# Anti-Alpha-2-Macroglobulin-Like-1 Autoantibodies Are Detected Frequently and May Be Pathogenic in Paraneoplastic Pemphigus

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Paraneoplastic pemphigus (PNP) shows autoantibodies mainly to plakin and desmosomal cadherin family proteins. We have recently identified alpha-2-macroglobulin-like-1 (A2ML1), a broad range protease inhibitor, as a unique PNP antigen. In this study, we tested a large number of PNP sera by various methods. Forty (69.0%) of 58 PNP sera recognized A2ML1 recombinant protein expressed in COS7 cells by immunofluorescence (IF) and/or immunoprecipitation (IP)/immunoblotting (IB). IP/IB showed higher sensitivity than IF. In addition, 22 (37.9%) PNP sera reacted with A2ML1 by IB of cultured normal human keratinocytes (NHKs) under non-reducing conditions. Statistical analyses using various clinical and immunological data showed that the presence of anti-A2ML1 autoantibodies was associated with early disease onset and absence of ocular lesions. Next, to investigate the pathogenic role of anti-A2ML1 antibody, we performed additional functional studies. Addition of anti-A2ML1 polyclonal antibody to culture media decreased NHK cell adhesion examined by dissociation assay, and increased plasmin activity detected by casein zymography, suggesting that anti-A2ML1 antibody may decrease NHK cell adhesion through plasmin activation by inhibition of A2ML1. This study demonstrates that autoantibodies to A2ML1 are frequently and specifically detected and may have a pathogenic role in PNP.

## INTRODUCTION

Paraneoplastic pemphigus (PNP) is an autoimmune multi-organ syndrome with intractable stomatitis and polymorphous cutaneous lesions (Anhalt *et al.*, 1990), and underlying neoplasias are mainly lymphoproliferative tumors (Wolff

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Abbreviations: A2ML1, alpha-2-macroglobulin-like-1; AP, alkaline phosphatase; BP, bullous pemphigoid; cDNA, complementary DNA; Dsg, desmoglein; EPL, envoplakin; ESTs, expressed sequence tags; HRP, horseradish peroxidase; IB, immunoblotting; IF, immunofluorescence; IP, immunoprecipitation; IP–IB, immunoprecipitation and immunoblotting; NHK, normal human keratinocyte; PA, plasminogen activator; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PF, pemphigus foliaceus; PIC, protease inhibitor cocktail; PNP, paraneoplastic pemphigus; PPL, periplakin; PV, pemphigus vulgaris; RP, recombinant protein

*et al.*, 1999; Hsiao *et al.*, 2001; Kimyai-Asadi and Jih, 2001; Mimouni *et al.*, 2002).

PNP sera recognize various autoantigens by immunoprecipitation (IP) or immunoblotting (IB) (Oursler *et al.*, 1992; Hashimoto *et al.*, 1995). IgG antibodies in PNP sera typically bind to plakin family proteins, including desmoplakins I and II, BP230, periplakin (PPL) (Kiyokawa *et al.*, 1998), and envoplakin (EPL) (Ruhrberg *et al.*, 1996; Kim *et al.*, 1997; Kiyokawa *et al.*, 1998), as well as desmoglein 3 (Dsg3) and/or Dsg1 (Hashimoto *et al.*, 1995; Amagai *et al.*, 1998). Finally, we have recently identified the 170 kDa antigen as alpha-2-macroglobulin-like-1 (A2ML1) (Schepens *et al.*, 2010). A2ML1 is a broad range protease inhibitor belonging to alpha-2-macroglobulin family. Northern blot and reverse transcriptase-PCR studies revealed *A2ML1* messenger RNA in epidermal keratinocytes.

Keratinocyte differentiation was found to associate with increased expression levels of *A2ML1* (Galliano *et al.*, 2006; Schepens *et al.*, 2010). By immunohistochemistry, A2ML1 was detected within keratinocytes between the uppermost granular layers and the cornified cell layers of the epidermis. Specifically, A2ML1 is secreted into the extracellular space, and inhibits *in vitro* various proteases, including chymotrypsin, papain, thermolysin, and subtilisin A (Galliano *et al.*, 2006). In addition, A2ML1, in analogy with alpha-2-macroglobulin, is thought to have a role in defense mechanisms,

maintenance of epidermal homeostasis, and regulation of cytokine and growth factor releases (Galliano *et al.*, 2006).

However, the prevalence of autoantibodies to A2ML1 was not studied in large series of PNP sera, and clinical and immunological significance and pathogenic role of anti-A2ML1 autoantibodies in PNP are still unknown (Schepens *et al.*, 2010). In this study, we have systematically examined 58 PNP sera by various techniques to search for the presence of anti-A2ML1 autoantibodies, and performed statistical analysis between the presence of anti-A2ML1 antibodies and clinical and immunological characteristics. Furthermore, we performed dissociation assay and protease assay to investigate the pathogenic role of anti-A2ML1 antibody.

## RESULTS

### A2ML1 was expressed in the normal epidermis but not in the rat bladder

Expression profile of A2ML1 in the skin and rat bladder was verified by immunofluorescence (IF) using anti-A2ML1 mouse polyclonal antibody (pAb). A2ML1 was expressed in the granular layers of the normal human epidermis (Figure 1a), as described previously (Galliano *et al.*, 2006; Schepens *et al.*, 2010). In contrast, A2ML1 was not expressed in the rat bladder, whereas IgG antibodies in PNP serum clearly reacted with transitional epithelia (Figure 1b).

### COS7 cells expressing A2ML1 recombinant protein (RP) were recognized by PNP sera in IF

We then assessed the reactivity of all 58 PNP sera by IF using transiently transfected COS7 cells expressing c-Myc-tagged human A2ML1 RP. Both anti-c-Myc rabbit pAb and anti-A2ML1 pAb stained the cytoplasm of transfected COS7 cells in a granular pattern (Figure 2a). The obtained signals showed perfect colocalization with those by PNP sera, indicating that the PNP sera recognized A2ML1 (Figure 2a). In the IF study for all 58 PNP sera, 30 (51.7%) sera recognized A2ML1 RP on COS7 cells (Figure 2b).

### c-Myc-tagged recombinant A2ML1 RP was detected inside of transfected COS7 cells but not in culture media

Proteins in COS7 cells transfected with A2ML1 complementary DNA (cDNA) were separated into four different fractions; i.e., culture medium, washing buffer (cold PBS (phosphate-buffered saline)) extract, IP buffer extract, and final pellet. Although protein bands corresponding to the 172 kDa A2ML1 RP (including c-Myc tag) were seen only faintly in IP buffer extract and pellet by protein staining with amido-black, IB using anti-c-Myc pAb clearly showed the RPs in IP buffer extract and pellet but not in culture medium or washing buffer extract (Figure 3a). Additional experiments confirmed that the same A2ML1 RP in IP buffer extract was detected by both anti-A2ML1 pAb and anti-c-Myc pAb (Figure 3b).

### IgG anti-A2ML1 autoantibodies were detected in PNP sera by IP and IB (IP–IB) of COS7 cells transfected with A2ML1 cDNA

By preliminary IB studies of either A2ML1-expressing COS7 cell extracts or normal human epidermal extracts under standard reducing condition, none of PNP, pemphigus vulgaris (PV), pemphigus foliaceus (PF), bullous pemphigoid (BP), or normal control sera detected A2ML1 proteins (data not shown), confirming the results of our previous studies that PNP sera do not react with A2ML1 in IB under standard reducing conditions.

Then, we examined all 58 PNP sera, diseases control sera, and normal sera by IP–IB of extracts of A2ML1 RP-expressing COS7 cells. Proteins immunoprecipitated with sera were separated on SDS-PAGE and immunoblotted with anti-c-Myc pAb. In this study, 35 (60.3%) of 58 PNP sera reacted with c-Myc-tagged A2ML1 RP (Figure 3c). None of the 10 sera each from PV, PF, and BP patients and 30 normal human sera reacted with A2ML1 RP (Figure 3c).

### PNP sera recognized A2ML1 in differentiated cultures of normal human keratinocytes (NHKs) by IB under non-reducing condition

To examine whether PNP sera detect disulfide bond–related conformational epitopes, we performed IB of NHK extracts
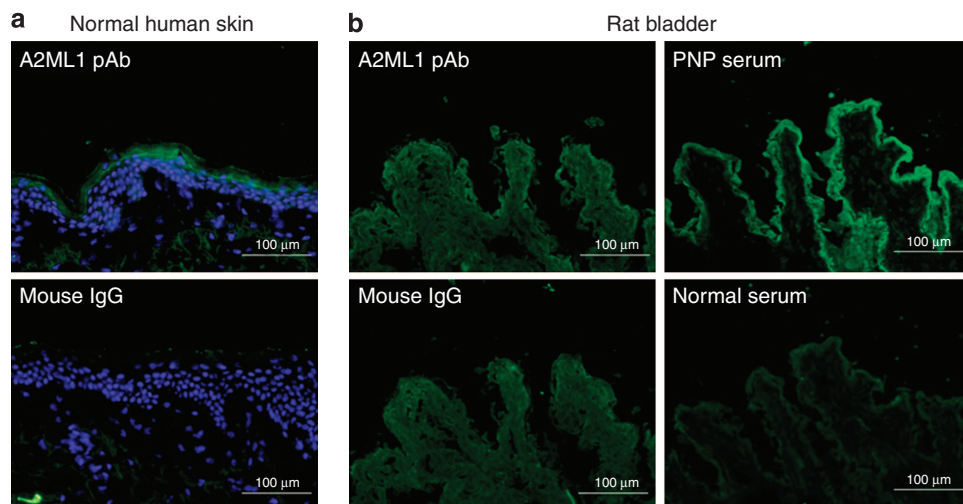


**Figure 1. Immunofluorescence (IF) for expression of alpha-2-macroglobulin-like-1 (A2ML1) in the human skin and rat bladder.** (**a**) IF of normal human skin sections. (**b**) IF of rat bladder sections. Anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb), purified normal mouse IgG, paraneoplastic pemphigus (PNP) serum, and normal human serum were used. Bar = 100 μm.
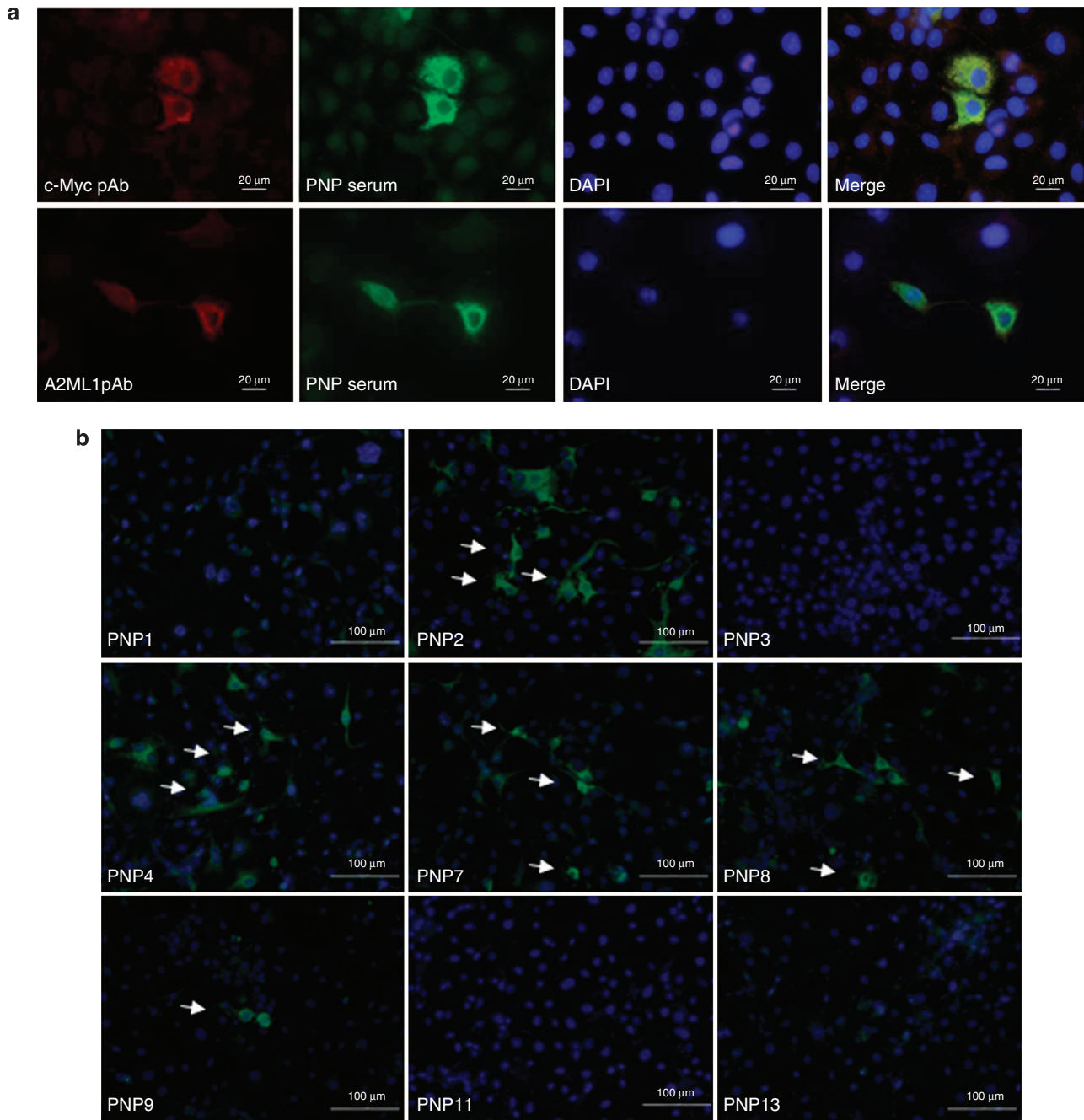
**Figure 2. Immunofluorescence (IF) studies of alpha-2-macroglobulin-like-1 (A2ML1) complementary DNA (cDNA)-transfected COS7 cells.** (**a**) IF of COS7 cells expressing c-Myc-tagged A2ML1, triple-stained with a representative paraneoplastic pemphigus (PNP) serum and 4′,6-diamidino-2-phenylindole (DAPI), as well as with either anti-c-Myc pAb or anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb). Bar = 20 μm. (**b**) IF of A2ML1-expressing COS7 cells, double-stained with 58 PNP sera and DAPI. The case numbers are the same as the case numbers in immunoprecipitation and immunoblotting (IP–IB) figure and Supplementary Table S1 online. PNP2, PNP4, PNP7, PNP8, and PNP9 showed positive reactivity, while PNP1, PNP3, PNP11, and PNP13 were negative. Positive cells are indicated by arrows. Bar = 100 μm.

under both reducing and non-reducing conditions for all 58 PNP sera. Twenty-two (37.9%) PNP sera recognized a protein comigrating with A2ML1 detected by anti-A2ML1 pAb under non-reducing condition (Figure 3d). None of the 5 PV sera, 5 PF sera, 5 BP sera, and 15 normal human sera showed this reactivity (Figure 3d). In contrast, under reducing condition, none of the same set of PNP and control sera recognized the

170 kDa A2ML1 protein band, which was strongly detected by anti-A2ML1 pAb (Figure 3e).

**Statistical comparison of clinical and immunological conditions between anti-A2ML1 antibody–positive and –negative PNP patients**
We statistically analyzed the relationship between the presence of anti-A2ML1 autoantibodies and various clinical and
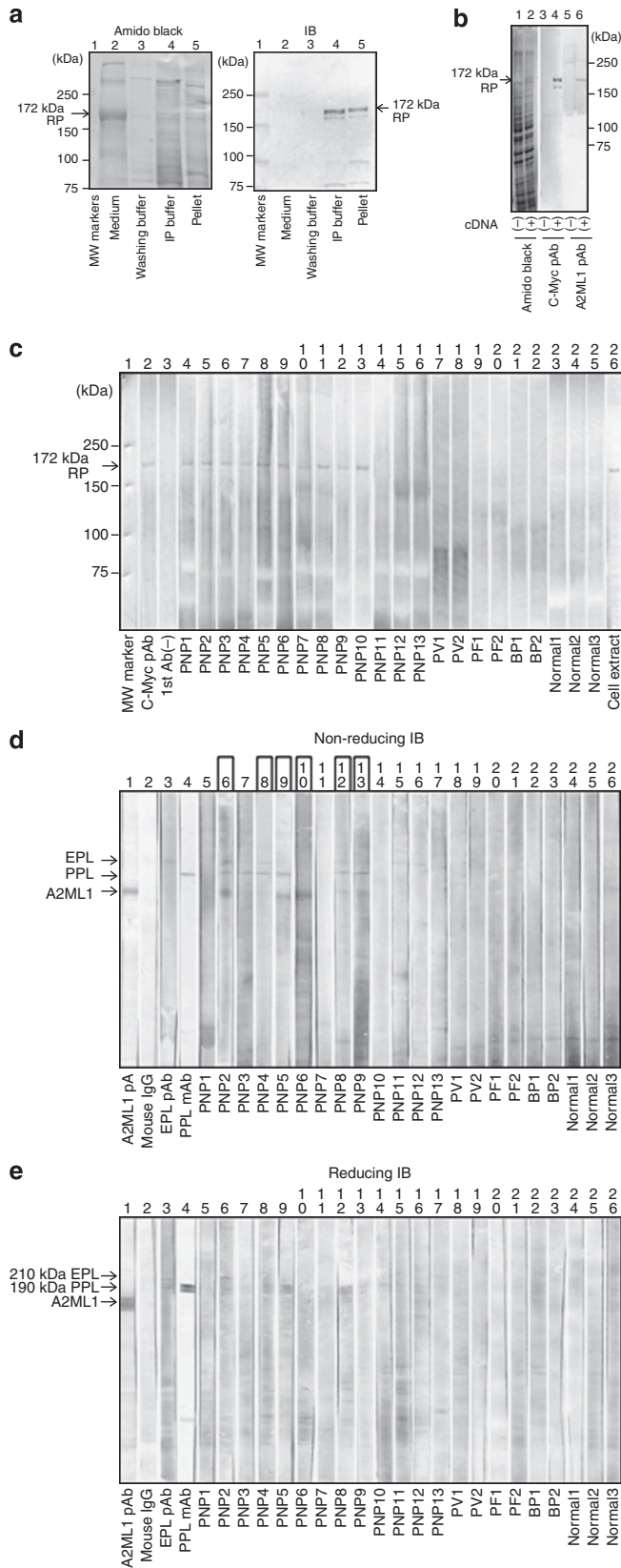
immunological findings in PNP (Figure 4 and Table 1). The 40 PNP sera (69.0%) with positive reactivity in IP–IB and/or COS7 cell IF were considered as anti-A2ML1 antibody-positive (A2ML1-positive). A2ML1-postive PNP cases had significantly earlier disease onset than A2ML1-negative PNP cases ($P = 0.026$) (Figure 4). Interestingly, ocular mucous membrane lesions occurred less frequently in A2ML1-postive PNP cases ($P = 0.050$) (Figure 4). There was no significant correlation of the presence of anti-A2ML1 antibodies with any other parameters (Figure 4 and Table 1). In Supplementary Table S1 online, relevant clinical parameters and the results for all studies of anti-A2ML1 antibodies are summarized.

## A2ML1 was present in NHK culture medium

As the first step of functional studies of anti-A2ML1 antibody, we examined the presence of A2ML1 in NHK culture medium by IB of NHKs at low and high levels of calcium. No A2ML1 was detected in non-concentrated NHK culture media (data not shown). In $100\times$ concentrated culture media, A2ML1 was detected in NHKs cultured at both low and high calcium levels (Figure 5a). A2ML1 was also detected in NHK extracts weakly at low calcium and strongly at high calcium (Figure 5a), as described previously (Galliano *et al.*, 2006).

## Anti-A2ML1 pAb bound to A2ML1 in NHK culture media

To examine whether anti-A2ML1 pAb actually bound to A2ML1 in culture media, we performed IB of NHK culture media treated with Protein G agarose. After IP with Protein G, A2ML1 was detected in NHK culture medium incubated with normal mouse IgG, but not with anti-A2ML1 pAb (Figure 5b).

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**Figure 3. Immunoblotting (IB) study of four fractions of COS7 cells, immunoprecipitation and immunoblotting (IP–IB) of alpha-2-macroglobulin-like-1 (A2ML1) complementary DNA (cDNA)–transfected COS7 cells for various sera, and IB of normal human keratinocyte (NHK) extracts under non-reducing and reducing conditions.** (**a**) Blots of four fractions obtained from A2ML1 cDNA–transfected COS7 cells, stained with amido-black (left panel), and processed for IB for anti-c-Myc pAb (right panel). (**b**) IP buffer extracts of COS7 cells transfected with or without A2ML1 cDNA were stained with amido-black, and processed for IB using both anti-c-Myc pAb and anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb). (**c**) IP buffer extracts of transfected COS7 cells were immunoprecipitated with the representative paraneoplastic pemphigus (PNP) sera, disease control sera, and normal sera, as well as anti-c-Myc pAb, and immunoblotted with anti-c-Myc pAb. The A2ML1 recombinant protein (RP) was detected by sera of PNP1–PNP10 (lanes 4–13), but not by sera of PNP11–PNP13 (lanes14–16). In lane 26, IP buffer extract of transfected COS7 cell was directly stained by IB with anti-c-Myc pAb. The positions of molecular weight (MW) markers and A2ML1 RP are shown by bars and arrows, respectively, in the left of each panel. (**d**) The results of IB under non-reducing condition. MWs are not shown, because MW markers cannot be used in this condition. The PNP sera with numbers surrounded by squares, but no control sera, reacted with the protein band corresponding to A2ML1. Some PNP sera also reacted with envoplakin (EPL) and periplakin (PPL). (**e**) IB under reducing condition. No sera reacted with the 170 kDa A2ML1, while some PNP sera reacted with EPL or PPL. Positions of EPL, PPL, and A2ML1 are shown in the left of each panel. BP, bullous pemphigoid; PF, pemphigus foliaceus.
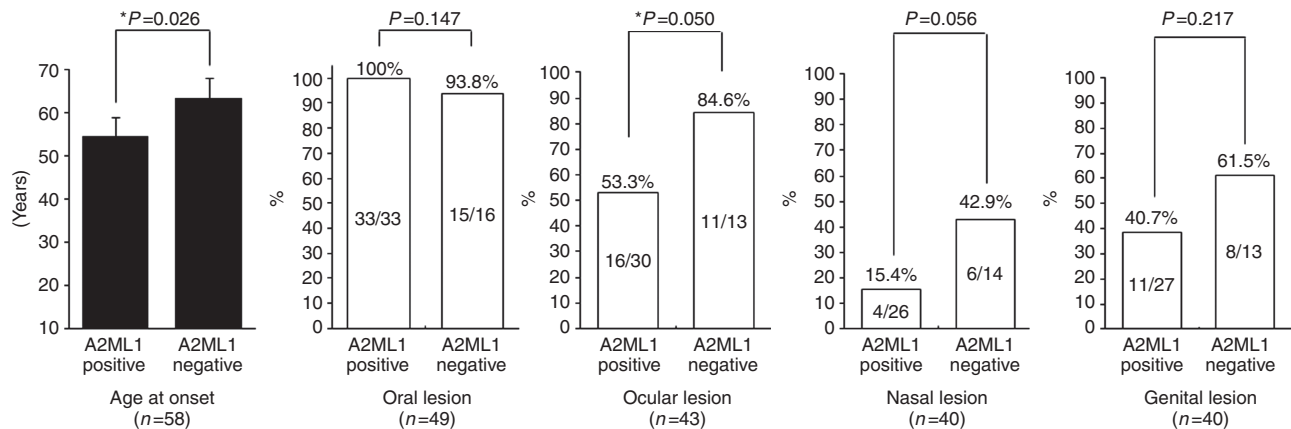
**Figure 4. Influence of the presence of anti-alpha-2-macroglobulin-like-1 (A2ML1) antibodies on age at onset of paraneoplastic pemphigus (PNP) and the appearance of various mucosal lesions.** Statistically significant differences were seen in age at disease onset and ocular mucosal lesion, but not in oral, nasal, or genital mucosal lesions. *$P = 0.05$ (after Bonferroni adjustment).

## Table 1. Clinical and immunological features in 58 PNP cases, differences between anti-A2ML1-antibody–positive and –negative PNP cases, and statistical analyses

| Parameters (n = cases with description) | A2ML1 positive | A2ML1 negative | Total |
|---|---|---|---|
| *Gender* (n = 53) | | | |
| Females | 24/36 (66.7%) | 11/17 (64.7%) | 35/53 |
| Males | 12/36 (33.3%) | 6/17 (35.3%) | 18/53 |
| *Cutaneous lesions* | | | |
| Trunk (n = 48) | 21/33 (63.6%) | 10/15 (66.7%) | 31/48 |
| Extremities (n = 49) | 18/33 (54.5%) | 10/16 (62.5%) | 28/49 |
| Bronchiolitis obliterans (n = 58) | 7/40 (17.5%) | 3/18 (22.2%) | 10/58 |
| Castleman's disease (n = 58) | 6/40 (15.0%) | 2/18 (11.1%) | 8/58 |
| Dsg1, indices (n = 58) cutoff index: >20 | 26.52 ± 38.78 | 11.86 ± 21.99 | 21.96 ± 34.93 |
| Dsg3, indices (n = 58) cutoff index: >20 | 86.14 ± 76.47 | 75.95 ± 70.70 | 82.97 ± 74.26 |
| Human skin indirect IF (n = 58) | 30/40 (75.0%) | 14/18 (77.8%) | 44/58 |
| Rat bladder indirect IF (n = 58) | 29/40 (72.5%) | 11/18 (61.1%) | 40/58 |
| Acantholysis (n = 43) | 17/27 (63.0%) | 6/16 (37.5%) | 23/43 |
| Necrotic cells (n = 43) | 12/27 (44.4%) | 4/16 (25.0%) | 16/43 |
| Liquefaction degeneration (n = 43) | 4/27 (14.8%) | 3/16 (18.8%) | 7/43 |
| Good response to therapies (n = 48) | 16/32 (50.0%) | 8/16 (50.0%) | 24/48 |
| *Outcome* (n = 43) | | | |
| Alive | 11/30 (36.7%) | 3/13 (23.1%) | 14/43 |
| Dead | 19/30 (63.3%) | 10/13 (76.9%) | 29/43 |

Abbreviations: A2ML1, alpha-2-macroglobulin-like-1; Dsg, desmoglein; IF, immunofluorescence; PNP, paraneoplastic pemphigus.

**Anti-A2ML1 pAb reduced cell–cell adhesion of cultured NHKs**

We determined the effect of anti-A2ML-1 pAb on NHK cell adhesion by dissociation assay. Remarkable increase in fragmentation of culture sheets of NHKs was induced by incubation with anti-A2ML1 pAb, but not with anti-PPL mAb or normal mouse IgG (Figure 5c).

**Anti-A2ML1 pAb increased plasmin activity in NHK culture medium**

Although we tried to detect protease activity using colorimetric protease assay kit, the sensitivity is too low to detect the protease activity (data not shown). Then, we examined protease activity in culture medium by casein gel zymography. Increased serine protease activity, corresponding to plasmin, was detected in culture medium of NHK incubated with anti-A2ML1 pAb, but not with anti-PPL mAb, normal mouse IgG, or no antibody addition (Figure 5d). No activity of other proteases was detected.

## DISCUSSION

In this study, double-staining IF of COS7 cells transiently transfected with human A2ML1 cDNA confirmed the specific reactivity of PNP sera with A2ML1. By this IF, 30 (51.7%) of 58 PNP sera reacted with A2ML1-expressing COS7 cells. By IP–IB using lysates of A2ML1-expressing COS7 cells, A2ML1 was detected by 35 (60.3%) of 58 PNP sera, but not by any disease and normal control sera. Precisely, the IF showed negative results in 10 IP–IB–positive sera, and conversely positive results in 5 IP–IB–negative sera. Thus, 40 (69.0%) of 58 PNP sera recognized A2ML1, when the results in IP–IB and IF studies of A2ML1-expressing COS7 cells were combined.

This frequency of anti-A2ML1 antibodies is higher than the positive reactivity in 10 (50%) of 20 PNP sera in our previous IP–IB study (Schepens *et al.*, 2010). The detection rate of about 70% is similar to those obtained by conventional IP with radiolabeled-cultured NHK extracts in previous case reports and smaller population systematic studies (Nishibori *et al.*, 1995; Izaki *et al.*, 1996; Mahler *et al.*, 1998; Williams *et al.*, 2000; Inaoki *et al.*, 2001; Fujimoto *et al.*, 2002; Mimouni *et al.*, 2002; Martinez De Pablo *et al.*, 2005; Marzano *et al.*, 2005; Santi *et al.*, 2005; Lee *et al.*, 2006).

Thus, by using a larger series of unselected PNP sera, this study unequivocally and independently confirms that A2ML1 is one of the major PNP antigens. Although the sensitivity of IF of A2ML1-expressing COS7 cells was slightly lower than that of IP–IB, the easier IF may become a useful method to detect anti-A2ML1 autoantibodies after adequate technical improvements.
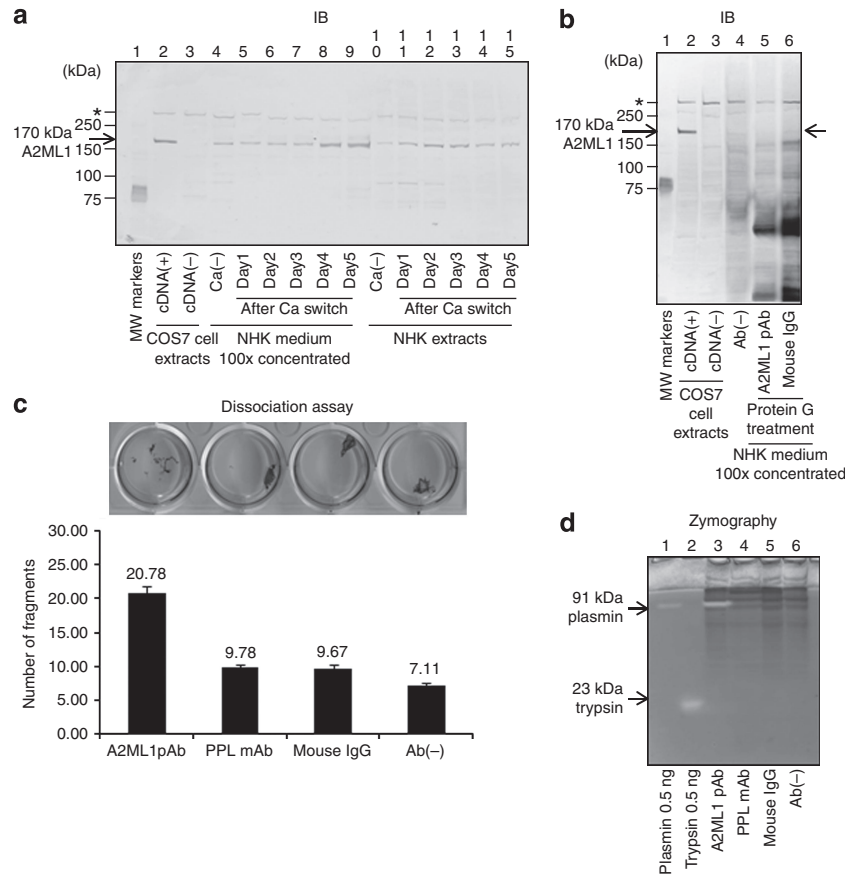
**Figure 5. Anti-alpha-2-macroglobulin-like-1 (A2ML1) polyclonal Ab (pAb) caused cell–cell dissociation and plasmin activation in cultured normal human keratinocytes (NHKs).** (**a**) A2ML1 expression in cultured NHKs. Immunoblotting (IB) using anti-A2ML1 mouse polyclonal antibody (A2ML1 pAb) was performed for either 100 × concentrated culture media or cell extracts of NHKs cultured in low calcium (at day 1) and in high calcium (at days 1–5). The position of A2ML1 recombinant protein (RP) is shown by an arrow in the left. (**b**) Anti-A2ML1 pAb bound to A2ML1 in NHK culture medium. IB using anti-A2ML1 pAb was performed for either 100 × concentrated culture media of NHKs. Lane 4 was for culture medium without treatments of antibody and protein G, lane 5 for culture medium incubated with anti-A2ML1 pAb and precipitated with protein G, and lane 6 for culture medium incubated with negative control mouse IgG and precipitated with protein G. Although the reactivity was weak, A2ML1 disappeared in culture medium incubated with anti-A2ML1 pAb after precipitation with protein G agarose (lane 5). The position of A2ML1 RP is shown by arrows in both sides. Extracts of COS7 cells with or without transfection of A2ML1 complementary DNA (cDNA) were used as positive and negative controls, respectively, in both panels (**a**) and (**b**). The positions of molecular weight (MW) markers and a nonspecific band are indicated by bars and asterisks (∗), respectively, in the left of both panels (**a**) and (**b**). (**c**) Anti-A2ML1 pAb decreased cell–cell adhesion of NHKs. Upper panel shows culture dishes after the process of dissociation assay, and lower panel shows numbers of fragments by histograms, in which each bar corresponds to the dish in the upper panel. NHKs were cultured with anti-A2ML1 pAb, anti-periplakin (PPL) mAb, or normal mouse IgG at a concentration of $1 \mu g \, ml^{-1}$, or without antibody (Ab( − )), for one day after calcium shift. After treatment with dispase II, detached cell sheets were pipetted and fragments were counted. (**d**) Anti-A2ML1 pAb increased plasmin activity. Casein zymography was performed using culture media of NHKs cultured with anti-A2ML1 pAb, anti-PPL mAb, or normal mouse IgG at a concentration of $1 \mu g \, ml^{-1}$, or without antibody (Ab( − )), for one day after calcium shift. The arrows in the left indicate the position of the 91 kDa plasmin and the 23 kDa trypsin.

The results in our previous study suggested that intramolecular disulfide bonds within A2ML1 molecule are important for conformational epitopes for autoantibodies in PNP sera (Schepens et al., 2010). This assumption was further confirmed by the results in the present study that 22 (37.9%) PNP sera reacted with A2ML1 in IB of NHKs under non-reducing, but not reducing condition. To the best of our knowledge, A2ML1 is the antigen, which has such unique epitopes that have not been reported previously.

However, the positive rate in non-reducing IB of NHK extracts was much lower than that in IP–IB using A2ML1-expressing COS7 cells. Noteworthy, all PNP sera with positive A2ML1 reactivity in non-reducing IB were also positive in IP–IB, whereas none of IP–IB–negative sera showed positive

reactivity by non-reducing IB (Supplementary Table S1 online), concluding that IP–IB using A2ML1 RP is more sensitive than non-reducing IB of NHK extracts.

In the second part of this study, we presented the evidence of the pathogenic role of anti-A2ML1 antibodies in PNP by functional studies, which to our knowledge is previously unreported. We first confirmed the presence of A2ML1 in NHK culture media by IB of concentrated samples. Then, we also confirmed actual binding of anti-A2ML1 pAb by IB of culture media immunoprecipitated with protein G agarose.

NHKs incubated with A2ML1 pAb showed decreased cell adhesion by dissociation assay, and increased activity of plasmin was detected by casein zymography. The results of these functional studies suggested that anti-A2ML1 pAb

decreased NHK cell adhesion through plasmin activation by inhibition of A2ML1.

Relationship between proteases and acantholysis was previously described (Morioka *et al.*, 1981), and plasmin and plasminogen activator (PA) were reported to be involved in the pathogenesis of pemphigus (Hashimoto *et al.*, 1983; Seishima *et al.*, 1997). However, this hypothesis was later negated by a study using PA-knockout mice (Mahoney *et al.*, 1999).

Plasmin activation is controlled by at least two different systems, i.e., inhibition of processing from plasminogen to plasmin by PA inhibitor and direct inhibition of plasmin by anti-plasmin and alpha-2-macroglobulin family inhibitors, including A2ML1. In addition, A2ML1 may inhibit kallikrein, which also processes from plasminogen to plasmin, although kallikrein activation was not found in our study. We suggest that A2ML1 has strong anti-plasmin activity and anti-A2ML1 autoantibodies may have a role in PNP lesions, probably through decrease in cell adhesion. However, new approaches using anti-A2ML1 autoantibodies in PNP sera are needed to unravel this hypothesis.

In the preliminary IF studies using anti-A2ML1 pAb, A2ML1 was confirmed to be present in uppermost layers of the normal human epidermis. By IF of rat bladder, which is one of the diagnostic criteria for PNP, although PNP sera clearly reacted with transitional epithelia, no positive reactivity was found by anti-A2ML1 pAb, indicating that A2ML1 is not responsible for the positive reactivity with rat bladder by PNP sera.

As PNP sera react with multiple antigens, their expression patterns in various tissues should be important to speculate the pathogenicity of each antigen. Expressed sequence tags (ESTs) profile for A2ML1 (Hs.620532, NCBI Unigene, EST profile viewer) suggests high A2ML1 transcript levels in the esophagus, mouth, pharynx, intestine, and muscle. At protein level, A2ML1 is also expressed in non-epithelial tissues, including thymus and testis. Therefore, future studies of the precise expression of A2ML1 in various tissues should be required to understand the role of anti-A2ML1 antibodies in the development of extra-cutaneous lesions.

In this study, the relationship of the presence of anti-A2ML1 antibodies with various clinical and immunological findings in PNP patients was also statistically examined. In our analyses, the presence of anti-A2ML1 autoantibodies was more frequently associated with early onset ($P < 0.05$) and absence of ocular lesion ($P = 0.05$), while no significant difference was found in any other parameters, including associated malignancies and bronchiolitis obliterans.

In previous IP studies, antibodies to the 170 kDa antigen appeared to be detected in young PNP patients (Williams *et al.*, 2000; Inaoki *et al.*, 2001; Fujimoto *et al.*, 2002; Mimouni *et al.*, 2002; Niimi *et al.*, 2010), which is in line with our results. The reason why the presence of anti-A2ML1 antibodies correlates with absence of ocular involvement is unknown, because the expression of A2ML1 in conjunctivae has not been studied.

In conclusion, the pathogenic role of anti-A2ML1 antibodies is still unclear. Anti-A2ML1 antibodies are already detectable in the initial stage of disease (Reich *et al.*, 1999; Borradori *et al.*, 2001; Heizmann *et al.*, 2001; Schepens *et al.*, 2010),

and preferentially react with the N-terminal domain of A2ML1 where target proteases bind (Schepens *et al.*, 2010). These findings raise the possibility that anti-A2ML1 autoantibodies are involved in the development of PNP lesions, through various effects, including inhibition of keratinocyte cell adhesion by activating plasmin, which was suggested in this study.

## MATERIALS AND METHODS

All studies followed the guidelines of the Medical Ethics Committees of Kurume University School of Medicine, and were conducted according to the Declaration of Helsinki Principles. Written informed consents were obtained from all patients and the controls.

### Patients and antibodies

Serum samples were obtained from 58 patients with PNP, none of which were used in our previous study (Schepens *et al.*, 2010). The diagnosis of PNP was made by the characteristic clinical and histopathological features, direct IF, indirect IF of normal human skin sections and rat bladder sections, enzyme-linked immunosorbent assays of Dsg1 and Dsg3, and recognition of autoantibodies against the EPL and PPL by IB of normal human epidermal extracts. All clinical data were collected from clinical chart of each patient, which are shown in Figure 4, Table 1, and Supplementary Table S1 online. All sera were taken at the time before therapy or on the incipient stage of PNP. Age at onset ranged 11–80 years (average, 57.4 years). Disease control sera were obtained from 10 PV, 10 PF, and 10 BP patients, and normal control sera were obtained from 30 healthy volunteers. See Supplementary Materials and Methods for details for antibodies used in this study.

### Indirect IF of normal human skin and rat bladder sections for A2ML1 protein expression

See Supplementary Materials and Methods for technical details.

### IF of A2ML1 cDNA-transfected COS7 cells

The cDNA coding entire human A2ML1 was cloned into pcDNA3 eukaryotic expression vector (Invitrogen, Palo Alto, CA) with c-Myc tagged in carboxy-terminus, as described previously (Ruhrberg *et al.*, 1996; Schepens *et al.*, 2010). A2ML1-harboring plasmids were transfected into COS7 cells using Lipofectamine (Invitrogen). After 48 hours, the cells were fixed with cold 100% methanol and blocked with 1% BSA/PBS. Cells were double-stained with 1:20 diluted patient serum, together with either 1:100 diluted anti-c-Myc pAb or 1:50 diluted anti-A2ML1 pAb. Then, the cells were incubated with appropriate secondary antibodies (1:100 diluted FITC-conjugated anti-human IgG, 1:1,000 diluted Texas Red-conjugated anti-rabbit IgG, or 1:1,000 diluted Alexa Fluor 568-conjugaed anti-mouse IgG) and 4′,6-diamidino-2-phenylindole (Nacalai, Kyoto, Japan).

### IB of normal human epidermal extracts and transfected COS7 cell extracts

See Supplementary Materials and Methods for technical details (Hashimoto *et al.*, 1990).

### Preparation of fractions of A2ML1 protein

Forty eight hours after transfection with A2ML1 construct, the culture medium was harvested. After washing the cells with cold PBS, COS7

cells were scraped in washing buffer (cold PBS) and centrifuged. After supernatant was stored, PBS containing 1% protease inhibitor cocktail (PIC, Sigma-Aldrich, St Louis, MO) and 1% polyoxyethylene-9-octylphenyl ether (NP40, Sigma-Aldrich) (IP buffer) was added to pellet, mixed gently, and incubated for 20 minutes on ice. After centrifugation, both supernatant and pellet resuspended with IP buffer were stored. All the four materials were separated by SDS-PAGE on 5–20% gel (ATTO, Tokyo, Japan) and transferred onto nitrocellulose membrane by semi-dry transfer method using iBlot (Invitrogen). Membrane was blocked with 3% skim milk for 1 hour at room temperature and incubated over night at 4 °C with 1:100 diluted anti-c-Myc pAb or 1:100 diluted anti-A2ML1 pAb, washed, and probed with 1:100 diluted horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or 1:2,000 diluted alkaline phosphatase (AP)-conjugated anti-mouse IgG. Protein bands were visualized using AP and HRP detection systems.

### IP–IB of A2ML1-transfected COS7 cell extracts

IP–IB was performed with transfected COS7 cell extracts as substrate for PNP, PV, PF, BP, and normal control sera. In this study, A2ML1 immunoprecipitated with these sera was detected by IB using anti-c-Myc pAb. IP–IB for eukaryotic recombinant A2ML1 protein was performed as described previously (Ruhrberg et al., 1996; Schepens et al., 2010). From preliminary IB results, cell extracts in IP buffer were used for IP–IB. For preclearance, cell extracts were incubated with prewashed protein A agarose (Bio-Rad Laboratories, Hercules, CA) in IP buffer for 30 minutes at 4 °C. After centrifugation, patient serum or anti-c-Myc pAb (positive control) was added to supernatant and incubated with rotation overnight at 4 °C. Then, prewashed protein A agarose was added and incubated for 3 minutes at 4 °C. After centrifugation, the sepharose were washed with wash buffer (PBS containing PIC) with rotation for 3 minutes at 4 °C. Proteins bound to the protein A agarose were eluted by boiling in SDS sample buffer and processed for IB using 1:100 diluted anti-c-Myc pAb as described above, except that protein-A/G–HRP conjugate (Pierce, Rockford, IL) diluted 1:5,000 was used.

### IB of NHK extracts under reducing and non-reducing conditions

See Supplementary Materials and Methods for technical details.

### Statistical analysis

Differences among qualitative results were compared using the $\chi^2$ test and Bonferroni adjustment. Differences among quantitative parameters between groups were assessed using the Mann–Whitney test. All data are expressed as means ± SD. All analyses were performed using SPSS (SPSS, Chicago, IL).

### Detection of A2ML1 protein in cultured NHKs

A2ML1 expression in cultured NHKs was examined by IB. NHKs were cultured in EpiLife medium (Invitrogen) with NHK growth supplement (Invitrogen). After NHKs were reaching confluency, medium was changed to the medium containing 1.2 mM calcium without growth factor and phenol red. We harvested both culture media and cell extracts of NHKs in low calcium (at day 1) and in high calcium (at days 1–5). Culture media were concentrated 100 × using Amicon Ultra-0.5–30K (Millipore, Billerica, MA), and cell extracts were prepared by suspending the NHKs in 1 × SDS sample buffer. IB for A2ML1 was performed as described above.

### IB of protein G immunoprecipitated NHK culture media to confirm binding of anti-A2ML1 pAb to A2ML1

After NHKs were cultured as described above, antibodies were added to the concentration of 1 µg ml$^{-1}$ and incubated at 37 °C for 24 hours. Protein G agarose (Millipore) and binding buffer (20 mM NaPO$_4$) were added to NHK culture media, and incubated for 30 minutes at 4 °C. After centrifugation, the supernatant was concentrated as above. IB for A2ML1 was performed by the same method described above.

### Dissociation assay

Dissociation assay was performed as described previously (Ishii et al., 2005; Saleh et al., 2012). NHKs were cultured as described above. After washing with PBS with calcium and magnesium (PBS (+)), the cells were incubated with 2.4 U ml$^{-1}$ dispase II (Roche Diagnostics, Mannheim, Germany). Released monolayer was washed with PBS (+) and pipetted five times in 1 ml PBS (−) using 1 ml pipette. The cells were fixed for 10 minutes with 5% formaldehyde. Twenty microliter of 0.02% crystal violet was added and incubated overnight to stain completely. Three pictures were taken and number of fragments was counted manually.

### Protease assays

Protease assays using Pierce Colorimetric Protease Assay Kit (Thermo, Rockford, IL) and Serine-Protease detection zymo-electrophoresis Kit (Cosmo Bio, Tokyo, Japan) were performed according to the instruction from manufacturers. Culture medium was prepared and concentrated as above. In zymography, areas of caseinolytic activity appeared as clear zones against a dark blue background. Plasmin and trypsin (0.5 ng each) were also loaded as controls.

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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