SHORT COMMUNICATION

Polycystic Kidney Disease with Steatocystoma Multiplex: Evidences for a Disruptive Effect of Mutated Polycystin-1 on Keratin 17 Polymerisation

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Steatocystoma multiplex (SM) is a rare benign tumour of the pilosebaceus unit. It is characterised by the development of numerous sebum-containing dermal cysts (1). Although most cases of SM are sporadic, pachyonychia congenita type 2 patients with SM inherit it as an autosomal dominant trait (1). In 1997, the responsible mutation for this disease was reported in the keratin 17 (*KRT17*) gene (2).

Autosomal dominant polycystic kidney disease (ADPKD) is a common inherited disorder that is characterised by the formation of multiple cysts in the kidneys and liver and, less frequently, in the pancreas. The two genes responsible for polycystic kidney disease are *PKD1* and *PKD2* (3). Here, we report a female ADPKD patient with SM, and provide *in vitro* evidence for a negative influence of the *PKD1* mutation on keratin 17 (K17) polymerization in HaCaT cells.

CASE REPORT AND *IN VITRO* RESULTS

A 48-year-old Japanese woman presented with about 100, asymptomatic, round to oval, well-defined, yellowish papules of around 5 mm in diameter over her entire body surface that started to appear in puberty (Fig. 1a). Her maternal grandmother had similar lesions. The patient had mild nail dystrophies and she had been diagnosed with ADPKD after an investigation of flank pain when she was 35 years old. Since then, she had suffered from recurrent cyst infections. Her mother also had ADPKD. The patient underwent kidney transplantation from her husband when she was 48 years old.

On clinical examination, the dermal cysts were found to be round to oval, well-defined, and smooth-surfaced without a punctum (Fig. 1a). Sonography revealed multiple nodules which were oval in shape. These nodules were well-marginated, hypoechoic, and showed posterior enhancement. Histological examination of a cyst showed that it was situated in the mid-dermis (Fig. 1b). The wall was thin and composed of keratinising epithelium. Lobules of sebaceous glands could be seen near the cyst wall (Fig. 1c). Surgical excision was conducted on the patient's request.

Genomic DNA was extracted from peripheral blood leucocytes for genetic testing after obtaining informed consent (4). Genomic DNA was PCR-amplified for the analysis of exons 1–33 of the *PKD1* gene and their flanking splice sites (4). Sequence analysis revealed that the proband was heterozygous for c.9855_9856insAC, which is a novel mutation not found in 100 control alleles. There was no mutation found in the *KRT17* gene.

We then investigated the effects of the *PKD1* mutation on keratin filament organisation *in vitro*. We obtained cDNA of human *PKD1* from OriGene (Rockville, MD, USA) and introduced the c.9855_9856insAC mutation. We also constructed an expression vector of *KRT17* in the pcDNA3.1/V5-His vector (Life Technologies, Carlsbad, CA, USA). HaCaT cells were nucleofected using the Amaxa Cell Line Optimization Nucleofector Kit (Lonza, Walkersville, MD, USA), as previously described (5, 6). At 48 h after nucleofection, cells were fixed with 4% paraformaldehyde and permeabilised using 0.1% Triton X-100 in phosphate-buffered saline. The cells were labelled and photographed as described previously (5, 6). When HaCaT cells were doubly nucleofected with wild-type (WT) *PKD1* and *KRT17* genes, part of the *PKD1* gene product looked filamentous, and this protein co-localised with K17 (Fig. 2). In contrast, when HaCaT cells were doubly nucleofected with mutant (Mut) *PKD1* and WT *KRT17* genes, the resulting network of K17 was markedly different. Depletion of keratin filaments near the cytoplasmic membrane of cells, together with cytoplasmic aggregates and a near-complete disruption of

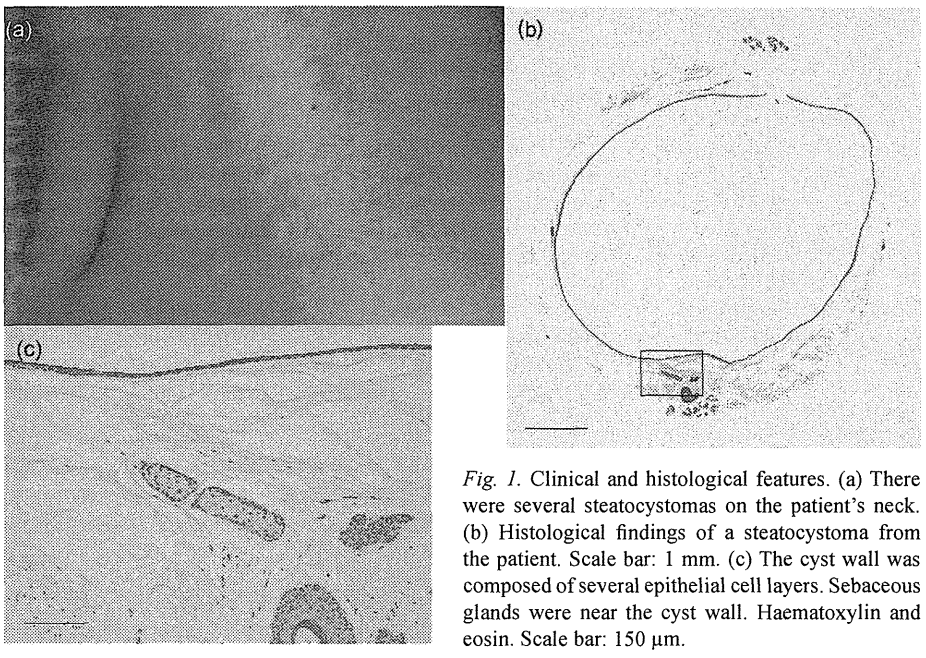

Fig. 1. Clinical and histological features. (a) There were several steatocystomas on the patient's neck. (b) Histological findings of a steatocystoma from the patient. Scale bar: 1 mm. (c) The cyst wall was composed of several epithelial cell layers. Sebaceous glands were near the cyst wall. Haematoxylin and eosin. Scale bar: 150 μm.

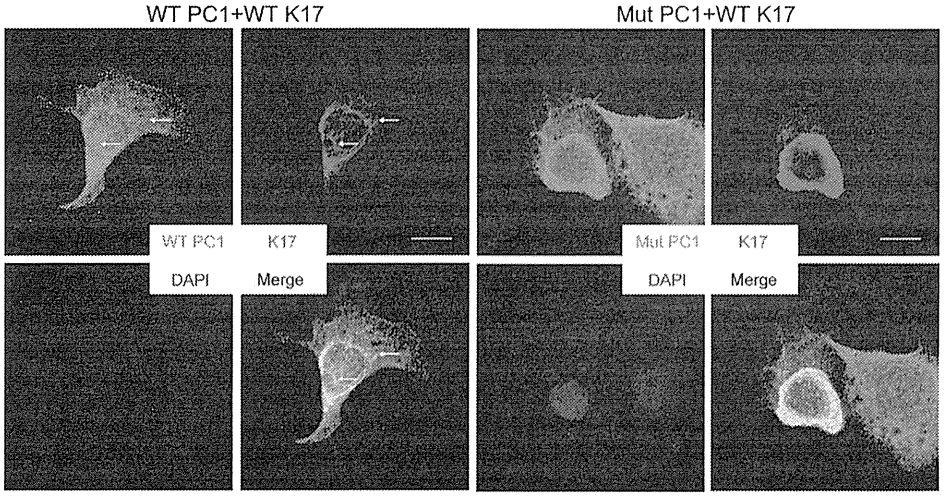

Fig. 2. When HaCaT cells were doubly nucleofected with WT *PKD1* and WT *KRT17* genes, part of the WT PC1 protein looked filamentous, and this protein co-localised with WT K17 (arrows). The anti-V5 antibody (Life Technologies, Carlsbad, CA) and the rabbit polyclonal anti-FLAG (F7425) antibodies (Sigma-Aldrich, St Louis, MO) were used to detect K17 protein and PC1, respectively. In contrast, when HaCaT cells were doubly nucleofected with Mut *PKD1* and WT *KRT17* genes, the resulting network of K17 was as follows; depletion of keratin filaments near the cytoplasmic membrane of cells and cytoplasmic aggregates around nucleus were observed. Near-complete disruption of the keratin filament network occurred. DAPI-staining of the nuclei. Scale bar: 25 μm.

the keratin filament network was observed in many cells (Fig. 2). We counted about 10,000 nucleofected HaCaT cells and found 42% with signs of a collapsed keratin filament network.

DISCUSSION

Sequence analysis of the *PKD1* gene showed that the patient was heterozygous for a novel c.9855_9856insAC mutation. There was no mutation found in the *KRT17* gene. As we hypothesised that her SM might be caused by the *PKD1* gene mutation, we conducted a double transfection experiment. When keratinocytes were transfected with Mut *PKD1* and WT *KRT17* genes, the network of K17 was markedly collapsed. Similar changes were also observed in keratinocytes from patients with SM associated with pachyonychia congenita type 2 (1).

Polycystin-1 (PC1) is a large (~4,302 residues) integral membrane with 11 transmembrane domains (7). The PC1-Pacsin 2-N-Wasp complex is thought to contribute to the formation and maintenance of normal kidney tubular structures (7). The putative role of PC1 in epidermis is unknown (8). We speculate that cysts formed because one *PKD1* allele contained a germline c.9855_9856insAC mutation and the other *PKD1* allele later acquired a somatic mutation.

Interestingly, when HaCaT cells were doubly nucleofected with Mut *PKD1* and WT *KRT17* genes, the resulting network of K17 collapsed. The precise molecular mechanism how K17 aggregates induce cyst formation is currently not clear. The altered signal transduction due to cytoplasmic K17 aggregates may, like K14 aggregates in epidermolysis bullosa simplex (9), impair normal organisation of the epidermal cytoskeleton and hence contribute to cyst formation.

Future studies should be performed to detect molecular pathomechanism underlining the association of polycystic kidney disease with SM.

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REFERENCES

1. Kanda M, Natsuga K, Nishie W, Akiyama M, Nagasaki A, Shimizu T, et al. Morphological and genetic analysis of steatocystoma multiplex in an Asian family with pachyonychia congenital type 2 harbouring a KRT17 missense mutation. Br J Dermatol 2008; 160: 465–468.
2. Smith FJ, Corden LD, Rugg EL, Ratnavel R, Leigh IM, Moss C, et al. Missense mutation in keratin 17 cause either pachyonychia congenital type 2 or a phenotype resembling steatocytoma multiplex. J Invest Dermatol 1997; 108: 220–223.
3. Chapin HC, Caplan MJ. The cell biology of polycystic kidney disease. J Cell Biol 2010; 191: 701–710.
4. Yoneda K, Demitsu T, Kubota Y. Novel ATPA2 mutation in a patient with Darier's disease. J Dermatol 2014; 41: 349–350.
5. Yoneda K, Demitsu T, Nakai K, Moriue T, Ogawa W, Igarashi J, et al. Activation of vascular endothelial growth factor receptor 2 in a cellular model of loricrin keratoderma. J Biol Chem 2010; 285; 16184–16194.
6. Yoneda K, Nakagawa T, Lawrence OT, Huard J, Demitsu T, Kubota Y, et al. Interaction of the profilaggrin N-terminal domain with loricrin in human cultured keratinocytes and epidermis. J Invest Dermatol 2012; 132: 1206–1214.
7. Yao G, Su X, Nguyen V, Roberts K, Li X, Takakura A, et al. Polycystin-1 regulates actin cytoskeleton organization and directional cell migration through a novel PC1 Pacsin 2-N-Wasp complex. Hum Mol Genet 2014; 23: 2769–2779.
8. Lu W, Peissel B, Babakhanlou H, Pavlova A, Geng L, Fan X, et al. Perinatal lethality with kidney and pancreas defects in mice with a targeted Pkd1 mutation. Nat Genet 1997; 17: 179–181.
9. Yoneda K, Furukawa T, Zheng Y-J, Momoi T, Izawa I, Inagaki M, et al. An autocrine/paracrine loop linking keratin 14 aggregates to tumor necrosis factor a-mediated cytotoxicity in a keratinocyte model of epidermolysis bullosa simplex. J Biol Chem 2004; 279: 7296–7303.

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SHORT COMMUNICATION

Detection of Autoantibodies to Precursor Proteins of Desmogleins in Sera of a Patient with Bowen Carcinoma

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Non-pathogenic autoantibodies against desmogleins (Dsg), which do not reflect disease activity, might be detected during disease courses of pemphigus, but are rarely shown in healthy individuals (1). To clarify the significance of these antibodies, using various techniques we examined sera of a patient with Bowen carcinoma, which showed high titres of circulating anti-Dsg1 and anti-Dsg3 IgG antibodies by commercially available enzyme-linked immunosorbent assays (ELISA).

CASE REPORT AND RESULTS

A 78-year-old Japanese female had suffered from an erosive erythematous lesion on the right hand for 9 months. She visited a dermatologist and pemphigus was suspected as a differential diagnosis. Because ELISA analysis showed positive IgG autoantibodies to Dsg3 (index value 117; normal <7), the patient was referred to us for further examination. Physical examination revealed a brownish erythema with infiltration 40 × 18 mm in diameter on the dorsum of the right hand (Fig. 1). Histopathology of the skin lesion demonstrated proliferation of numerous dysplastic cells with abnormal nuclei in the epidermis. Tumour cells showed subepidermal invasion (Fig. S1[1]). These findings were consistent with Bowen carcinoma. Direct immunofluorescence of both the normally appearing skin and the tumour showed no positive reactivity. Re-examination by ELISA confirmed positive IgG reactivity with both Dsg1 (index value 280; normal <14) and Dsg3 (index value 113; normal <7). Anti-Dsg1 antibody titres were higher than anti-Dsg3 antibody titres.

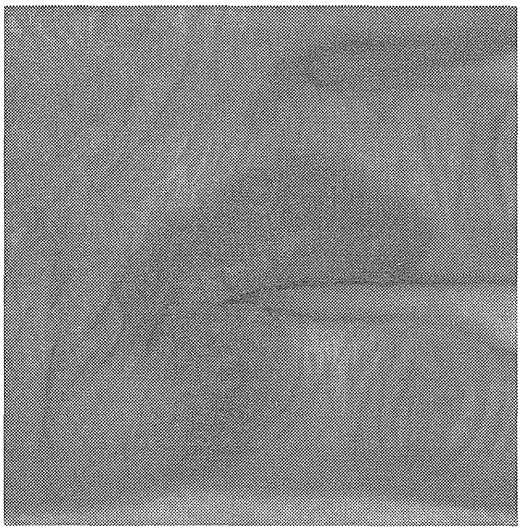

Fig. 1. A brownish, erythematous plaque with scales and crusts on the right dorsal hand.

The study of non-pathogenic pemphigus antibodies by EDTA-treated ELISA. Pathogenic pemphigus antibodies recognise calcium-dependent conformational epitopes of Dsgs, whereas non-pathogenic antibodies react with calcium-independent linear epitopes of Dsgs (1, 2). To investigate whether the patient sera reacted with calcium-dependent or independent epitopes, we measured titres of anti-Dsg IgG autoantibodies in the patient sera by both conventional and EDTA-treated ELISA. ELISA analysis was carried out as previously reported using ELSA kits (MBL, Co., Ltd., Japan) (2, 3). The titres of IgG anti-Dsg1 and anti-Dsg3 autoantibodies in the patient sera were not influenced by the treatment of EDTA, as both sera taken before and after surgical resection of the tumour (Table S1[1]). This result suggested that the patient had calcium-independent non-pathogenic antibodies to Dsgs. Therefore, we considered that antibodies in our case might recognise calcium-independent linear epitopes or precursor domain on Dsgs. In the pipetting assay, the patient serum was not capable of inducing the cell dissociation of the keratinocytes *in vitro* (data not shown) (2).

The study of antibodies against precursor domains of Dsgs by immunoprecipitation-immunoblotting (IP-IB). To clarify whether the patient IgG antibodies reacted with the precursor forms of Dsg1 and Dsg3, we performed IP-IB using recombinant proteins (RPs) of mutated forms of Dsg1 and Dsg3, in which the original endoproteolytic cleavage site of Dsg is replaced (4). The original RPs of Dsg1 and Dsg3 were also used as mature forms. These RPs were expressed in Chinese hamster ovary (CHO) cells, which have more efficient post-translational modification than insect cells (baculovirus expression system) used for standard Dsg ELISA analysis. Because the pure precursor and mature forms of Dsg were prepared in CHO cells from their respective plasmid constructs (Fig. S2[1], left panel), we could investigate reactions with the precursor and mature forms of Dsg1 and Dsg3, respectively (5). The IP-IB clearly demonstrated that the patient serum reacted with the precursor forms of Dsg1 and Dsg3, but not with the mature forms of both Dsgs (Fig. S2[1], right panel). Immunoreactivity of Dsg3 was much stronger than that of Dsg1.

The study of antibodies against extracellular (EC) domains of Dsgs by IP-IB using domain-swapped molecules. The domain-swapping approach is useful for conformational epitope mapping in pemphigus (6). To analyse specific domains of Dsgs reacted by the patient serum, IP-IB using swapped molecules of Dsg1/Dsg2 and Dsg3/Dsg2 containing one each of EC1-5 domains of Dsg1 or Dsg3 on the backbone of Dsg2, whose endoproteolytic cleavage sites were not replaced (7, 8), was performed as described previously (9–11), with the following

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1776

modifications. We used anti-His-tag mAb, anti-6xHis (28–75) (WAKO, Osaka, Japan) instead of anti-E-tag mAb for IP. In IB, precipitated proteins, which were considered to contain both precursor and mature forms of Dsgs (12), were visualized by a chemiluminescence detection system. IP-IB was performed using 20 µl of the patient serum, and anti-His tag mAb and normal serum were used as positive and negative controls, respectively. The results indicated that the patient serum failed to react with any domains of Dsg1. In contrast, the patient serum reacted with both the full-length protein and EC1 domain of Dsg3 (Fig. S3[1]).

Direct immunohistochemical study of the skin tumour using anti-Dsg1 and -3 antibodies. To examine expression of Dsgs in the tumour, we have performed immunohistochemical staining on formalin-fixed and paraffin-embedded tissue sections. Anti-Dsg1 antibody (prediluted, Progen Biotechnik GmbH, Heidelberg, Germany) and anti-Dsg3 antibody (1:50 diluted, Invitrogen Corporation, Camarillo, CA, USA) were used in the experiment with histofine new fuchsin substrate kit (Nichirei, Tokyo, Japan). Immunohistochemical staining of the tumour tissue showed positive intracytoplasmic staining with weak cell surface staining for both Dsg1 and Dsg3 (Fig. 2). In contrast, surrounding normal epidermis demonstrated much weaker staining than the tumour portion (data not shown). Negative controls were obtained by switching primary antibody to normal mouse IgG (Fig. 2C) or by omitting the primary antibody (Fig. 2D).

DISCUSSION

The results of IP-IB study of RPs of precursor and mature forms suggested that the patient IgG antibodies reacted with precursor segments of Dsg1 and Dsg3, and were non-pathogenic. Furthermore, IP-IB study of domain-swapped molecules showed that the patient serum reacted with both the full-length and EC1-containing RPs of Dsg3. These 2 RPs contain the precursor domain of Dsg3, further suggesting positive reactivity of the patient serum with precursor fragment of Dsg3. The results of EDTA-treated Dsg ELISA analysis indicated that the patient serum reacted with non-conformational epitopes.

In IP-IB studies, the patient serum reacted more strongly with Dsg3 RPs than Dsg1 RPs, and reacted swapped molecule of only Dsg3 but not Dsg1. These results indicated that the patient's IgG antibodies reacted with different types of epitopes on the precursor fragments on Dsg1 and Dsg3.

However, these results were inconsistent with the stronger reactivity with Dsg1 in ELISA analysis. To account for this discrepancy between IP-IB and ELISA methods the following (unproven) are proposed: (*i*) ELISA is solid phase reaction in which dried antigen proteins fixed to the bottom of wells react with patient serum, whereas IP-IB is a liquid phase reaction between antigen and serum; (*ii*) RPs used in ELISA analysis originated from insect cells, while RPs used in IP-IB were produced by CHO cells; (*iii*) experimental conditions, including molecular structures of RPs, reaction time and secondary antibodies, are different. Accordingly, ELISA index value may not necessarily reflect intensity of the signals in IP-IB.

Immunohistochemical analysis demonstrated that the tumour portion expressed intracytoplasmic Dsg1 and Dsg3 stronger than adjacent normal epidermis. Therefore, the cancer cells may contribute to the development of these proteins.

Because Dsg precursor proteins are not exposed to the immune system, immune tolerance to precursor segments may not be induced. Therefore, since the presence of specific B cells against precursor of Dsg1 in a normal population has been demonstrated previously (13), antibodies to precursor segments may be produced in some specific individuals. Alternatively, large amounts of Dsg precursor proteins were released from Bowen carcinoma cells and were exposed to immune system, causing production of anti-Dsg antibodies. Further studies are needed to clarify the nature of anti-Dsg antibodies in non-pemphigus cases.

REFERENCES

1. Yokoyama T, Amagai M. Immune dysregulation of pemphigus in humans and mice. J Dermatol 2010; 37: 205–213.
2. Kamiya K, Aoyama Y, Shirafuji Y, Hamada T, Morizane S, Fujii K, et al. Detection of antibodies against the non-calcium-dependent epitopes of desmoglein 3 in pemphigus vulgaris and their pathogenic significance. Br J Dermatol 2012; 167: 252–261.
3. Amagai M, Komai A, Hashimoto T, Shirakata Y, Hashimoto K, Yamada T, et al. Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus. Br J Dermatol 1999; 140: 351–357.
4. Yokouchi M, Saleh MA, Kuroda K, Hachiya T, Stanley JR, Amagai M, et al. Pathogenic epitopes of autoantibodies in pemphigus reside in the amino-terminal adhesive region of

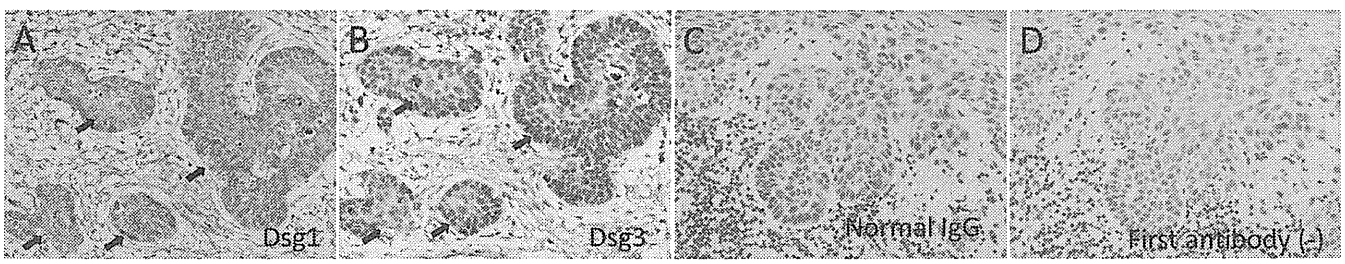

Fig. 2. Immunohistochemical studies of tumour tissue for Dsg1 and Dsg3. Immunohistochemically, Dsgs showed strong cytoplasmic staining with weak cell surface staining. (A) Intracytoplasmic staining of Dsg1 in tumour cells (×200). (B) Intracytoplasmic expression of Dsg3 in tumour cells (×200). (C) Negative control obtained by switching primary antibody to normal mouse IgG (×200). (D) Negative control obtained by skipping primary antibody (×200).

desmogleins which are unmasked by proteolytic processing of prosequence. J Invest Dermatol 2009; 129: 2156–2166.
5. Toumi A, Saleh MA, Yamagami J, Abida O, Kallel M, Masmoudi A et al. Autoimune reactivity against precursor form of desmoglein 1 in healthy Tunisians in the area of endemic pemphigus foliaceus. J Dermatol Sci 2013;70: 19–25.
6. Futei Y, Amagai M, Sekiguchi M, Nishifuji K, Fujii Y, Nishikawa T. Use of domain-swapped molecules for conformational epitope mapping of desmoglein 3 in pemphigus vulgaris. J Invest Dermatol 2000;115: 829–834.
7. Ishii K, Amagai M, Hall RP, Hashimoto T, Takayanagi A, Gamou S, et al. Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. J Immunol 1997; 159: 2010–2017.
8. Ota T, Amagai M, Watanabe M, Nishikawa T. No involvement of IgG autoantibodies against extracellular domains of desmoglein 2 in paraneoplastic pemphigus or inflammatory bowel diseases. J Dermatol Sci 2003; 32: 137–141.
9. Koga H, Ohyama B, Tsuruta D, Ishii N, Hamada T, Dainichi T, et al. Five Japanese cases of anti-Dsg1 antibody positive and anti-Dsg3 antibody negative pemphigus with oral lesions. Br J Dermatol 2012; 166: 976–980.
10. Ohyama B, Nishifuji K, Chan PT, Kawaguchi A, Yamashita T, Ishii N, et al. Epitope spreading is rarely found in pemphigus vulgaris by large-scale longitudinal study using desmoglein 2-based swapped molecules. J Invest Dermatol 2012; 132: 1158–1168.
11. Chan PT, Ohyama B, Nishifuji K, Yoshida K, Ishii K, Hashimoto T, et al. Immune response towards the amino-terminal of desmoglein 1 prevails across different activity stages in nonendemic pemphigus foliaceus. Br J Dermatol 2010; 162: 1242–1250.
12. Yokouchi M, Saleh MA, Kuroda K, Hachiya T, Stanley JR, Amagai M, et al. Pathogenic epitopes of autoantibodies in pemphigus reside in the amino-terminal adhesive region of desmogleins which are unmasked by proteolytic processing of prosequence. J Invest Dermatol 2009; 129: 2156–2166.
13. Yamagami J, Kacir S, Ishii K, Payne AS, Siegel DL, Stanley JR. Antibodies to the desmoglein 1 precursor proprotein but not to the mature cell surface protein cloned from individuals without pemphigus. J Immunol 2009; 183: 5615–5621.

Diagnosis of oral mucous membrane pemphigoid by means of combined serologic testing

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Objective. Mucous membrane pemphigoid (MMP) is a rare autoimmune bullous disease caused by various autoantibodies. This study aimed to evaluate the diagnostic value of MMP-specific autoantibodies in patient sera.

Study Design. We analyzed sera from 30 MMP-suspected patients with intractable oral mucosal lesions using a combination of indirect immunofluorescence with 1M NaCl–split skin, immunoblot analysis, and ELISAs. We also analyzed clinical features among different types of MMP.

Results. Seventeen, 4, and 3 patients were diagnosed with anti-BP180-type MMP, anti-laminin-332-type MMP, and combined anti-BP180/anti-laminin-332-type MMP, respectively.

Conclusions. Our results indicated that a combination of immunologic testing for circulating autoantibodies is useful for the diagnosis of MMP. (Oral Surg Oral Med Oral Pathol Oral Radiol 2014;117:483-496)

Autoimmune bullous diseases (ABDs) are a group of heterogeneous blistering disorders that may involve the oral cavity.[1,2] ABDs are divided into the pemphigus group and subepidermal bullous disease group. Current classification of ABDs with their autoantigens is summarized in Table I. Major diseases of the pemphigus group are pemphigus vulgaris (PV), pemphigus foliaceus (PF), and paraneoplastic pemphigus (PNP). The subepidermal bullous disease group includes bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), and epidermolysis bullosa acquisita (EBA). ABDs show autoantibodies to various epithelial adhesion molecules or extracellular matrices. Pemphigus diseases are characterized by the presence of autoantibodies against components of cell-cell adhesion, whereas pemphigoid diseases have autoantibodies against components at the dermoepidermal junction (Figure 1). Recently developed assays for autoantibodies and autoantigens enable serologic diagnosis in 90% of ABD cases.[3-9]

Previous studies using biochemical and molecular biologic techniques have identified autoantigens and epitopes in various ABDs.[10-13] These autoantigens were originally examined by immunoprecipitation and immunoblotting (IB).[14-16] In particular, noncollagenous 16a (NC16a) domain, which localizes adjacent to the transmembrane portion in the extracellular region of BP180 (type XVII collagen), has been found to be highly antigenic in BP.[17-19] To facilitate the testing of larger numbers of sera simultaneously, various enzyme-linked immunosorbent assays (ELISAs) were developed.[3-6,8,9] ELISAs of recombinant proteins (RPs) of the extracellular domains of desmoglein 1 (Dsg1) and Dsg3, which were produced by the baculovirus-expression system,

Statement of Clinical Relevance

Mucous membrane pemphigoid (MMP) is a rare autoimmune bullous disease caused by various autoantibodies. Our results suggest that a combination of multiple immunologic tests can be used in the diagnosis of MMP.

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Table I. Classification and autoantigens in various ABDs

Diseases	*Ig subtypes*	*Autoantigens*
**Pemphigus vulgaris (PV)	IgG	
**Mucosal dominant type PV	IgG	Dsg3
**Mucocutaneous type PV	IgG	Dsg3 + Dsg1
*Pemphigus vegetans	IgG	Dsg3, Dsg1, (**Dsc3**)
Pemphigus foliaceus	IgG	Dsg1
**Oral pemphigus foliaceus*	*IgG*	*Dsg1*
Pemphigus erythematosus	IgG	Dsg1
Herpetiform pemphigus	IgG	Dsg1, (Dsg3, **Dsc1**)
Drug-induced pemphigus	IgG	**Mainly Dsg1**
**Paraneoplastic pemphigus	IgG	Plectin, **epiplakin**, desmoplakin, BP230, envoplakin, periplakin, *A2ML1*, Dsg1, Dsg3, **Dsc2, Dsc3**
Atypical pemphigus (Anti-Dsc pemphigus)	IgG/IgA	Dsc1-3
IgA pemphigus		
SPD type IgA pemphigus	IgA	Dsc1
IEN type IgA pemphigus	IgA	**Unknown**
*Bullous pemphigoid (BP)	IgG	BP180, BP230
BP230-type BP	**IgG**	**BP230**
*Herpes gestationis	IgG	BP180
**Mucous membrane pemphigoid (MMP)		
**Anti-BP180-type MMP	IgG/IgA	BP180
**Anti-laminin332-type MMP	IgG	Laminin-332 (laminin-5, epiligrin)
**Anti-p168 MMP	IgG	**168-kDa unknown protein**
Ocular MMP	*IgG/IgA*	*β4 integrin*, **unknown**
***Oral MMP*	*IgG*	*α6 integrin*
Dermatitis herpetiformis	IgA	*Epidermal transglutaminase*
C3 dermatitis herpetiformis	C3	Unknown (no autoantigen?)
Linear IgA dermatosis (LABD)		
Lamina lucida type LABD	IgA	97-kDa/120-kDa LAD-1
Sublamina densa type LABD	IgA	**Unknown** (Type VII collagen, *Laminin γ1*)
**Epidermolysis bullosa acquisita	IgG	Type VII collagen
Bullous SLE	IgG	Type VII collagen
Anti-laminin γ1 pemphigoid	IgG	*Laminin γ1 (p200)*

Italic text: recently characterized.
Bold text: autoatigens have not been characterized to date.
ABDs, autoimmune bullous diseases; *Dsc*, desmocollin; *Dsg*, desmoglein; *IEN*, intraepidermal neutrophilic; *Ig*, immunoglobulin; LAD-1, linear IgA bullous dermatosis antigen-1; *SLE*, systemic lupus erythematosus; *SPD*, subcorneal pustular dermatosis.
*Diseases that may show oral lesions.
**Diseases that show extensive oral lesions.

were established for use in the diagnosis of PF and PV.[8,9] Subsequently, ELISAs of RP of BP180 NC16a domain and RPs of N- and C-terminal domains of BP230 were developed for diagnosis of BP.[20,21] Recently, ELISA of RP of combined NC1 and NC2 domains of type VII collagen was developed for the diagnosis of EBA.[22] These 5 ELISAs are now commercially available in Japan. Furthermore, in Japan at least, ELISAs for Dsg1, Dsg3, and BP180 are now covered by the National Health Insurance. Thus, most patients with PV, PF, BP, and EBA can be assessed using commercial ELISA kits.[3-6,8,9,22,23]

ABDs with predominant mucosal lesions were previously referred to as cicatricial pemphigoid. However, although ocular or laryngeal lesions frequently result in scarring, oral mucosal lesions are not commonly associated with scarring. Therefore, at an international consensus meeting on MMP held in 2002, cicatricial pemphigoid was renamed MMP.[24] MMP most commonly affects oral mucosa, followed by ocular, nasal, genital, pharyngeal, esophageal, laryngeal, and anal mucosae.[24-26] In addition, a variable number of MMP patients may show skin lesions. These variable clinical features lead to frequent misdiagnoses of patients with MMP.

On indirect immunofluorescence (IIF) of various substrates, MMP demonstrates the presence of immunoglobulin G (IgG) antibodies, IgA antibodies, or both (IgG/IgA) against the epithelial basement membrane zone (BMZ).[26,27] However, MMP sera will occasionally show negative or very low reactivity in either IIF of normal human skin sections or IIF of 1M NaCl–split skin sections (ss-IIF), because of low titers of circulating antibodies.[28-31] MMP sera react with a number of additional BMZ antigens. Among these, 70% of MMP patients exhibit IgG/IgA antibodies against the BP180 C-terminus domain and are diagnosed as anti-BP180-type MMP.[24,28,30,31] Most patients with anti-BP180-type MMP are negative by ELISA for the BP180 NC16a domain. In addition, approximately 20% of MMP patients show IgG antibodies against various subunits of laminin-332, previously referred to as laminin-5 or epiligrin,[32] and thus are diagnosed as anti-laminin-332-type MMP.[33-35] Anti-BP180-type and anti-laminin-332-type MMP sera react to the epidermal and dermal sides on ss-IIF, respectively.[35,36]

Furthermore, there are reports of MMP patients exhibiting IgG/IgA antibodies against BP230,[37] the 97-/120-kDa linear IgA bullous dermatosis antigen-1 (LAD-1; soluble truncated extracellular domain of BP180),[38] laminin-331,[39] type VII collagen,[24] and integrin α6 and integrin β4.[40] Thus, MMP is very heterogeneous, which may lead to difficulty in its diagnosis. The relationship between clinical features and different autoantigens has not been completely studied, and autoantigens associated with several MMP types have not been identified.

As PV and BP patients may show blistering skin lesions in addition to oral lesions, they often present initially to dermatology. In contrast, because most MMP patients present initially with oral mucosal lesions as their

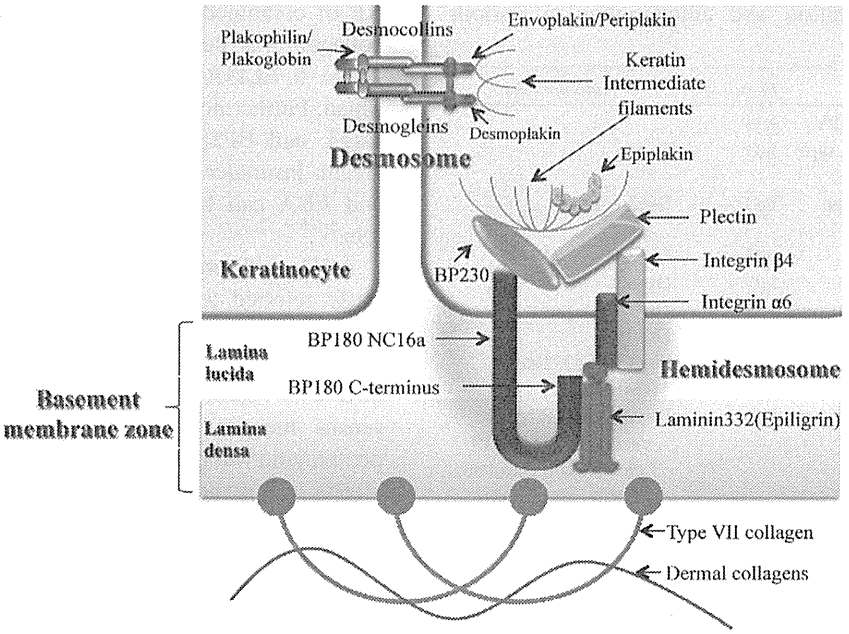

Fig. 1. Schematic structure and component proteins of desmosomes and hemidesmosomes. Desmosomes connect neighboring keratinocytes and hemidesmosomes connect the epidermis to the dermis. Keratin intermediate filaments bind to the cell membrane containing plakoglobins, plakofilins, and desmoplakins at desmosomal attachment plaques. These proteins bind to desmogleins and desmocollins, cadherin-type desmosomal cell adhesion molecules. Hemidesmosomal cytoplasmic proteins (BP230, plectin) bind transmembrane proteins BP180 and α6β4-integrin, which connect to laminin-332. Type VII collagen in the lamina densa and in the uppermost dermis composes anchoring fibrils and binds dermal collagens.

principal complaint, significant numbers of these patients initially present to their dentists. However, because the oral lesions are not specific to ABDs, many patients are misdiagnosed as having other acute or chronic conditions.

Because ABDs are typically intractable and affect the patient's quality of life, both dermatologists and dentists should always consider ABDs, including PV, MMP, and less commonly PNP or EBA, in patients with chronic oral erosive lesions. Serologic tests for autoantibodies and autoantigens are important in distinguishing ABDs from other oral mucosal disorders. As mentioned earlier, most patients with PV, PNP, and EBA can be diagnosed by means of commercially available ELISAs.[3-6,8,9,22,23] In addition, commercial ELISA of BP180 NC16a domain RP shows negative results in typical MMP cases, because most MMP sera contains predominantly antibodies against BP180 C-terminal domain or laminin-332 but not against the BP180 NC16a domain.

In this study, we examined 3 immunologic methods to analyze circulating antibodies in 30 patients with suspected MMP. Our results suggest that a combination of multiple immunologic tests for circulating autoantibodies is useful in the diagnosis of MMP.

MATERIALS AND METHODS

Patients

We enrolled 30 patients with suspected oral mucosal lesions of MMP. In these patients, clinical, histopathologic, and ELISA studies found no findings suggestive of PV or oral lichen planus. We excluded patients who were clinically diagnosed as bacterial, viral, or fungal infection; physical, heat, or chemical injuries; malignant tumors; erythema multiforme; Stevens-Johnson syndrome; toxic epidermal necrolysis; or systemic lupus erythematosus. Sera from patients with different ABDs, including PV, BP, PF, MMP, PNP, EBA, and linear IgA bullous dermatosis (LABD), were used as positive controls. Normal sera were used as negative controls.

All studies were performed in accordance with the guidelines of the Medical Ethics Committees of Aichi-Gakuin University School of Dentistry and Kurume University School of Medicine. All patients or their legal guardians provided informed consent before participating in this study. The study was conducted in compliance with the principles of the Declaration of Helsinki.

Indirect immunofluorescence

IIF of normal human skin sections was performed using standard methods.[41] ss-IIF was also performed, using previously described methods.[42] For immunostaining, skin sections were first blocked with 1% bovine serum albumin for 30 minutes at 4°C, followed by incubation with patient sera, diluted to 1:10 to 1:160 for IgG antibodies and to 1:10 to 1:40 for IgA

Table II. Summaries of clinical features and immunologic results in 30 cases of suspected MMP

		Clinical features												Immunological results: IIF	
				Location/Sites of lesions						Treatment				Normal skin	
				Oral cavity											
Cases	MMP type	Age	Gender	Gingiva	Buccal mucosa	Palate	Tongue	Lip	Other	Steroids	ASG	Others	Outcomes	IgG	IgA
1	BP180	72	M	U	U	U	-	U	-	S	-	DDS	1	+	+
2	BP180	80	M	B/U	-	-	-	-	Skin	T	-	-	2	+	-
3	BP180	75	M	B/U	B/U	B/U	-	-	Skin	T/S	+		2	-	-
4	BP180	37	M	-	A	A	A	-	-	T	+	M/N	2	-	-
5	BP180	35	F	-	U/E	U/E	-	U/E	-	T	+	M/N	2	-	-
6	BP180	60	F	U/E	U/E	-	-	-	-	T	+	M/N	1	-	-
7	BP180	49	F	B/U/E	-	-	-	-	-	S	+	DDS	2	-	-
8	BP180	82	M	U	-	-	-	-	-	T/S	-	-	2	-	-
9	BP180	85	M	-	U/E	U/E	-	-	-	T	+	-	2	-	-
10	BP180	72	F	B	-	-	-	-	-	T	+	-	2	-	-
11	BP180	67	F	B/U/E	-	-	-	-	-	T	+	DDS	2	+	-
12	BP180	82	M	B/U	-	-	-	-	-	T	+	-	1	-	-
13	BP180	72	F	-	A	-	A	-	-	T	+	-	3		
14	BP180	69	M	U	U	-	-	-	-	T	+	-	1	-	-
15	BP180	61	M	U/E	U/E	-	-	-	-	T	+	M/N	2	-	-
16	BP180	87	M	-	U	-	-	-	-	T	+	-	2	-	-
17	BP180	47	F	U/E	-	-	-	-	-	T	+	M/N	2	-	-
18	L332	82	M	U	-	U	-	-	-	T	+	-	3	+	+
19	L332	61	F	U	U	-	-	-	-	T	-	M/N	2	-	-
20	L332	72	F	U/E	-	-	-	-	-	T	-	-	3	-	-
21	L332	62	F	-	B/U	-	-	-	Pharynx	T	+	-	2	-	-
22	BP180+L332	74	M	U	U	-	-	-	-	T	+	-	3	-	-
23	BP180+L332	58	F	B/U	-	-	-	-	-	-	+	-	1	-	-
24	BP180+L332	71	F	U/E	U/E	-	-	-	-	T	+	M/N	2	-	+
25	Unlikely	46	M	B	-	-	-	-	-	-	-	-	1	-	-
26	Unlikely	17	M	-	U	-	-	-	-	T	+	-	1	-	-
27	Unlikely	85	F	-	U	-	-	-	Skin	T	+	-	2	-	-
28	Unlikely	62	F	U	-	-	-	-	-	T	-	-	1	-	-
29	Unlikely	62	F	U	U	U	U	U	Pharyngolarynx	T	+	-	1	-	-
30	Unlikely	42	M	-	A	A	A	A	-	T	-	M/N	2	-	-

L332, laminin-332; *F*, female; *M*, male; *B*, blisters; *U*, ulcers and erosions; *E*, erythema; *A*, aphthous-like; *T*, topical steroid therapy; *S*, systemic steroid therapy; *ASG*, azulene sulfonate gargling; *M/N*, minocycline and nicotinic acid; *DDS*, diaminodiphenyl sulfone; *A*, IgA; *E*, epidermal side; *D*, dermal side; *MMP*, mucous membrane pemphigoid; *IIF*, indirect immunofluorescence; *IB*, immunoblottting; *ELISA*, enzyme-linked immunosorbent assay; *HaCaT*, cell line; *BP*, bullous pemphigoid.

antibodies, for 1.5 hours at room temperature. Subsequently, sections were washed 3 times with phosphate-buffered saline (PBS) and incubated with 1:100 diluted fluorescein isothiocyanate–conjugated anti-human IgG or IgA antiserum (Dako, Glostrup, Denmark) for 1 hour at room temperature. Sections were again washed 3 times with PBS, mounted, and examined under a BX51 microscopy system (Olympus, Tokyo, Japan).

Immunoblotting

IB analysis performed for all 30 cases. For IB sources, normal human epidermal extract,[16] RP of BP180 NC16a domain,[19] RP of BP180 C-terminal domain,[43] concentrated supernatant from culture of HaCaT cell line cells,[44] normal human dermal extract,[45] and purified human laminin-332[46] were prepared as described previously.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the method of Laemmli,[47] with 6% separating gels (Bio Craft, Tokyo, Japan) for epidermal and dermal extracts, 9% separating gels (Bio Craft) for concentrated HaCaT cell supernatant and purified human laminin-332, and 12% separating gels (Bio Craft) for BP180 RPs. Separated proteins were electrophoretically transferred onto nitrocellulose membranes.

For immunostaining, strips of blotted sheets were first blocked with 3% skim milk (Morinaga, Tokyo, Japan) in Tris-buffered saline (TBS) for 1 hour at room temperature and were then incubated for 3 hours at room temperature with sera diluted at 1:20 with 3% skim milk in TBS. Strips were subsequently incubated with horseradish peroxidase–conjugated rabbit anti-human IgG or IgA antiserum (Dako) diluted at 1:100 with 3% skim milk in TBS for 2 hours at room temperature. Between each treatment, strips were washed for 5 minutes with 3 changes of TBS containing 0.05%

Immunological results																			
IIF		*IB*														*ELISA*			
		Epidermal extracts				*BP180 RP*										*BP180*			
Split skin		*BP180*		*BP230*		*NC16a*		*C-Term*		*HaCaT (LAD-1)*		*Dermal extracts*		*Laminin -332*		*NC16a*		*BP230*	
IgG	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*	*IgG*	*IgA*
E	E	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-
E	E	+	-	-	-	+	-	+	-	+	-	-	-	-	-	+	-	-	-
E	-	+	+	+	-	+	-	+	-	+	-	-	-	-	-	+	-	-	-
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Tween 20 (Wako, Osaka, Japan) and with 1 change of TBS. Finally, color was developed with 4-chloro-1-naphthol (Sigma-Aldrich, St Louis, MO, USA) in the presence of hydrogen peroxide.

Enzyme-linked immunosorbent assay

ELISA of BP180 NC16a domain RP using MESACUP BP180 test[20] (MBL, Nagoya, Japan) and ELISA of BP230 N- and C-terminal domain RPs using BP230 ELISA Kit[21] were performed according to protocols provided by the supplier (MBL).

Statistical analysis

Data were statistically analyzed with the Student *t* test and Pearson χ^2 test using SigmaPlot (Hulinks Inc, Tokyo, Japan). We assessed the clinical features among different MMP groups. Differences were considered significant at $P < .05$.

RESULTS

Clinical features of 30 patients with suspected MMP

The present study included 15 male and 15 female patients with ages ranging from 17 to 87 years (mean, 64.2 years; Table II). Additional complicating diseases included hypertension, diabetes mellitus, chronic renal failure, angina pectoris, sarcoidosis, drug induced hepatitis, hepatitis C, ovarian tumor, benign thyroid tumor, collagen disease, bronchial asthma, and several allergic diseases. Five patients (17%) had significant histories of malignant tumors: 2 cases (7%) of oral cancer and 1 case (3%) each of stomach, liver, and skin cancer. Three (10%) of these represented prior histories of cancer, and 2 (7%) developed cancer during follow-up.

Twenty-five patients (83%) showed bullous, ulcerative, or erythematous lesions suspicious for MMP involving only the oral mucosa. The gingiva (70%

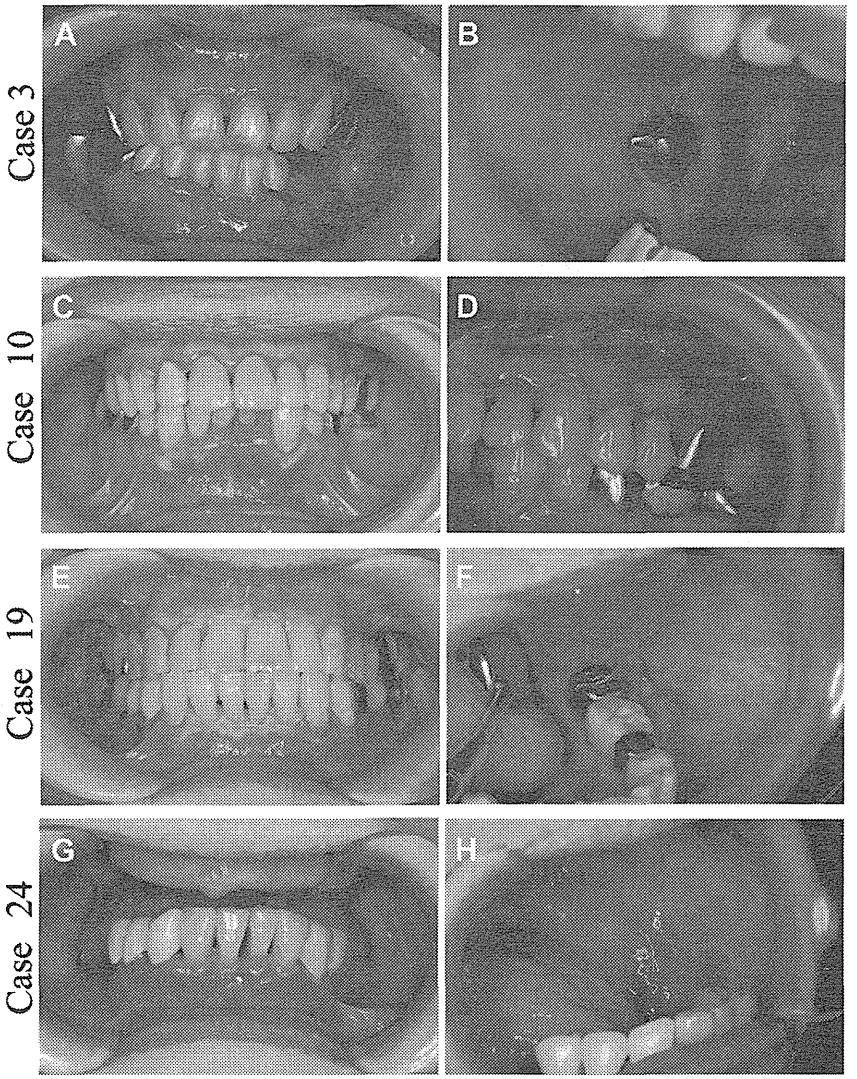

Fig. 2. Clinical appearance of oral mucosal lesions in 4 representative MMP patients. A-B, anti-BP180-type MMP, case 3. C-D, BP180-type MMP, case 10. E-F, Anti-laminin-332-type MMP, case 19. G-H, Concurrence of anti-BP180-type MMP and anti-laminin-332-type MMP, case 24. (*MMP*, mucous membrane pemphigoid.)

[21 of 30]) and buccal mucosa (60% [18 of 30]) were most commonly affected, with 10 patients showing only gingival lesions. Less commonly affected sites included the palate (27% [8 of 30]), tongue (13% [4 of 30]), and lip (13% [4 of 30]). Two patients (7%) had pharyngeal or laryngeal lesions, and 3 patients (10%) had skin lesions (see Table II). No ocular mucosal involvement was seen. The clinical appearances of 4 representative cases are shown in Figure 2.

Topical corticosteroid medication was used as first-line therapy for most patients (87% [26 of 30]) to reduce oral symptoms. For poor responders to topical corticosteroid therapy, oral minocycline (26.7% [8 of 30]; dosage range, 100-300 mg; mean, 175 mg), oral nicotinic acid (26.7% [8 of 30]; dosage range, 150-600 mg; mean, 262.5 mg), and oral 4,4′-diaminodiphenyl sulfone (DDS) (10% [3 of 30]; dosage range, 50-75 mg; mean, 58.3 mg) were administered simultaneously or independently. For patients with more severe disease, systemic corticosteroids were administered (13% [4 of 30]; dosage range, 5-30 mg; mean, 15 mg). Two patients were treated with a combination of oral steroids and DDS.

Indirect immunofluorescence

IIF of normal human skin sections found IgG and IgA anti-BMZ antibodies at titers of 1:10 to > 1:160 in 4 and 3 of the 30 cases, respectively (see Table II). Because 2 patients were positive for both IgG and IgA antibodies, IIF of normal human skin sections detected anti-BMZ antibodies in only 5 sera (17%). In contrast, ss-IIF showed much higher sensitivity, and

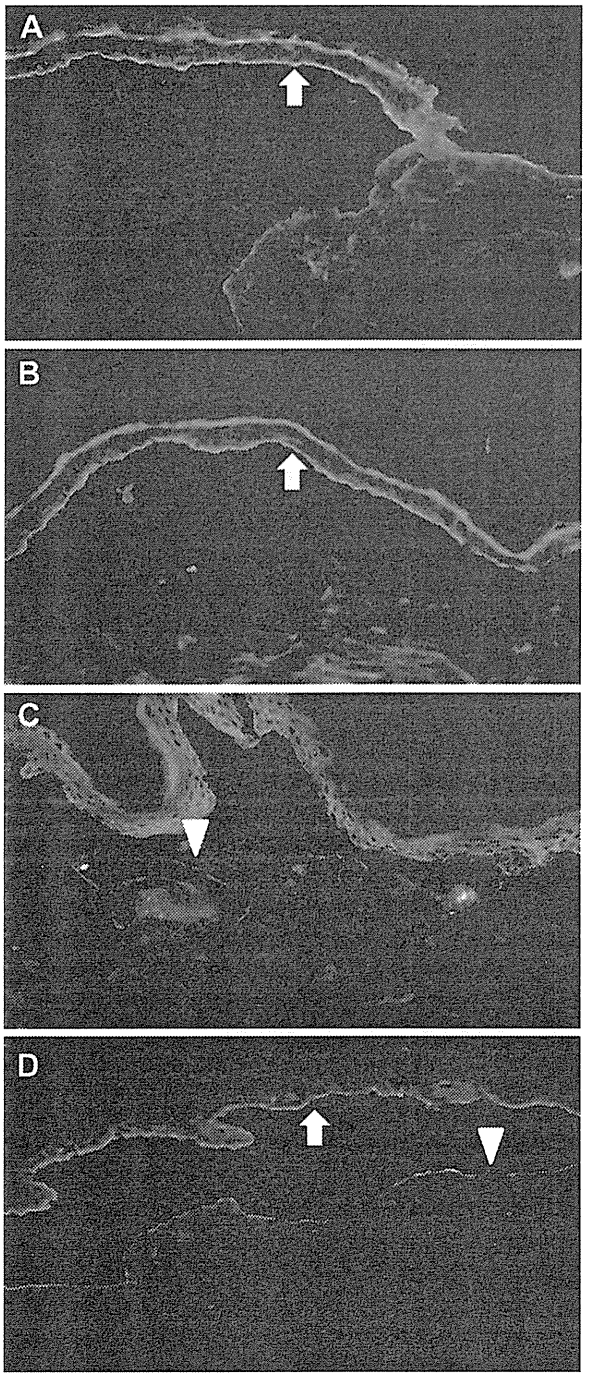

Fig. 3. Results of ss-IIF. A, IgG reactivity only with epidermal side of split skin in anti-BP180-type MMP, case 12. B, IgA reactivity only with epidermal side in anti-BP180-type MMP, case 12. C, IgG reactivity only with dermal side in case 20 of anti-laminin-332-type MMP. D, IgG reactivity with both epidermal and dermal sides in case 22 of concurrence of both MMP types. Arrows and arrowheads indicate positive reactivity with epidermal side and dermal sides, respectively. (*Ig*, immunoglobulin; *MMP*, mucous membrane pemphigoid; ss-IIF, indirect immunofluorescence of split skin section.)

demonstrated positive reactivity in 20 (67%) of the 30 patients, with 7 distinct staining patterns (see Table II; Figure 3). These included positive IgG reactivity with epidermal side (30% [9 of 30]), IgG and IgA with epidermal side (13% [4 of 30]), IgA with epidermal side (3% [1 of 30]), IgG with both epidermal and dermal sides (3% [1 of 30]), IgG with both epidermal and dermal sides and IgA with epidermal side (7% [2 of 30]), IgA with both epidermal and dermal sides (3% [1 of 30]), and IgG with dermal side (3% [1 of 30]).

Staining patterns of ss-indirect immunofluorescence in different MMP types

The ss-IIF demonstrated positive IgG/IgA reactivity in 20 of 30 cases (Figure 4; see Table II). In 17 cases of anti-BP180-type MMP, 10 (59%) showed IgG reactivity with epidermal side of split skin, 4 cases (24%) showed IgG and IgA reactivity with epidermal side, 1 case (6%) showed IgA reactivity with epidermal side, and 2 cases showed no reactivity. In 4 anti-laminin-332-type MMP cases, 1 case showed IgG reactivity with dermal side, 1 case showed IgG reactivity with both epidermal and dermal sides, 1 case showed IgG reactivity with both epidermal and dermal sides and IgA reactivity with epidermal side, and 1 case showed no reactivity. In 3 cases with concurrence of both types, 1 case showed IgA reactivity with both epidermal and dermal sides, 1 case showed IgG reactivity with both epidermal and dermal sides and IgA reactivity with epidermal side, and 1 case showed no reactivity.

Immunoblotting

On IB of normal human epidermal extracts, IgG antibodies in 6 cases (20%) and IgA antibodies in 2 cases (7%) reacted with BP180, whereas IgG antibodies in 2 cases (7%) and IgA antibodies in 3 cases (10%) reacted with BP230 (Figure 5, A). IB of epidermal extracts also showed IgG reactivity with the 210-kDa envoplakin in 1 case (3%), whereas IgG and IgA reactivity with the 190-kDa periplakin was detected in 5 cases (17%) and 6 cases (20%), respectively. No cases reacted with envoplakin and epiplakin simultaneously, which is the major diagnostic criterion for PNP.[48] No IgG/IgA reactivity with the 160-kDa Dsg1 or 130-kDa Dsg3 was observed.

The reactivity with BP180 observed in IB of epidermal extracts was confirmed by IB of BP180 RPs. RP of BP180 NC16a domain reacted with IgG, but not IgA, antibodies in 8 patient sera (27%) (see Figure 5, B). RP of BP180 C-terminal domain reacted with IgG

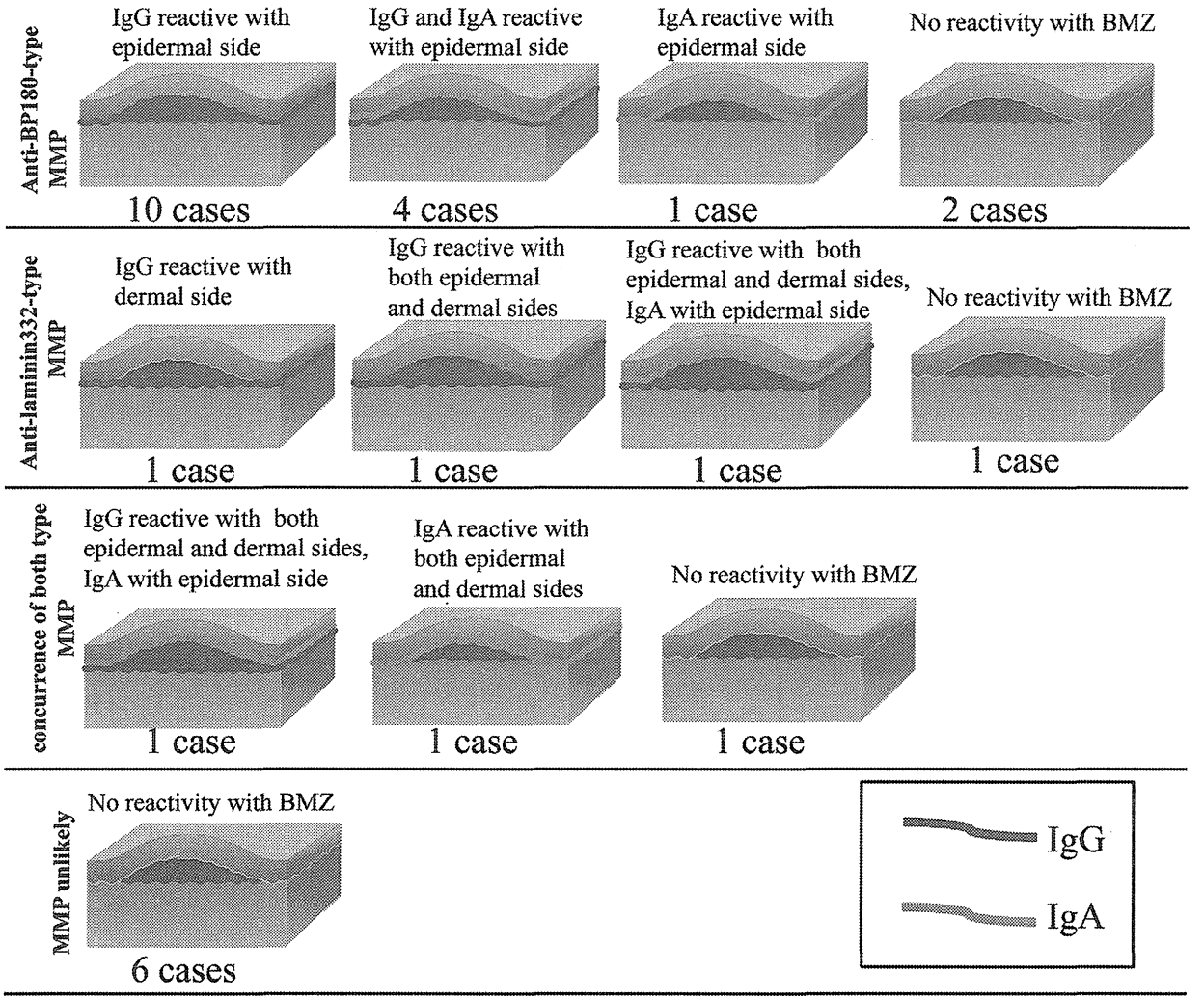

Fig. 4. Schematic summary of ss-IIF results in 4 different MMP groups. Red lines indicate positive IgG reactivity, and green lines indicate positive IgA reactivity. (*BMZ*, basement membrane zone; *Ig*, immunoglobulin; *MMP*, mucous membrane pemphigoid; *ss-IIF*, indirect immunofluorescence of split skin sections.)

antibodies in 11 sera (37%) and by IgA antibodies in 5 sera (17%) (see Figure 5, C).

The 120-kDa LAD-1 in concentrated HaCaT cell culture supernatant reacted with IgG antibodies in 5 sera (17%) and by IgA antibodies in 1 sample (3%) (see Figure 5, D). In IB of normal human dermal extracts, no sera showed any significant reactivity with either 290-kDa type VII collagen or 200-kDa laminin γ1 (p200 antigen) (see Figure 5, E).

On IB of purified human laminin-332, IgG antibodies in 1 serum (3%) and IgA antibodies in another serum reacted with doublets of the 165-kDa and 145-kDa laminin α3 subunits (see Figure 5, F). In addition, IgG antibodies in 5 sera (17%) reacted with the 105-kDa laminin γ2 subunit. One of the 5 sera also reacted with the 165-kDa form, but not with the 145-kDa form, of laminin α3 subunit.

Enzyme-linked immunosorbent assay

IgG and IgA ELISAs for both BP230 and BP180 were performed on all 30 serum samples. IgG antibodies in 9 sera (30%), but no IgA antibodies, were positive for BP180. No positive reactions were observed on ELISA for BP230 (see Table II).

Definitive diagnosis based on serologic examinations

To make a definitive diagnosis of MMP, we used the results of IIF, IB, and ELISA. When IgG/IgA reactivity with RP of BP180 C-terminal domain was positive on IB, a diagnosis of anti-BP180-type MMP was made. When IgG reactivity was positive with at least 1 subunit of lamin-332 on IB for purified human laminin-332, a diagnosis of anti-laminin-332-type MMP was made.

Thus, when both tests were positive, the patient was diagnosed as having concurrence of both MMP types.

Patients without any positive reactivity on IB tests were diagnosed based on the results of ss-IIF. When IgG/IgA reactivity to the epidermal side was seen on ss-IIF, a diagnosis of anti-BP180-type MMP was made, as this ss-IIF pattern is characteristic of anti-BP180-type MMP.[24,49] When IgG reactivity on the dermal side was observed, a diagnosis of anti-laminin-332-type MMP was made.[35,36] When reactivity was positive on both sides of split skin, the patient was diagnosed as concurrence of both MMP types. Remaining patients with negative results in both IB and ss-IIF studies were diagnosed as "MMP-unlikely."

All 30 patients with suspected MMP were diagnosed by these criteria. Twelve sera were positive for antibodies against BP180 C-terminal domain RP in IB, and 7 sera were positive for antibodies against anti-laminin-332. Among the 14 IB-negative sera, 8 sera reacted with epidermal side on ss-IIF. The remaining 6 sera showed no positive reactivity on either IB or ss-IIF. Three sera reacted with both BP180 C-terminal domain RP and laminin-332. Four sera showed reactivity with both the epidermal and dermal sides on ss-IIF. However, in these 4 samples, which showed reactivity with both epidermal and dermal sides on ss-IIF, 2 sera reacted only with laminin-332, but not with the BP180 C-terminal domain, on IB. As 2 sera had very weak reactivity with the epidermal side on ss-IIF and no anti-BP180-antibodies were detected on IB, we diagnosed these as anti-laminin-332-type MMP. Eventually, we diagnosed 17 cases as anti-BP180-type MMP, 4 cases as anti-laminin-332-type MMP, 3 cases as concurrence of both MMP types, and 6 cases as MMP-unlikely (see Table II).

Finally, we analyzed the results for ELISA of the BP180 NC16a domain RP. Eight of the 17 anti-BP180-type MMP cases and 1 of the 3 concurrent MMP cases, but none of the 4 anti-laminin-332-type MMP cases, were positive on BP180 ELISA. These results suggested that BP180 ELISA may be useful in the diagnosis of anti-BP180-type MMP, although to date BP180 ELISA has mainly been used for the diagnosis of BP.

Statistical analyses of relationships between clinical features and MMP types

We statistically compared the clinical features, including age at onset, gender, lesional sites, lesional status, presence of extraoral lesions, presence of malignant tumor, and clinical outcome, among the different MMP types. The mean age at onset in MMP-unlikely patients was 52.3 years, which was significantly younger than the 67.2 years in patients diagnosed as any MMP type ($P = .0271$). Gingiva and buccal mucosa were most commonly affected in both MMP and MMP-unlikely patients, but no significant differences in affected sites were observed among different MMP types. Frequency of malignant complications was similar among different MMP types ($P > .05$). Stomach cancer and liver cancer were associated with 1 case each of anti-laminin-332-type MMP, although the difference in frequency of association of malignant tumors between anti-laminin-332-type MMP and anti-BP180-type MMP was not statistically significant ($P = .079$).

Outcome was determined by improvement of oral symptoms: grade 0 for deterioration, grade 1 for no change, grade 2 for moderate improvement, and grade 3 for marked improvement. Nine (30%) of the 30 patients were refractory to topical or systemic therapies and were rated as grade 0 or 1. Nineteen (79%) patients showed good response to topical and systemic treatments and were rated as grade 2 or 3. In contrast, only 2 of 6 MMP-unlikely patients improved with topical treatment. When we compared patients with grades 2 and 3 with those with grades 0 and 1, improvement in both anti-laminin-332-type MMP and all MMP was significantly better than in MMP-unlikely patients ($P = .035$ and $P = .028$, respectively).

Relationship between immunoglobulin isotypes and BP180 domains

Domain specificity of anti-BP180 antibodies was classified based on the results of IB and ELISA studies using RPs of BP180 NC16a and C-terminal domains, and the relationship with immunoglobulin isotypes was examined (Table III). Five sera reactive with NC16a domain showed only IgG antibodies. Among the 5 sera reactive with both NC16a and C-terminal domains, 4 sera showed only IgG antibodies and 1 serum showed both IgG and IgA antibodies. Among the 7 sera reactive only with the C-terminal domain, 1 serum showed only IgA antibodies, 2 sera showed only IgG antibodies, and 4 sera showed both IgG and IgA antibodies. Statistical analysis found that IgA antibodies had significantly higher reactivity with BP180 C-terminal domain than NC16a domain ($P = .004678$). Furthermore, no patients showed IgA antibodies against BP180 NC16a domain in this study.

DISCUSSION

In this study, when the results of ss-IIF and IB studies were combined, 24 patients with suspected MMP (80%) demonstrated anti-BMZ autoantibodies and were diagnosed as 1 of 3 MMP types. In contrast, the remaining 6 cases were negative on all tests and were considered unlikely to have MMP. We detected circulating IgG/IgA anti-BMZ antibodies in the sera of 20 of 30 patients with suspected MMP by ss-IIF. Further IB studies found that 21 sera had IgG/IgA reactivity with

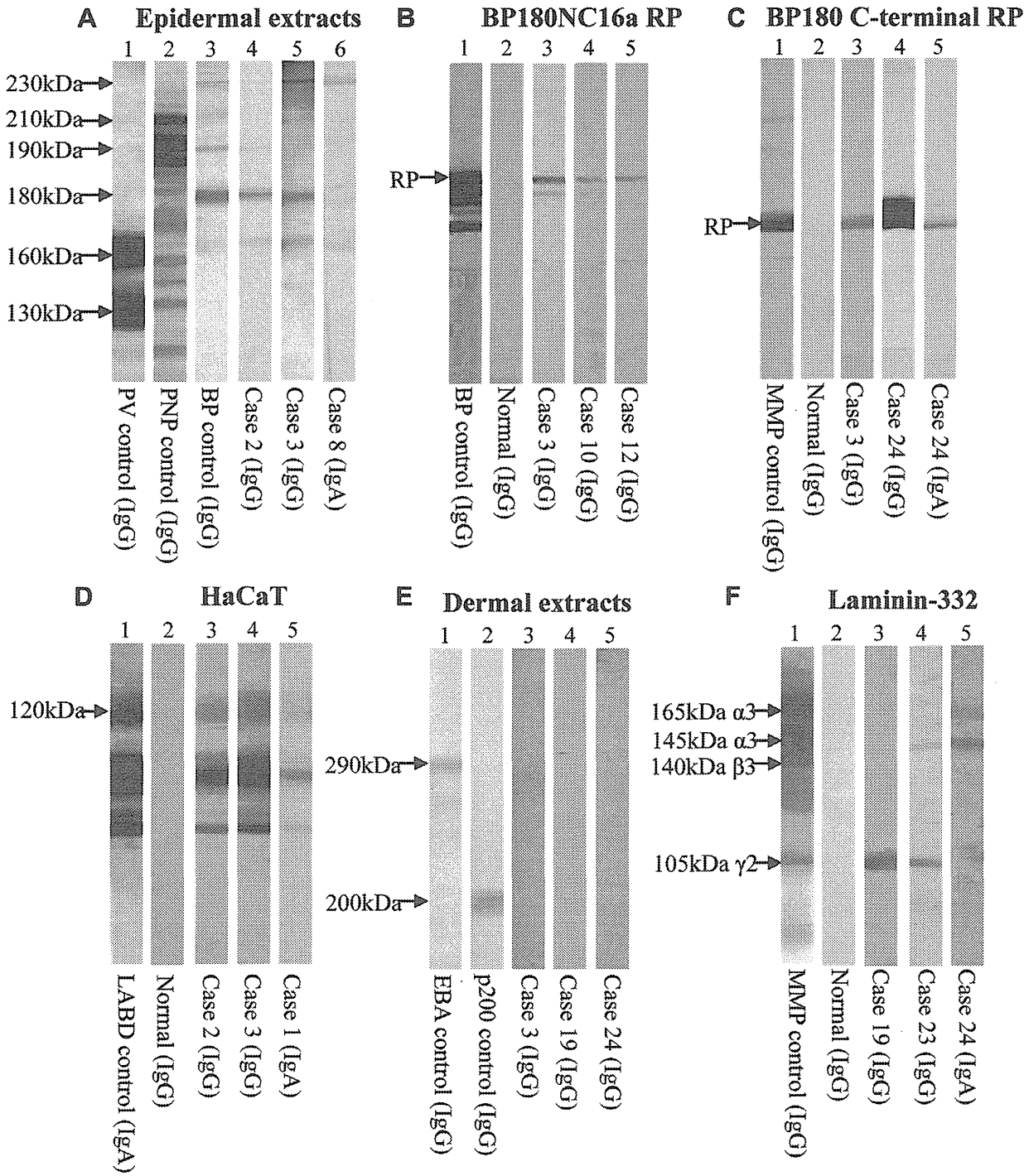

Fig. 5. Results of IB studies using various antigen sources for representative MMP sera. A, IB of normal human epidermal extracts. Control PV serum reacted with the 160-kDa Dsg1 and the 130-kDa Dsg3 (lane 1), control PNP serum reacted with both the 210-kDa envoplakin and the 190-kDa periplakin (lane 2), and control BP serum reacted with the 180-kDa BP180 and the 230-kDa BP230 (lane3). IgG antibodies of anti-BP180-type MMP, case 2, reacted with BP180 (lane 4). IgG antibodies of anti-BP180-type MMP, case 3, reacted with BP230 and BP180 (lane 5). IgA antibodies of anti-BP180-type MMP, case 8, reacted with BP230 (lane 6). B, IB of RP of BP180 NC16a domain. Control BP serum (lane 1), but not normal control serum (lane 2), reacted with this RP. IgG antibodies of cases 3, 10, and 12 of anti-BP180-type MMP reacted with this RP (lanes 3-5). C, IB of RP of BP180 C-terminal domain. Control anti-BP180-type MMP serum (lane 1), but not normal control serum (lane 2), reacted with this RP. IgG antibodies of anti-BP180-type MMP, case 3 (lane 3), and IgG (lane 4) and IgA (lane 5) antibodies of case 24 of concurrence of both MMP types reacted with this RP. D, IB of concentrated HaCaT cell culture supernatant. IgA antibodies of control LABD serum, but not IgG antibodies of normal control serum, reacted with the 120-kDa LAD-1. IgG antibodies of case 2 (lane 3) and case 3 (lane 4) of

various BMZ autoantigens. Specifically, 17 sera (56.7%) reacted with epidermal side on ss-IIF or with BP180 C-terminal domain RP and were diagnosed as anti-BP180-type MMP. Nine of 17 anti-BP180-type MMP patients had IgG/IgA autoantibodies against BP180 C-terminal domain. In contrast, 7 sera (23.3%) reacted with laminin-332 and were diagnosed as anti-laminin-332-type MMP. This incidence of anti-laminin-332-type MMP was consistent with previous findings.[35]

Although there are differences between anti-BP180-type MMP and anti-laminin-332-type MMP, the 2 types are difficult to distinguish by clinical features alone, particularly in patients presenting only with oral symptoms. Thus, ss-IIF is commonly used to differentiate the 2 types. However, in our study, 4 MMP patients were negative on ss-IIF, and the diagnosis was confirmed by IB results.

One patient (case 24) with concurrence of both types showed IgA, but not IgG, antibodies reactive with both sides of split skin on ss-IIF and with the 165-kDa and 145-kDa forms of laminin α3 subunit on IB. This case would be diagnosed as LABD based on the results of serologic tests. However, because the patient had only oral lesions, we diagnosed this case as IgA anti-laminin-332-type MMP.

Two anti-laminin-332-type MMP cases (cases 18 and 19) showed reactivity with both epidermal and dermal sides of split skin. However, because the 2 sera showed much weaker reactivity with the epidermal side and did not react with the BP180 C-terminal domain RP, we ultimately diagnosed them as anti-laminin-332-type MMP, although the possibility that the sera contained low titer antibodies against BP180 not be excluded.

Sensitivity of ELISA for the BP180 NC16a domain RP in the diagnosis of MMP has not been well documented, although this ELISA is highly sensitive and reliable for the diagnosis of BP.[20,50] In our study, 8 of 17 anti-BP180-type MMP patients were positive on ELISA for the BP180 NC16a domain. These 8 cases were convincingly diagnosed as anti-BP180-type MMP based on typical clinical features and findings on IB and ss-IIF. In addition, all patients with anti-laminin-332 type MMP and MMP-unlikely were negative by BP180 ELISA. Therefore, although sensitivity was only around 50%, positive BP180 ELISA in patients suspected for MMP strongly suggests anti-BP180-type MMP.

Table III. Relationship between immunoglobulin isotopes and BP180 domains

	IgG	*IgG + IgA*	*IgA*
NC16a	5	0	0
NC16a + C-terminus	4	1	0
C-terminus	2	4	1

BP180, type XVII collagen; Ig, immunoglobulin; NC16a, noncollagenous 16a domain.

In the present study, sera reactive with BP180NC16a tended to have only IgG antibodies, whereas sera reactive with C-terminus tended to have IgG/IgA antibodies (see Table III), which is consistent with previous results.[18,19,51] These findings suggest that distinct immunoglobulin isotypes target different epitopes on BP180, and this may contribute to different pathophysiology between BP and MMP.

Six patients, who showed no positive reactions on any serologic tests, were diagnosed as MMP-unlikely. However, it is possible that these patients react with other untested antigens. For example, MMP patients with ocular and oral lesions were reported to react with integrin β4 and integrin α6, respectively, by IB using bovine gingival lysate.[40] We have also confirmed positive reactivity with integrin β4 in pure ocular MMP sera using novel IB with hemidesmosome-rich fraction (Li et al, manuscript in preparation).

After MMP types were determined, we analyzed the clinical findings in the different subtypes. Anti-BP180-type MMP tended to show diffuse and erythematous stomatitis with shallow ulcers, whereas anti-laminin-332-type MMP tended to show gingivitis and stomatitis with deep ulceration and necrosis (see clinical features in Figure 2). These different clinical presentations may be caused by different targets in the epidermal BMZ between BP180 and laminin-332.[52,53] In patients with suspected MMP, clinical findings of deep

anti-BP180-type MMP and IgA antibodies of case 1 of anti-BP180-type MMP (lane 5) reacted with LAD-1. E, IB of normal human dermal extracts. Control EBA serum reacted with the 290-kDa type VII collagen (lane 1), and control anti-laminin γ1 (p200) pemphigoid serum reacted with the 200-kDa laminin γ1 (p200) (lane 2). IgG antibodies of case 3 (lane 3) of anti-BP180-type MMP and case 19 (lane 4) of anti-laminin-332-type MMP, and IgG antibodies of case 24 of concurrence of both MMP types (lane 5), did not show any reactivity. F, IB of purified human laminin-332. Control anti-laminin-332 type MMP serum reacted with the 165-kDa and 145-kDa forms of α3 subunit, the 140-kDa β3 subunit, and the 105-kDa γ1 subunit of laminin-332 (lane 1), whereas normal control serum did not react with any subunits (lane 2). IgG antibodies of case 19 of anti-laminin-332-type MMP reacted with the 105-kDa γ2 (lane 4). IgG antibodies of case 23 of concurrence of both MMP types reacted with the105-kDa γ2 (lane 5). IgA antibodies of case 24 of concurrence of both MMP types reacted with the 165-kDa and 145-kDa forms of α3 subunit (lane 5). (*BP*, bullous pemphigoid; *EBA*, epidermolysis bullosa acquisita; *HaCaT*, cell line; *IB*, immunoblotting; *Ig*, immunoglobulin; *LABD*, linear IgA dermatosis; *LAD-1*, linear IgA bullous dermatosis antigen-1; *MMP*, mucous membrane pemphigoid; *PNP*, paraneoplastic pemphigus; *PV*, pemphigus vulgaris; *RP*, recombinant protein.)

ulceration on the gingival and buccal mucosa may suggest a diagnosis of anti-laminin-332-type MMP, even before specific autoantibodies and autoantigens are detected. Previous studies indicated that anti-laminin-332-type MMP shows more severe clinical features than BP180-type MMP, and is characterized by the presence of lesions involving additional mucous membranes.[54] However, our study did not reflect this tendency, probably because most patients showed only oral mucosal lesions.

It has been well documented that anti-laminin-332-type MMP patients frequently show internal malignancies, particularly cancers of the digestive tract.[54] In this context, it is important to distinguish between the 2 MMP types. Indeed, our study found that 2 patients with anti-laminin-332-type MMP had a history of digestive malignancies (stomach and liver cancers), although no significant differences in associated malignant diseases were observed between anti-BP180-type MMP and anti-laminin-332-type MMP.

In addition to a frequent association with malignant neoplasms,[55] anti-laminin-332-type MMP is known to show significant complications, including blindness and laryngeal stenosis.[56,57] However, our study did not find clear differences in these clinical features and therapeutic response between the 2 MMP types.

For histopathologic examination, biopsy specimens taken from gingival lesions may cause periodontal defects. In addition, chronic inflammation in the gingivitis may interfere with histologic evaluation.[58] In this study, we were unable to confirm the usefulness of histopathologic study, owing to the inadequate quality of our biopsy specimens. In addition, we did not perform direct immunofluorescence (DIF) studies. However, DIF using biopsy samples taken from normal-looking mucosa should be performed in future studies, because positive deposition of immunoglobulins and C3 by DIF may be the only diagnostic finding in cases without positive results from serologic tests.

PV and BP are generally controlled by systemic corticosteroids in dermatologic practice. However, most of our patients had only mild and limited oral symptoms. Therefore, to control the oral symptoms, we used azulene gargle and topical application of corticosteroid ointments. Most cases showed good clinical course with only topical steroids as both initial and maintenance treatments, and the few refractory patients were treated with intermittent systemic steroids at minimal dose.

In conclusion, when assessing patients with erosive oral lesions, the possibility of MMP should be considered in the differential diagnosis, and patients should be referred to a clinician experienced in the treatment of ABDs. To facilitate diagnosis of MMP, ELISA-based systems for both BP180 C-terminal domain and laminin-332 should be developed. We are now preparing an ELISA using bacterial RP of BP180 C-terminal domain. However, our study indicates that the currently available ELISA against the BP180 NC16a domain RP is able to help render a diagnosis in half of anti-BP180-type MMP patients. Therefore, the current ELISA should be used in patients with suspected MMP regardless of its subtypes. However, even if a diagnosis of MMP is suggested on the basis of the results from the currently available ELISA, further examination is still necessary to detect other autoantigens to confirm the MMP subtype.

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REFERENCES

1. Darling MR, Daley T. Blistering mucocutaneous diseases of the oral mucosa—a review, part 1: mucous membrane pemphigoid. *J Can Dent Assoc.* 2005;71:851-854.
2. Darling MR, Daley T. Blistering mucocutaneous diseases of the oral mucosa—a review, part 2: pemphigus vulgaris. *J Can Dent Assoc.* 2006;72:63-66.
3. Blöcker IM, Dähnrich C, Probst C, et al. Epitope mapping of BP230 leading to a novel enzyme-linked immunosorbent assay for autoantibodies in bullous pemphigoid. *Br J Dermatol.* 2012;166:964-970.
4. Charneux J, Lorin J, Vitry F, et al. Usefulness of BP230 and BP180-NC16a enzyme-linked immunosorbent assays in the initial diagnosis of bullous pemphigoid: a retrospective study of 138 patients. *Arch Dermatol.* 2011;147:286-291.
5. Roussel A, Benichou J, Randriamanantany ZA, et al. Enzyme-linked immunosorbent assay for the combination of bullous pemphigoid antigens 1 and 2 in the diagnosis of bullous pemphigoid. *Arch Dermatol.* 2011;147:293-298.
6. Hashimoto T. Recent advances in the study of the pathophysiology of pemphigus. *Arch Dermatol Res.* 2003;295(suppl 1):S2-S11.
7. Bracke S, Speeckaert R, Van Geel N, De Bacquer D, Lambert J. Evaluation of commercially available ELISA assays as a tool for monitoring and managing pemphigus patients: a prospective study. *Eur J Dermatol.* 2013;23:33-39.
8. Amagai M, Komai A, Hashimoto T, et al. Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus. *Br J Dermatol.* 1999;140:351-357.
9. Ishii K, Amagai M, Hall RP, et al. Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol.* 1997;159:2010-2017.
10. Beutner EH, Jordon RE. Demonstration of skin antibodies in sera of pemphigus vulgaris patients by indirect immunofluorescent staining. *Proc Soc Exp Biol Med.* 1964;117:505-510.
11. Jordon RE, Beutner EH, Witebsky E, Blumental G, Hale WL, Lever WF. Basement zone antibodies in bullous pemphigoid. *JAMA.* 1967;200:751-756.
12. Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell.* 1991;67:869-877.
13. Diaz LA, Ratrie H, Saunders WS, et al. Isolation of a human epidermal cDNA corresponding to the 180-kD autoantigen

recognized by bullous pemphigoid and herpes gestationis sera: immunolocalization of this protein to the hemidesmosome. *J Clin Invest.* 1990;86:1088-1094.
14. Stanley JR, Yaar M, Hawley-Nelson P, Katz SI. Pemphigus antibodies identify a cell surface glycoprotein synthesized by human and mouse keratinocytes. *J Clin Invest.* 1982;70:281-288.
15. Stanley JR, Koulu L, Thivolet C. Distinction between epidermal antigens binding pemphigus vulgaris and pemphigus foliaceus autoantibodies. *J Clin Invest.* 1984;74:313-320.
16. Hashimoto T, Ogawa MM, Konohana A, Nishikawa T. Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. *J Invest Dermatol.* 1990;94:327-331.
17. Giudice GJ, Emery DJ, Diaz LA. Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180. *J Invest Dermatol.* 1992;99:243-250.
18. Giudice GJ, Emery DJ, Zelickson BD, Anhalt GJ, Liu Z, Diaz LA. Bullous pemphigoid and herpes gestationis autoantibodies recognize a common non-collagenous site on the BP180 ectodomain. *J Immunol.* 1993;151:5742-5750.
19. Matsumura K, Amagai M, Nishikawa T, Hashimoto T. The majority of bullous pemphigoid and herpes gestationis serum samples react with the NC16a domain of the 180-kDa bullous pemphigoid antigen. *Arch Dermatol Res.* 1996;288: 507-509.
20. Kobayashi M, Amagai M, Kuroda-Kinoshita K, et al. BP180 ELISA using bacterial recombinant NC16a protein as a diagnostic and monitoring tool for bullous pemphigoid. *J Dermatol Sci.* 2002;30:224-232.
21. Yoshida M, Hamada T, Amagai M, et al. Enzyme-linked immunosorbent assay using bacterial recombinant proteins of human BP230 as a diagnostic tool for bullous pemphigoid. *J Dermatol Sci.* 2006;41:21-30.
22. Saleh MA, Ishii K, Kim YJ, et al. Development of NC1 and NC2 domains of type VII collagen ELISA for the diagnosis and analysis of the time course of epidermolysis bullosa acquisita patients. *J Dermatol Sci.* 2011;62:169-175.
23. Kim JH, Kim YH, Kim S, et al. Serum levels of anti-type VII collagen antibodies detected by enzyme-linked immunosorbent assay in patients with epidermolysis bullosa acquisita are correlated with the severity of skin lesions. *J Eur Acad Dermatol Venereol.* 2013;27:e224-e230.
24. Chan LS, Ahmed AR, Anhalt GJ, et al. The first international consensus on mucous membrane pemphigoid: definition, diagnostic criteria, pathogenic factors, medical treatment, and prognostic indicators. *Arch Dermatol.* 2002;138:370-379.
25. Thorne JE, Anhalt GJ, Jabs DA. Mucous membrane pemphigoid and pseudopemphigoid. *Opthalmology.* 2004;111:45-52.
26. Ahmed AR, Kurgis BS, Rogers RS. Cicatricial pemphigoid. *J Am Acad Dermatol.* 1991;24:987-1001.
27. Zillikens D. Acquired skin disease of hemidesmosomes. *J Dermatol Sci.* 1999;20:134-154.
28. Schmidt E, Skrobek C, Kromminga A, et al. Cicatricial pemphigoid: IgA and IgG autoantibodies target epitopes on both intra- and extracellular domains of bullous pemphigoid antigen 180. *Br J Dermatol.* 2001;145:778-783.
29. Setterfield J, Shirlaw PJ, Kerr-Muir M, et al. Mucous membrane pemphigoid: a dual circulating antibody response with IgG and IgA signifies a more severe and persistent disease. *Br J Dermatol.* 1998;138:602-610.
30. Oyama N, Setterfield JF, Powell AM, et al. Bullous pemphigoid antigen II (BP180) and its soluble extracellular domains are major autoantigens in mucous membrane pemphigoid: the pathogenic relevance to HLA class II alleles and disease severity. *Br J Dermatol.* 2006;154:90-98.
31. Balding SD, Prost C, Diaz LA, et al. Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain. *J Invest Dermatol.* 1996;106:141-146.
32. Aumailley M, Bruckner-Tuderman L, Carter WG, et al. A simplified laminin nomenclature. *Matrix Biol.* 2005;24:326-332.
33. Chan LS, Hammerberg C, Cooper KD. Cicatricial pemphigoid: identification of two distinct sets of epidermal antigens by IgA and IgG class circulating autoantibodies. *Arch Dermatol.* 1990;126: 1466-1468.
34. Hisamatsu Y, Nishiyama T, Amano S, Matsui C, Ghohestani R, Hashimoto T. Usefulness of immunoblotting using purified laminin 5 in the diagnosis of anti-laminin 5 cicatricial pemphigoid. *J Dermatol Sci.* 2003;33:113-119.
35. Domloge-Hultsch N, Gammon WR, Briggaman RA, Gil SG, Carter WG, Yancey KB. Epiligrin, the major human keratinocyte integrin ligand, is a target in both an acquired autoimmune and an inherited subepidermal blistering skin disease. *J Clin Invest.* 1992;90:1628-1633.
36. Domloge-Hultsch N, Anhalt GJ, Gammon WR, et al. Anti-epiligrin cicatricial pemphigoid: a subepithelial bullous disorder. *Arch Dermatol.* 1994;130:1521-1529.
37. Leverkus M, Bhol K, Hirako Y, et al. Cicatricial pemphigoid with circulating autoantibodies to beta4 integrin, bullous pemphigoid 180 and bullous pemphigoid 230. *Br J Dermatol.* 2001;145:998-1004.
38. Chan LS, Soong HK, Foster CS, Hammerberg C, Cooper KD. Ocular cicatricial pemphigoid occurring as a sequela of Stevens-Johnson syndrome. *JAMA.* 1991;266:1543-1546.
39. Chan LS, Majmudar AA, Tran HH, et al. Laminin-6 and laminin-5 are recognized by autoantibodies in a subset of cicatricial pemphigoid. *J Invest Dermatol.* 1997;108:848-853.
40. Rashid KA, Gürcan HM, Ahmed AR. Antigen specificity in subsets of mucous membrane pemphigoid. *J Invest Dermatol.* 2006;126:2631-2636.
41. Beutner EH, Jordon RE, Chorzelski TP. The immunopathology of pemphigus and bullous pemphigoid. *J Invest Dermatol.* 1968;51: 63-80.
42. Woodley D, Sauder D, Talley MJ, Silver M, Grotendorst G, Qwarnstrom E. Localization of basement membrane components after dermal-epidermal junction separation. *J Invest Dermatol.* 1983;81:149-153.
43. Nie Z, Hashimoto T. IgA antibodies of cicatricial pemphigoid sera specifically react with C-terminus of BP180. *J Invest Dermatol.* 1999;112:254-255.
44. Ishii N, Ohyama B, Yamaguchi Z, Hashimoto T. IgA autoantibodies against the NC16a domain of BP180 but not 120-kDa LAD-1 detected in a patient with linear IgA disease. *Br J Dermatol.* 2008;158:1151-1153.
45. Ishii N, Yoshida M, Hisamatsu Y, et al. Epidermolysis bullosa acquisita sera react with distinct epitopes on the NC1 and NC2 domains of type VII collagen: study using immunoblotting of domain-specific recombinant proteins and postembedding immunoelectron microscopy. *Br J Dermatol.* 2004;150:843-851.
46. Amano S, Scott IC, Takahara K, et al. Bone morphogenetic protein 1 is an extracellular processing enzyme of the laminin 5 gamma 2 chain. *J Biol Chem.* 2000;275:22728-22735.
47. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680-685.
48. Poot AM, Diercks GF, Kramer D, et al. Laboratory diagnosis of paraneoplastic pemphigus. *Br J Dermatol.* 2013;169:1016-1024.
49. Kelly SE, Wojnarowska F. The use of chemically split tissue in the detection of circulating anti-basement membrane zone antibodies in bullous pemphigoid and cicatricial pemphigoid. *Br J Dermatol.* 1988;118:31-40.
50. Zillikens D, Mascaro JM, Rose PA, et al. A highly sensitive enzyme-linked immunosorbent assay for the detection of

circulating anti-BP180 autoantibodies in patients with bullous pemphigoid. *J Invest Dermatol.* 1997;109:679-683.
51. Hashimoto T, Han-Yaku H, Higashiyama M, et al. Four Japanese patients with cicatricial pemphigoid showing both IgG and IgA antibodies against the 180 kDa bullous pemphigoid antigen. *Br J Dermatol.* 1997;137:305-306.
52. McMillan JR, Akiyama M, Shimizu H. Ultrastructural orientation of laminin 5 in the epidermal basement membrane: an updated model for basement membrane organization. *J Histochem Cytochem.* 2003;51:1299-1306.
53. Powell AM, Sakuma-Oyama Y, Oyama N, Black MM. Collagen XVII/BP180: a collagenous transmembrane protein and component of the dermoepidermal anchoring complex. *Clin Exp Dermatol.* 2005;30:682-687.
54. Egan CA, Lazarova Z, Darling TN, Yee C, Coté T, Yancey KB. Anti-epiligrin cicatricial pemphigoid and relative risk for cancer. *Lancet.* 2001;357:1850-1851.
55. Sadler E, Lazarova Z, Sarasombath P, Yancey KB. A widening perspective regarding the relationship between anti-epiligrin cicatricial pemphigoid and cancer. *J Dermatol Sci.* 2007;47:1-7.
56. Kato K, Moriyama Y, Saito H, Koga H, Hashimoto T. A case of mucous membrane pemphigoid involving the trachea and bronchus with autoantibodies to β3 subunit of laminin-332 [published online August 27, 2013]. Acta Derm Venereol.
57. Dainichi T, Takeshita H, Moroi Y, et al. Cicatricial pemphigoid with autoantibodies against the laminin 5 gamma 2 subunit. *Eur J Dermatol.* 2005;15:189-193.
58. Siegel MA, Anhalt GJ. Direct immunofluorescence of detached gingival epithelium for diagnosis of cicatricial pemphigoid: report of five cases. *Oral Surg Oral Med Oral Pathol.* 1993;75:296-302.

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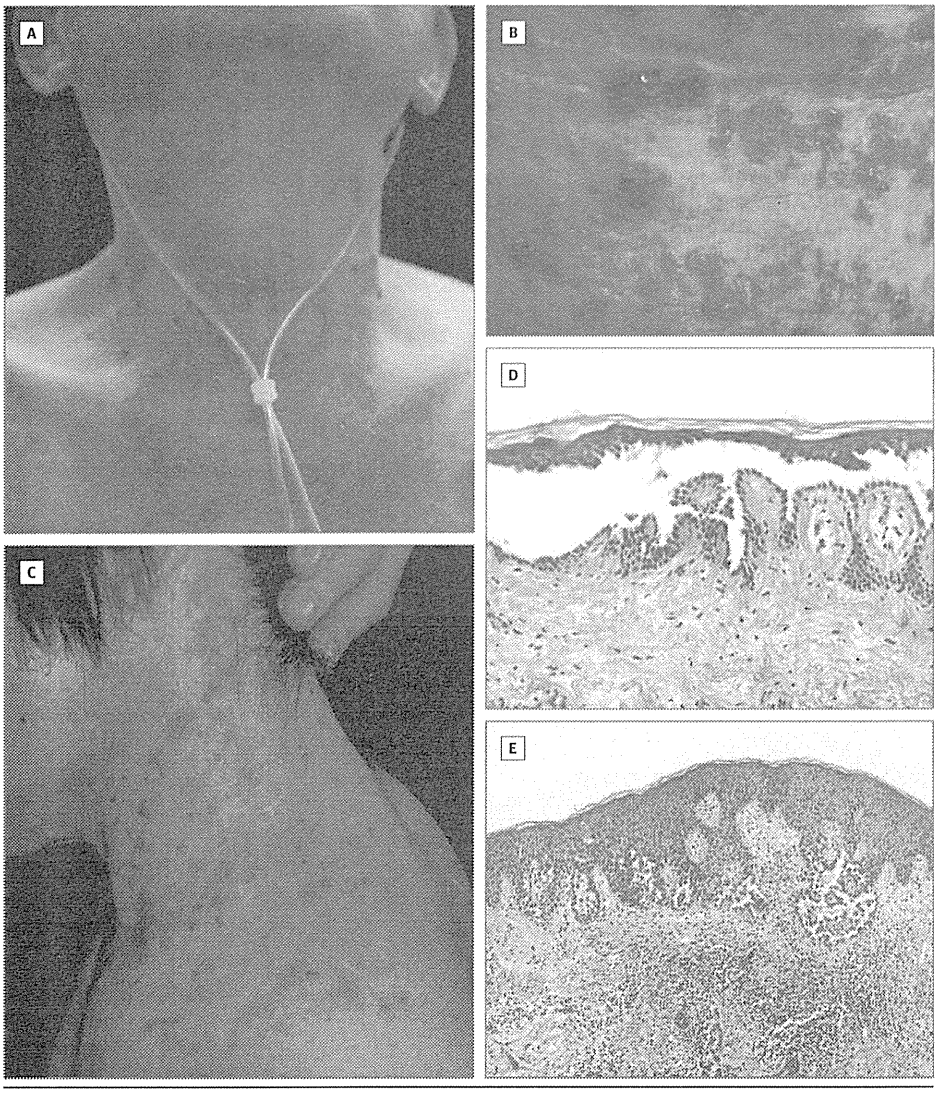
Sibling Cases of Hailey-Hailey Disease Showing Atypical Clinical Features and Unique Disease Course

Hailey-Hailey disease (HHD), a well-characterized autosomal dominant hereditary disease, is caused by mutation in *ATP2C1* gene and clinically shows characteristic erosive lesions predominantly on the intertriginous areas.[1-3] We herein report sibling cases of HHD with novel mutations in the *ATP2C1* gene that showed unique and atypical clinical phenotypes mimicking seborrheic dermatitis, pemphigus vulgaris, or pemphigus foliaceus as well as considerable alterations during the disease course.

Report of Cases | *Case 1.* A 61-year-old Japanese man with intractable pulmonary arterial hypertension, pulmonary fibrosis, and emphysema was receiving oxygen supply from a nasal

Figure 1. Clinical and Histopathologic Features of Case 1 and Case 2 at the First Visit



A and B, Skin lesions on the neck (A) and abdomen (B) in case 1. Clinical features on the neck in case 2 (C). D and E, Histopathologic features (hematoxylin-eosin) from the abdomen in case 1 (D, original magnification ×100) and the neck in case 2 (E, original magnification ×200)

tube at the time of presentation. Physical examination revealed seborrheic dermatitis-like diffuse scaly erythemas on the face and anterior neck, where the nasal tube of the oxygen supply contacted the skin (**Figure 1**A). In addition, extensive erosive erythemas with severe pain were seen on the abdomen, and a few pemphigus vulgaris–like flaccid bullae were also observed (Figure 1B). No skin lesions were seen in any intertriginous areas, including axillae and groin. No mucosal membranes were involved. Detailed medical history indicated that, beginning at age 19 years, the patient had occasional mild eczematous lesions on the axillae, groin, popliteal fossa, and head.

Case 2. A 57-year-old younger sister of patient 1 reported that, beginning at age 20 years, she had developed dermatitis-like skin lesions on the genital region during menstrual periods. Subsequently, itchy erosive lesions appeared on the axillae and groin, which worsened in the summer every year. The skin lesions on the axillae and groin gradually decreased, while skin lesions on the face, neck, and chest continued to develop.

Physical examination revealed erythemas with small erosions scattered on the forehead, neck, chest, and upper back (Figure 1C) but not on the intertriginous areas, except for minimum lesions on the left axilla. There were no apparent vesicles or bullae.

Comment | Histopathologic analysis of the seborrheic dermatitis-like lesion and pemphigus vulgaris-like lesion in case 1 and pemphigus foliaceus-like lesion in case 2 revealed acantholysis without any dyskeratotic keratinocytes in the lower epidermis (Figure 1D and E). Indirect immunofluorescence studies showed IgG antibodies to epithelia of monkey esophagus and rat bladder in case 1 (**Figure 2**A and B), but no conclusive pattern was seen in case 2. Genetic study revealed a novel splice-site mutation in the *ATP2C1* gene in both cases (Figure 2C).

The 2 cases were diagnosed as HHD by identification of a novel mutation in the *ATP2C1* gene. However, while both patients showed more typical HHD features when they were