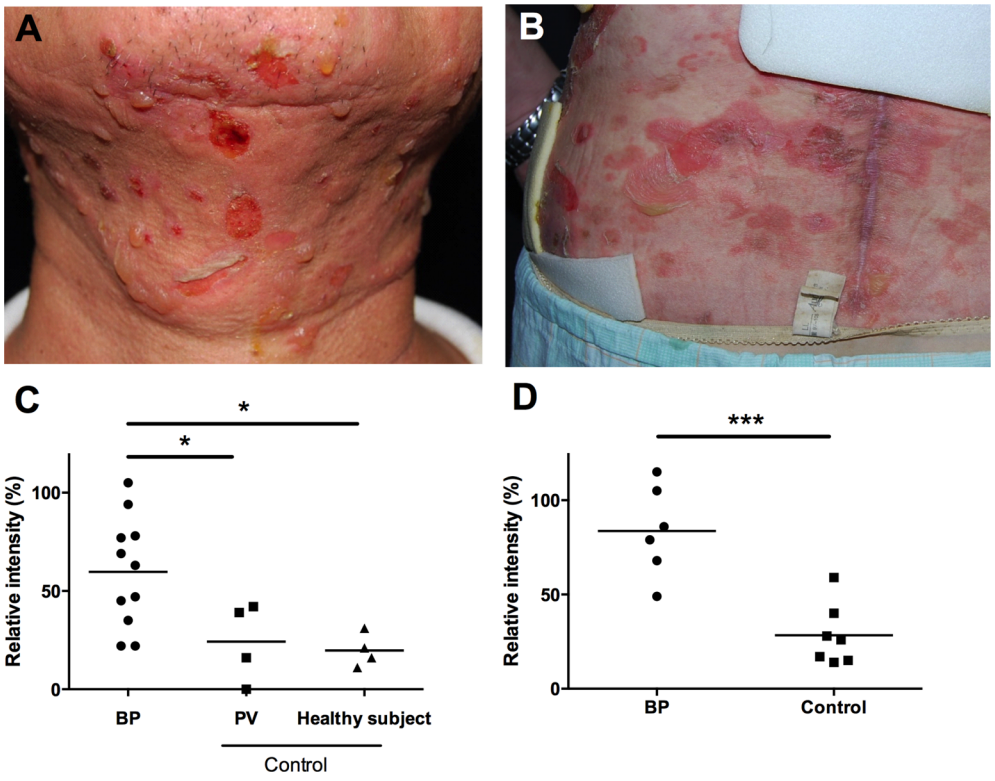

Figure 3. Clinical manifestation of patients with two conditions. (A) bullous pemphigoid (BP) (case BP no. 4): erythematous wheal plaques with blister formation. (B) pemphigus vulgaris (PV) (case PV no. 3): erythematous patches with eroded surface or flaccid blister formation. The detection of anti-NC16A autoimmune antibodies by P-ELISA in the (C) serum or (D) blister fluid of BP patients. Statistical analysis was performed by Student's *t* test. (*$P < 0.05$ and ***$P < 0.001$).

RESULTS AND DISCUSSION

In order to determine optimal antigen-coating concentrations, we placed 2 μL of NC16A at different concentrations (1, 10, 50, and 100 μg/mL) onto the test zone and examined reactions with the blister fluid from an index case (case 10). We found that 50 μg/mL (0.1 μg/test zone) of NC16A antigen provided the maximum color intensity (Figure 2A). The relative color intensity of P-ELISA decreased when blister fluids were diluted 25-fold and 625-fold (Figure 2B).

We detected autoimmune antibodies for NC16A in the serum and blister fluid of BP patients (Figure 3A) using our P-ELISA system. The patients with pemphigus vulgaris (PV), another type of autoimmune blistering disorder in which autoimmune antibodies target cell−cell junction (desmosome), were recruited as our control group (Figure 3B). Using patient serum, the relative intensity was significantly higher in the BP group ($N = 11$) than the PV group ($N = 4$) and the healthy subjects ($N = 4$) ($P < 0.05$, Student's *t* test) (Figure 3C). Using patient blister fluid, the relative intensity was much higher in the BP group ($N = 6$) than the control group ($N = 7$) ($P < 0.001$, Student's *t* test) (Figure 3D). The area under the receiver operating characteristic (ROC) curves for the diagnosis of BP by serum and blister fluids were 0.9091 (95% confidence interval 0.7789−1.039, $P = 0.002953$) and 0.9762 (95% confidence interval 0.9038 to 1.049, $P = 0.004275$), respectively (Figure S1 of the Supporting Information). In the serum experiment, a cutoff of 33.0 resulted in a sensitivity of 81.8% and a specificity of 75.0%. In the blister fluids experiment, the cutoff of 54.0 resulted in a sensitivity of 83.3% and a specificity of 85.7%.

A moderate positive correlation (rho = 0.5680, $P = 0.0011$) between the titer of the commercial ELISA kit and the relative intensity of our P-ELISA was observed (Figure 4). None of the results, including the relative intensity of P-ELISA, the titer of the commercial ELISA kit, and the titer of IIF, correlated with BPDAI score (data not shown).

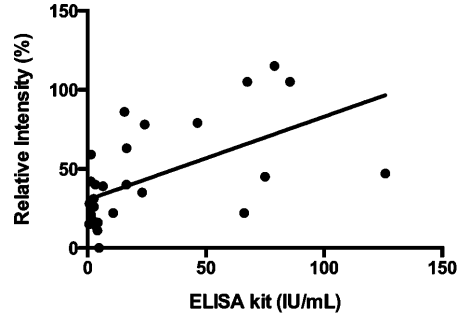

Figure 4. Correlation between the results of the commercial ELISA kit and P-ELISA. The results of ELISA kit exceeded 150 U/mL were excluded.

In the past, P-ELISA has been applied to detect an antigen or protein. For example, we previously applied P-ELISA to identify the dengue virus serotype-2 envelope proteins[26] and detect the vascular endothelial growth factor (VEGF) level in the aqueous humor of patients with ophthalmologic diseases.[19] To our knowledge, the present study is the first one applying P-ELISA to detect autoimmune antibodies in patients with autoimmune disorders. We believe that this model could be

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applied to many other autoimmune diseases, such as lupus erythematosus or scleroderma.

In this study, P-ELISA offers three principal advantages over conventional ELISA in plastic 96-well plates: (1) It is more rapid than commercial ELISA, as the entire P-ELISA procedure, from antigen immobilization to final quantitative result, can be completed within 70 min, whereas commercial 96-well antigen-coated plate ELISA requires at least 150 min. (2) It utilizes simple equipment (a pipet, a refrigerator for storing the reagents, and a desktop scanner) and inexpensive materials, primarily, paper. (3) It requires only small volumes (2 μL) of reagents.

Tense blisters often arise spontaneously in the active phase of BP. A few studies have reported that the antibody titer in blister fluid is the same or less than that in serum in BP.[27,28] Similar to a previous report by Patsatsi,[28] our data suggest that aspirated blister fluid could provide an alternative medium to diagnose BP. This approach will help us to collect specimens more easily, especially in elderly patients who refuse biopsy or have poor venous access. However, it is difficult to harvest blister fluid in BP patients during partial or complete remission stage. Therefore, we suggest that both serum and blister fluid samples be used for diagnosing BP, although a serum sample is highly preferred to monitor the titer of autoantibodies.

The most updated diagnostic criteria for BP are based on a combination of clinical manifestation, direct immunofluorescence microscopy of a perilesional specimen, and serology (commercially available ELISA tools for detecting antibodies to BP180 and BP230).[29] P-ELISA, we believe, would play an advantageous role in diagnosing BP and has the potential to be included as one of the diagnostic criteria for BP. In addition, it is uniquely poised as a potential screening test in clinics or nursing homes.

In our study, we could not see a correlation between BPDAI and the three immunoassays (IIF, P-ELISA, and ELISA). Several studies have shown that IIF titers do not necessarily correlate with disease activity.[11,30−32] Patsatsi et al. correlated the titers of anti-BP180 circulating autoantibodies by commercial ELISA kit (MBL, Japan) to BPDAI in 39 BP patients.[33] They found that titers of anti-BP180 autoantibodies were correlated with BPDAI (r = 0.557, P value <0.0001). In our study, we could not see a correlation between disease activity and the titers from P-ELISA, ELISA kit, or IIF. This may be due to the limited case number (14 BP patients) in our study.

However, we found that the results of P-ELISA were correlated with the titers of the commercial ELISA kit, which supports the idea that we may effectively use P-ELISA to monitor the disease activity of BP patients receiving treatment.[14,34−36]

CONCLUSION

P-ELISA provides a portable, inexpensive, and simple diagnostic tool to detect anti-NC16A autoantibodies in the serum or blister fluid of BP patients. We expect this translational medicine study (from fundamental studies to functional systems) to pave the path toward faster and less expensive diagnostic assays than have been previously available for the diagnosis of dermatological diseases or autoimmune diseases, ultimately, in different divisions of medicine.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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