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ORIGINAL PAPER

Detection of anti-envoplakin and anti-periplakin autoantibodies by ELISA in patients with paraneoplastic pemphigus

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Abstract Paraneoplastic pemphigus patients (PNP) develop a group of autoantibodies, among which those against envoplakin and periplakin are almost always found. Epitope mapping has indicated that the linker subdomains of the proteins harbor the major antigenic sites recognized by PNP sera. In order to detect specific autoantibodies for the diagnosis of PNP, we expressed recombinant proteins containing linker subdomains of human periplakin and envoplakin in a human kidney cell line, and used them as the antigens for ELISAs. We found that all of the sera from 16 PNP patients recognized these two recombinant proteins by ELISA, and sera from 20 pemphigus vulgaris (PV), 12 pemphigus foliaceus (PF), 20 bullous pemphigoid (BP), 2 Castleman's tumor without PNP and 20 normal controls showed negative results. We also expressed the extracellular domain of desmoglein 3 (Dsg3) in the cell line, and used this recombinant Dsg3 as the ELISA antigen. Only 11 of our 16 PNP sera were positive, and most PV sera were positive. Our findings indicate that ELISAs using the recombinant proteins containing linker subdomains of *envoplakin* and *periplakin* expressed in a human cell line as the antigens are highly sensitive and specific for the diagnosis of PNP.

Keywords Envoplakin · Periplakin · ELISA · PNP

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Introduction

Paraneoplastic pemphigus (PNP) is an autoimmune mucocutaneous disorder associated with neoplasms of lymphocytic origin, characterized by polymorphous mucocutaneous lesions, such as erythema, bullae, erosions, papulosquamous eruption and erythema multiforme-like lesions [5]. Clinically, PNP may mimic a variety of dermatological diseases including pemphigus vulgaris (PV), erythema multiforme, erosive lichen planus and acute lupus erythematosus, leading to misdiagnosis of PNP. If the diagnosis and treatment of PNP are delayed, the mortality rate approaches to 90% [10, 11].

PNP sera recognize a variety of autoantigens including desmogleins and members of the plakin family (desmoplakins, BP230, envoplakin, periplakin and plectin) [4, 7, 12]. Currently, immunoblot using protein extract of cultured human keratinocytes as the substrate and indirect immunofluorescence reactive with rodent urinary bladder epithelia for the detection of serum autoantibodies are the two commonly used diagnostic procedures. However, these procedures are time consuming and technically demanding, with the sensitivity and specificity of about 75 and 83%, respectively [8].

Among the autoantigens identified by PNP sera, envoplakin and periplakin are the most characteristic and consistently recognized proteins. Our epitope mapping results have shown that the linker subdomains of the two proteins harbor the major antigenic sites recognized by PNP patients [16]. However, the recombinant proteins used for the epitope mapping were expressed in *E. coli*, which may lack the proper processes required for protein folding and posttranslational modifications, and as a result, for the formation of conformation-dependent epitopes [1].

In order to overcome these problems and to get more specific and sensitive methods for the diagnosis of PNP, we

expressed the recombinant proteins containing linker subdomains of human envoplakin (EPL) and periplakin (PPL) in a mammalian cell line, and used them as the antigens for enzyme-linked immunosorbent assay (ELISA). Here we report that ELISAs using the recombinant EPL and PPL from mammalian cells as the antigens are highly sensitive and specific for the detection of circulating autoantibodies in PNP sera.

Materials and methods

Patients and their sera

Sera from a group of 16 well-characterized PNP patients (Table 1) were utilized for this study. These PNP patients had typical clinical and skin histopathological features, as well as underlying Castleman's tumor, thymoma or follicular dendritic cell sarcoma. All patients also had indirect immunofluorescence titer ≥1:20 against transitional epithelia on mouse urinary bladder. Sera from 20 cases of PV, 12 cases of pemphigus foliaceus (PF) and 20 cases of bullous pemphigoid (BP) were used as the controls. These sera were obtained when the diseases were active. Other controls included 2 Castleman's tumor patients without any PNP symptoms before and after surgical resection for more than 36 months, and 20 normal individuals. This study was conducted according to the Declaration of Helsinki Principles and approved by the Medical Ethical Committee of Peking University First Hospital. All patients gave informed consent.

Production and purification of recombinant proteins

The cDNAs of EPL, PPL and entire extracellular domain of desmoglein 3 (Dsg3) were obtained by reverse transcription-PCR from total RNA of human keratinocytes. PCR products of EPL cDNA (amino acid codons 1,543–1,784, NP_001979) and PPL cDNA (amino acid codons 1,509–1,756, NP_002696) were cloned in frame into the plasmid pCDNA3.0 (Invitrogen, Carlsbad, CA, USA) with signal peptide codons of human osteonectin cDNA and six histidine codons at their 5′ ends. The entire extracellular domain of Dsg3 cDNA (amino acid codons 1-615, NP_001935) was cloned in frame into plasmid pCDNA3.1-Myc-His-A (Invitrogen). Sequence accuracy of the resulting clones was confirmed by DNA sequencing from both directions. The recombinant constructs were then transfected into human embryonic kidney cells (HEK293) by the protocol of Chen and Okayama [6]. After G418 (Life Technologies, Gaithersburg, MD, USA) selection, the resistant cells were kept in serum-free DMEM for the ease of protein purification from cultured media.

The cell culture media were harvested and applied to Ni-NTA His-binding resin (Novagen, Madison, WI, USA) allowing binding of the recombinant proteins via their histidine tag. After washing with washing buffer (containing 40 mM imidazole), the proteins were eluted with elution buffer (containing 250 mM imidazole), and were then concentrated and dissolved in phosphate buffered saline (PBS) by ultrafiltration (Amicon, Millipore, Cork, Ireland).

Purified recombinant proteins were checked for their apparent molecular weight along with protein molecular

Table 1 Clinical manifestations of the 16 PNP patients

Patient no./sex/age (years)	Mucosal lesion			Skin rash	Tumor type
	Oral	Ocular	Anogenital		
1/M/30	+	+		EM + PV	Ct
2/F/17	+	+	+	EM + LP	Ct
3/M/29	+	+	+	EM + PV + PK	Ct
4/F/22	+	+	+	EM + LP + PV	Ct
5/F/48	+	+		LP + PV	Ct
6/F/41	+	+	+	EM + PK	Thymoma
7/F/57	+		+	EM + PV + PK	FDCS
8/F/28	+	+	+	EM + PK	Ct
9/M/46	+	+		PV	Ct
10/M/36	+	+	+	EM + LP + PV + PK	Ct
11/M/56	+	+	+	EM + PV + PK	Ct
12/M/22	+		+	EM + LP + PK	Ct
13/F/16	+				Ct
14/F/14	+	+		EM + PV + PK	Ct
15/F/19	+		+	EM + LP + PV	Ct
16/F/16	+	+	+	EM + LP + PV	Ct

Cases #1–8 and #10 have been published in our previous reports [14–16]

EM erythema multiforme, *LP* lichen planus, *PV* pemphigus vulgaris, *PK* palmoplantar keratosis, *Ct* Castleman's tumor, *FDCS* follicular dendritic cell sarcoma

The "+" symbol indicates present

weight markers and for their purity by immunoblotting and silver staining of the gels after loading 0.1 μg of the purified proteins for SDS-polyacrylamide electrophoresis (SDS-PAGE). In the purification and manipulation of recombinant Dsg3, all buffers including coating buffer for ELISA contained 1 mM calcium chloride for retaining its native conformation [9]. The purified recombinant proteins were also subjected to partial amino acid sequencing after trypsin digestion by mass spectrometry at The National Center for Biomedical Analysis, Beijing, China.

ELISA

The assay was performed at room temperature using 96-well ELISA plates (Corning, NY, USA). Each well was coated for 2 h with purified recombinant protein in 50 μl 0.1 M sodium bicarbonate buffer, pH 9.6. Wells were then washed with Tris-buffered saline containing 0.05% Tween-20, pH 7.2 (TBS-T). After blocking with TBS-T containing 1% bovine serum albumin for 1 h, wells were incubated for 1 h with 50 μl serum diluted in TBS-T. After washing as above, wells were incubated for 1 h with 50 μl alkaline phosphatase-conjugated goat anti-human IgG antibody. Following another series of washes, 50 μl substrate solution (1 mg/ml *P*-nitrophenyl phosphate in 1 M diethanolamine and 0.5 mM $MgCl_2$, pH 9.8) were added to each well and incubated for 15 min. The reaction was terminated by 1 M NaOH and measured at 405 nm by an ELISA reader (Bio-Rad, Hercules, CA, USA). All assays were run in duplicate.

Optimal concentrations of the reagents used in ELISA were determined by chessboard titrations [17]. We first took 1:100 dilution of normal sera to titrate the optimal amount of antigens used in the coating step and the appropriate dilution of secondary antibody with minimum background. We then used the optimal amount of antigens to determine the optimal dilution of normal sera and secondary antibody with minimum background.

The following formula established by Amagai et al. [2] was adopted to compare the results from different plates: index = (optical density [OD] of tested serum − OD of negative control)/(OD of positive control-OD of negative control) × 100. Where positive control indicates a serum from a confirmed PNP (for EPL and PPL) or PV (for Dsg3). The negative control was a normal human serum. The controls were the same in all plates.

The cut-off points for EPL and PPL were based on a receiver-operating-characteristic (ROC) analysis of the index values from sera of PNP and normal subjects. For ROC analysis of Dsg3, index values were from sera of PV and normal individuals.

Competitive ELISA was also performed to verify the specificity of the ELISA results. Ten microliters of PNP serum or control serum were mixed with purified EPL, PPL or Dsg3 to the amount approximately equivalent to the antigen/sera ratio used in the ELISAs, and incubated at 4°C overnight. The serum was then diluted to 1:100, and subjected to ELISA as described above.

Monitoring amount changes of the autoantibodies by the ELISAs in four PNP patients before and after resection of the tumors

The changes of clinical symptoms and levels of autoantibodies against EPL, PPL and Dsg3 could be followed-up in 4 (case 6, 12, 13 and 15) of our 16 PNP cases for more than 6 weeks after the resection of the tumors. Case 15 underwent two times of plasmapheresis before and after the surgery.

Results

Purity of recombinant PPL, EPL and Dsg3 proteins

The purity of EPL (~36 kDa), PPL (~36 kDa) and Dsg3 (~72 kDa) was examined by immunoblotting utilizing a monoclonal antibody against the 6x histidine tag (Novagen), and silver staining of the polyacrylamide gel after SDS-PAGE (Fig. 1). These results indicate that the three recombinant proteins were highly purified. Amino acid sequencing results of the 21 fragments from purified EPL digested with trypsin perfectly match the sequences of human envoplakin within the region of amino acid residues 1,543–1,784 (covering 36.0% of the sequences). Similarly, those of the 52 fragments from our purified PPL correctly fit the sequences in amino acid residues 1,509–1,756 of human periplakin (covering 70.6% of the sequences). We then used these proteins as the antigens for ELISA.

Optimal ELISA parameters for the detection of autoantibodies against EPL, PPL and Dsg3

Chessboard titration of the antigens showed that the optimal concentrations of PPL, EPL and Dsg3 for coating wells were 0.35, 0.4 and 0.6 μg/ml, respectively. Lower dilution of antigens resulted in higher background, and higher dilution of antigens caused lower sensitivity. In a similar way, we found that 1:100 dilution of the sera and 1:1,000 dilution of the secondary antibody presented the most optimal results.

A ROC analysis was performed to determine the cut-off values differentiating positive and negative results (Fig. 2). The one which gave the highest sum of sensitivity (%) and specificity (%) was defined as the cut-off value to be used. For EPL and PPL ELISAs, the cut-off index value was 4.3

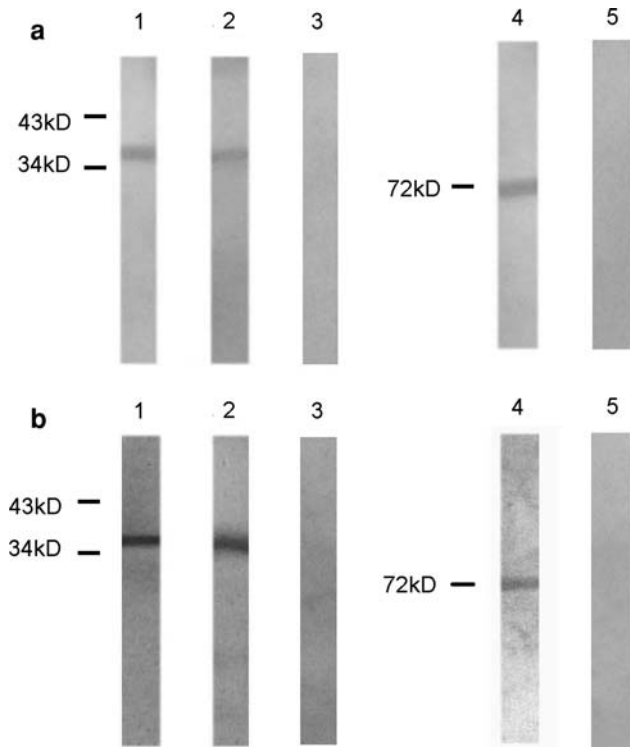

Fig. 1 **a** Purified recombinant proteins immunoblotted by anti-6xHis tag monoclonal antibody. *Lane 1* EPL; *lane 2* PPL; *lane 3* control from untransfected cells; *lane 4* Dsg3; *lane 5* control from untransfected cells. **b** Purified recombinant proteins in polyacrylamide gels visualized by silver staining. In **a** and **b**, 15% polyacrylamide gel was used for separating gel for lanes 1–3, and 8% polyacrylamide gel for lanes 4 and 5

and 6.4, respectively. For Dsg3 ELISA, the value was 5.4. With these cut-off values, the sensitivity and specificity for EPL and PPL were both 100%, and the sensitivity and specificity for Dsg3 were 95.0% (19/20) and 100% (20/20), respectively.

Autoantibodies against PPL, EPL and Dsg3 in 16 PNP patients by ELISA

All of the 16 PNP patients we tested recognized our recombinant PPL and EPL by ELISA using the cut-off values mentioned above. In contrast, none of the sera from 20 PV, 12 PF, 20 BP and 20 normal controls showed index values beyond the cut-off values (Fig. 3a–c). Interestingly, sera from two Castleman's tumor patients without PNP were also negative for the ELISAs. Thus, the ELISAs using our recombinant EPL and PPL as the antigens may have satisfactory sensitivity and specificity for the diagnosis of PNP. Using our purified recombinant Dsg3 as the antigen for ELISA, we found that 11 of the 16 PNP sera were positive, 95.0% (19/20) PV sera were positive, and 96.3% (52/54) of other control sera were negative.

When the tested PNP and control sera were pre-incubated with the recombinant protein same as that coated on the wells, almost all of the positive OD_{405} readings decreased to the normal range (Fig. 3d), and the control showed no significant change (data not shown), indicating that the positive OD_{405} readings are antigen-specific.

Amount change of autoantibodies against EPL and PPL evaluated by the ELISAs in four PNP patients after removal of the tumors

As seen in Fig. 4, the autoantibodies against EPL and PPL decreased after resection of the tumors, roughly corresponding to the improvement of their mucocutaneous lesions. In case 15 who underwent plasmapheresis twice, her autoantibodies decreased rapidly after the treatment.

The index value of anti-Dsg3 autoantibody was higher than the cut-off value before surgery and decreased after surgery in two cases (case 12 and 15), and was below the cut-off value before surgery in the other two cases.

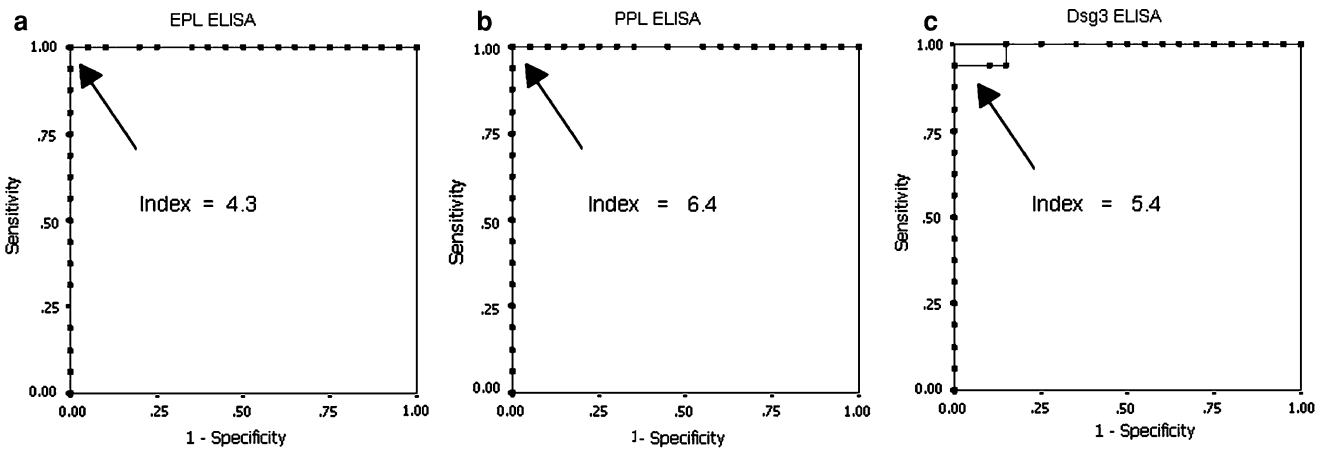

Fig. 2 Receiver-operating-characteristic curves to determine cut-off index values for EPL (**a**), PPL (**b**) and Dsg3 (**c**) ELISAs

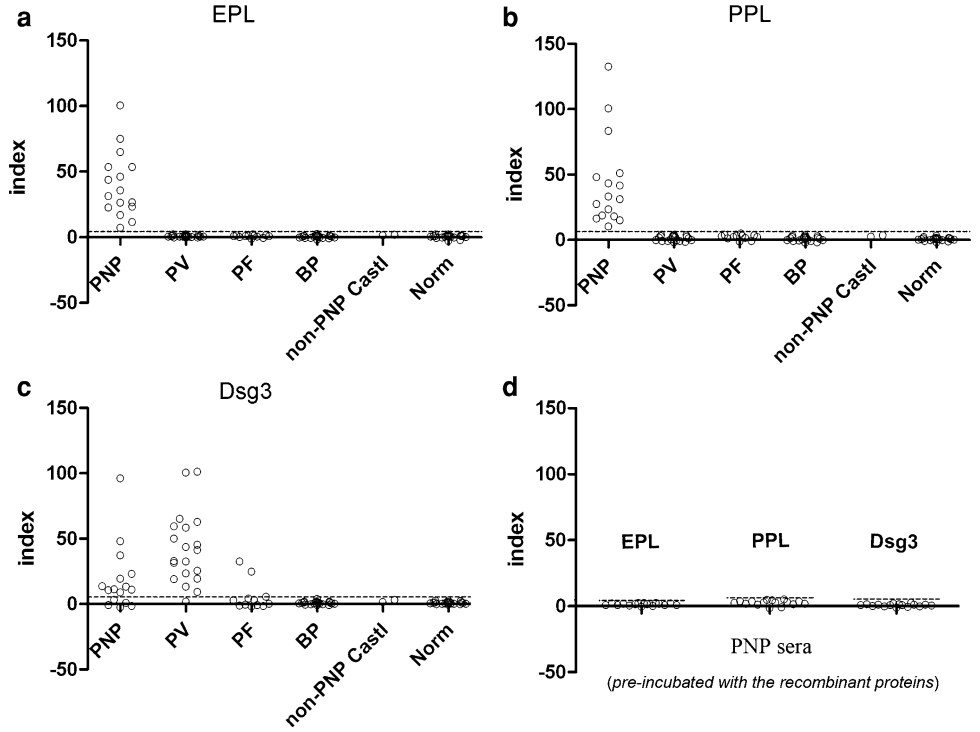

Fig. 3 ELISA results using our recombinant EPL (**a**), PPL (**b**) and Dsg3 (**c**) as the antigens. *Dotted horizontal lines* indicate cut-off values. *Circles* indicate the index value of each sample. *PNP* paraneoplastic pemphigus, *PV* pemphigus vulgaris, *PF* pemphigus foliaceus, *BP* bullous pemphigoid, *non-PNP Castl.* sera form 2 Castleman's tumor patients without PNP; Norm: normal human sera. All of the 16 PNP sera showed the index values to PPL and EPL beyond the cut-off values, but only 11 of the 16 PNP sera were positive to our recombinant Dsg3. **d** ELISA results using our recombinant PPL, EPL and Dsg3 as the antigens to examine the PNP sera pre-incubated with the recombinant proteins same as those coated on the wells show the decrease of the index values to normal range

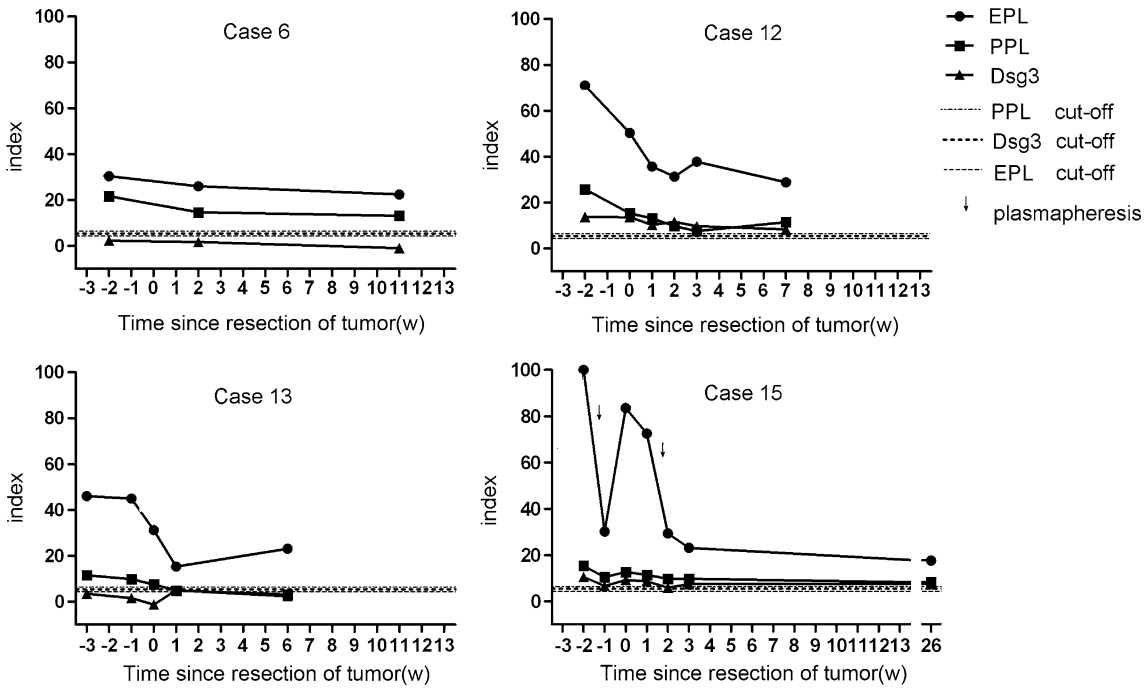

Fig. 4 Following up of amount change of the autoantibodies against EPL, PPL and Dsg3 by the ELISAs in 4 PNP patients after removal of the tumors. *w* weeks

Discussion

Here we report the development of highly sensitive and specific ELISAs for the detection of the autoantibodies in PNP. The antigens used for the ELISA contain linker subdomains of envoplakin and periplakin, which are highly homologous among the members of the plakin family [13]. To facilitate the identification of the recombinant proteins on SDS-PAGE, we extended about 135 amino acid residues of envoplakin and periplakin polypeptides to the amino-terminal direction as the recombinant EPL and PPL proteins.

Using the software at the website www.expasy.org, we obtained the theoretical molecular weight of 28.4 and 28.9 kDa for EPL, PPL respectively. A single band of our recombinant Dsg3 on western blot (Fig. 1) indicates that the signal peptide and prosequence have been completely cleaved [1]. For mature form of the recombinant Dsg3, the theoretical molecular weight is 63.0 kDa. However, the apparent molecular weight was approximately 36, 36 and 72 kDa for PPL, EPL and Dsg3, respectively, on SDS-PAGE (Fig. 1). Subsequently, the three recombinant proteins had undergone posttranslational modifications. ELISAs using these recombinant proteins as the antigens are expected to be more efficient for the detection of autoantibodies in PNP patients.

Using EPL and PPL as the ELISA antigens, we found that all of the 16 PNP sera were positive, and none of the sera from 20 PV, 12 PF, 20 BP and 2 castleman's tumor without PNP as well as 20 normal controls showed the index values beyond the cut-off values. The specificity of these results were further confirmed by the competitive ELISA (Fig. 3d). Therefore, the ELISAs we developed are highly sensitive and specific for the diagnosis of PNP. In contrast, in an ELISA study using the recombinant linker subdomains of EPL and PPL expressed in bacteria as the antigens to detect the autoantibodies in 14 of our 16 PNP sera, 2 of the 14 PNP sera were negative (manuscript in preparation). Subsequently, some of the autoantibodies against the linker subdomains of EPL and PPL may be conformation-specific or even specifically against their modification elements.

Studies have shown that anti-Dsg3 antibody is present in PNP sera and can induce the phenotype of PNP in passive transfer animal model [3]. In this study, we also expressed the extracellular domain of desmoglein 3 in mammalian cells, and used this recombinant Dsg3 as the ELISA antigen. However, only 11 of the 16 PNP sera were positive. Presumably, the inconsistency is partially caused by the different PNP samples being tested.

We followed up the amount change of the autoantibodies against EPL, PPL and Dsg3 by ELISA in four PNP patients before and after removal of the tumors. The decrease of autoantibodies against EPL and PPL was accompanied by the improvement of clinical symptoms, suggesting the possible usefulness of the ELISAs for the evaluation of the disease course.

In summary, the PPL and EPL ELISAs we developed are highly specific and sensitive for the diagnosis of PNP. However, only 16 PNP cases were available for the evaluation of the ELISAs, and more PNP cases are needed to further confirm the diagnostic value of the two ELISAs.

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Conflict of interest statement The authors state no conflict of interest.

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