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

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Directed by Professor Soo-Chan Kim

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<ABSTRACT>

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

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(Directed by Professor Soo-Chan Kim)

Epidermolysis bullosa acquisita (EBA) is a chronic autoimmune subepidermal bullous disease characterized by circulating autoantibodies against type VII collagen. Detecting these autoantibodies is crucial for the diagnosis of this disease, and is also useful for measuring disease severity. Enzyme linked immunosorbent assay (ELISA), a quantitative method to measure anti-type VII collagen antibody levels, is currently available to diagnose EBA. The aim of this study was to validate the usefulness of type VII collagen ELISA in diagnosing EBA and monitoring disease severity. Sera from patients with EBA (n=30), bullous pemphigoid (n=20), anti-laminin γ1 pemphigoid (n=9), and healthy donors (n=24) were tested by ELISA using the recombinant noncollagenous 1 (NC1) and 2 (NC2) domains of type VII collagen. Relationships between clinical characteristics, indirect immunofluoroscence (IIF) titers and ELISA values

were investigated. The sensitivity and specificity of the type VII collagen ELISA were 96.7% and 98.1%, respectively. There was no significant difference between ELISA results for classic and inflammatory types (72.6 vs. 47.4 U/ml, p=0.15). The severity of skin involvement was positively correlated with both ELISA index value (r=0.87, p<0.01) and IIF titer (r=0.59, p<0.01). Time sequence analysis in 4 patients with EBA showed that ELISA index values reflect disease severity better than IIF titers. Type VII collagen ELISA using the NC1 and NC2 domains is useful for diagnosing EBA and monitoring disease severity.

Key words: Epidermolysis bullosa acquisita, ELISA, Type VII collagen

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I. INTRODUCTION

Epidermolysis bullosa acquisita (EBA) is a chronic subepidermal bullous disease characterized by autoantibodies against type VII collagen, which is the major component of the anchoring fibrils that connect the basement membrane zone to the papillary dermis[1,2]. Clinically, the two main clinical types of EBA are characterized by classic mechanobullous and inflammatory vesiculobullous features. Mechanobullous EBA manifests as trauma-induced blistering, skin fragility, scarring, and milia, whereas inflammatory EBA manifests as non-traumatic blisters that mimic other bullous diseases, including bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), and linear IgA dermatosis (LAD).

Type VII collagen consists of a triple-helical domain flanked by a large 145 kDa non-collagenous amino-terminal (NC1) domain and a small 34 kDa non-collagenous carboxyl-terminal (NC2) domain[3-5]. Most EBA autoantibodies recognize epitopes within the NC1 domain of type VII collagen[6], while a small number of EBA autoantibodies recognize the NC2 and collagenous domains[7-10].

The routine screening test for diagnosing EBA is direct and indirect immunofluorescence (DIF and IIF) microscopy. Particularly, salt-split IIF in immunofluorescence can distinguish EBA from BP. Patients with EBA have immune deposits on the dermal side of salt-split skin, whereas in BP, deposits are on the epidermal side. This technique, however, does not distinguish EBA from anti-laminin 332 MMP or anti-laminin γ1 pemphigoid, as these diseases also show immune deposition on the dermal side of salt-split skin. EBA sera recognize the 290 kDa type VII collagen protein in immunoblotting studies. This reactivity, in fact, confirms the diagnosis of EBA, however, immunoblotting studies are practically difficult because they are time consuming and technically demanding. To overcome these problems, anti-type VII collagen enzyme-linked immunosorbent assays (ELISA) systems were developed.

Since ELISA was first introduced as a diagnostic tool of pemphigus[11,12], many ELISA studies have been conducted to diagnose various autoimmune bullous diseases including BP (bullous pemphigoid antigen 1 and 2)[13,14], paraneoplastic pemphigus (envoplakin and periplakin)[15], dermatitis herpetiformis (epidermal and tissue transglutaminase)[16,17], and anti-laminin γ1 pemphigoid (laminin γ1)[18]. Previous studies demonstrated that ELISAs for detecting autoantibodies in pemphigus and BP are more sensitive than immunoblotting and more related to disease severity than IIF[12,19-21]. ELISAs were also developed to detect autoantibodies in EBA using different recombinant proteins of the NC1 domain of type VII collagen[22-25]. Recently, recombinant NC1 and NC2 domains of type VII collagen were also used in a commercial ELISA for EBA[26]. Previous reports demonstrated that ELISAs showed high sensitivity and specificity, and that serial titers of each patient with EBA reflect disease severity. However, the relationship between titers of ELISA and clinical severity scores has not been characterized. In this study, we investigated the usefulness of type VII

collagen ELISA using the recombinant NC1 and NC2 domains of type VII collagen to diagnose EBA and measure disease severity.

II. MATERIALS AND METHODS

1. Patients and controls

Before beginning treatment, we obtained serum samples from 30 EBA patients showing (1) blisters and erosions on the skin and/or mucosa; (2) subepidermal blister formation by histopathology; (3) linear deposits of IgG autoantibodies along the dermal-epidermal junction by DIF; (4) circulating IgG autoantibodies on the dermal side of 1 mol/L salt-split skin by IIF; and (5) reactivity against the 290 kDa protein using dermal extract or recombinant NC1 protein by immunoblotting. The sera were also tested for laminin 332 and laminin γ1 immunoreactivity by immunoblotting to rule out other subepidermal bullous diseases that react to the dermal side of salt-split skin by IIF[18,27]. Furthermore, we performed IIF using HEK293 cells transfected with the NC1 recombinant domain (Euroimmun, Lübeck, Germany). Sera from healthy blood donors were used as controls (n=24). Additional controls included patients with BP (n=20) and anti-laminin γ1 pemphigoid (n=9).

A retrospective medical record review was performed on 24 EBA patients. We evaluated sex, age of onset, clinical type, oral mucosal involvement, IIF titer, methylprednisolone (MPD) dose, and disease severity score (Table 1). We further assessed ELISA values and IIF titers over a time course from four patients to investigate their sequential correlation with disease severity.

Table 1 Patient characteristics

No	Gender/Age (y)	Clinical type	Oral mucosal involvement	Initial MPD dose (mg/d)	Other IS drugs (mg/d)	Severity score	ELISA (U/ml)	NC1-transfected HEK cell
1	F/73	Inflammatory (BP-like)	Positive	8	None	31	70.2	Positive
2	F/67	Inflammatory (MMP-like)	Positive	8	None	10	25.2	Positive
3	F/38	Inflammatory (BP-like)	Positive	8	None	17	22.7	Positive
4	M/49	Mechanobullous	Positive	16	None	30	65.5	Positive
5	F/46	Inflammatory (BP-like)	Positive	72	MMF 150mg	28	41	Positive
6	M/40	Mechanobullous	Positive	20	CsA 100mg	26	99	Positive
7	F/79	Mechanobullous	Positive	0	None	13	20	Positive
8	F/42	Mechanobullous	Positive	2	None	17	32.8	Positive

9	F/67	Mechanobullous	Positive	8	None	16	32	Positive
10	F/69	Mechanobullous	Positive	24	None	22	126.8	Positive
11	M/61	Mechanobullous	Positive	32	None	18	95.8	Positive
12	F/68	Inflammatory (BP-like)	Positive	8	None	30	133.5	Positive
13	M/74	Mechanobullous	Negative	8	None	12	3	Negative
14	M/56	Inflammatory (BP-like)	Positive	8	None	22	37.5	Positive
15	M/41	Mechanobullous	Positive	8	None	38	152.9	Positive
16	F/68	Mechanobullous	Positive	16	None	38	78.3	Positive
17	F/59	Inflammatory (BP-like)	Positive	16	CsA 100mg	30	55	Positive
18	M/44	Inflammatory (BP-like)	Positive	16	None	11	14.4	Positive
19	F/71	Inflammatory (BP-like)	Positive	16	None	14	63.8	Positive
20	F/68	Mechanobullous	Positive	8	None	26	105.9	Positive

21	F/76	Mechanobullous	Positive	10	Rituximab 1 cycle	27	140.8	Positive
22	F/54	Mechanobullous	Negative	8	Rituximab 1 cycle	21	45.2	Positive
23	F/74	Mechanobullous	Positive	8	None	17	25.3	Positive
24	F/83	Mechanobullous	Positive	8	None	9	24.6	Positive

BP, bullous pemphigoid; MMP, mucous membrane pemphigoid; MPD, methylprednisolone; IS, immunosuppressive; MMF, mycophenolate mofetil; CsA, cyclosporine A

2. Clinical severity assessment and remission

Disease severity was evaluated based on retrospective chart reviews and photographs. We modified the pemphigus disease area index (PDAI) by assessing the cutaneous and mucosal disease extent before beginning treatment. The modified PDAI score is a continuous scale ranging from 0 to 40 (Table 2). Changes in skin involvement of one patient who had seven serial sera were measured using a 4-point scoring system: grade 0, quiescent (no lesion) status; grade 1, 0-10% skin involvement; grade 2, 10-30% skin involvement; grade 3, 30-60% skin involvement; grade 4, over 60% skin involvement. We defined complete remission (CR) as the absence of new or established lesions for at least two months with minimal therapy. Partial remission (PR) was defined as the presence of transient new lesions that heal within 1 week with minimal therapy for at least 2 months. Minimal therapy was defined as less than or equal to 8 mg/day of MPD with or without adjuvant therapy including dapsone and colchicine[28].

Table 2. Modified Pemphigus Disease Area Index (PDAI) for patients with epidermolysis bullosa acquisita

Anatomic location	**Erosion/blisters or new erythema** **<Skin>** **0 Absent** **1 1-3 lesions, none > 5 cm diameter** **2 >3 lesions, none > 5 cm diameter** **3 >3 lesions, and/or at least one > 5cm diameter** **<Mucosa>** **0 Absent** **5 1-3 lesions** **10 >3 lesions**
Scalp	/3
Face	/3
Neck	/3
Chest	/3
Abdomen	/3
Back, buttocks	/3
Arms	/3
Hands	/3
Legs	/3
Feet	/3
Mucosa	/10
Total points	**/40**

3. ELISA for detecting antibodies against type VII collagen

To measure the levels of antibodies against type VII collagen, we used the type VII collagen ELISA kit (MBL, Nagoya, Japan). Two purified recombinant antigens (NC1 and NC2) were combined and coated in the same well of an ELISA microplate. The antibody titer was measured according to the manufacturer's instructions. The following formula was used to compare the index values: Index (units per milliliter of serum) = (OD of tested serum – OD of negative control) / (OD of positive control – OD of negative control) X 100. The pre-determined cut-off value by manufacturer for anti-type VII collagen antibodies was 6.14 U/ml.

4. Statistical analysis

Continuous variables were summarized with median (range) or mean (standard deviation) based on the distribution. The Wilcoxon rank-sum test was performed for two group comparisons and Spearman correlation (r) was used to investigate associations. Statistical analyses were performed using SAS 9.2 and R 2.13.0 under the alpha level of 0.05.

III. RESULTS

1. ELISA using recombinant NC1 and NC2 proteins of type VII collagen

A total 30 EBA patients who presented at the Department of Dermatology in Gangnam Severance Hospital between April 1993 and June 2011 were included in this study. The mean±SD age of EBA patients was 57.9±15.4 years. Twenty nine of 30 sera had positive ELISA results. One of the sera from the control group showed ELISA values beyond the cut-off value (6.14 U/ml). The sensitivity and specificity of the ELISA were 96.7% and 98.1%, respectively (Figure 1).

To assess the relationship between clinical features and ELISA values, we divided EBA patients into mechanobullous and inflammatory type (Table 1). The median (min., max.) ELISA values of the 15 mechanobullous type and 9 inflammatory type EBA patients were 65.5 (3.0, 152.9) and 31.4 (0.9, 149.3), respectively. The ELISA values for mechanobullous and inflammatory type EBA were not significantly different (p=0.15).

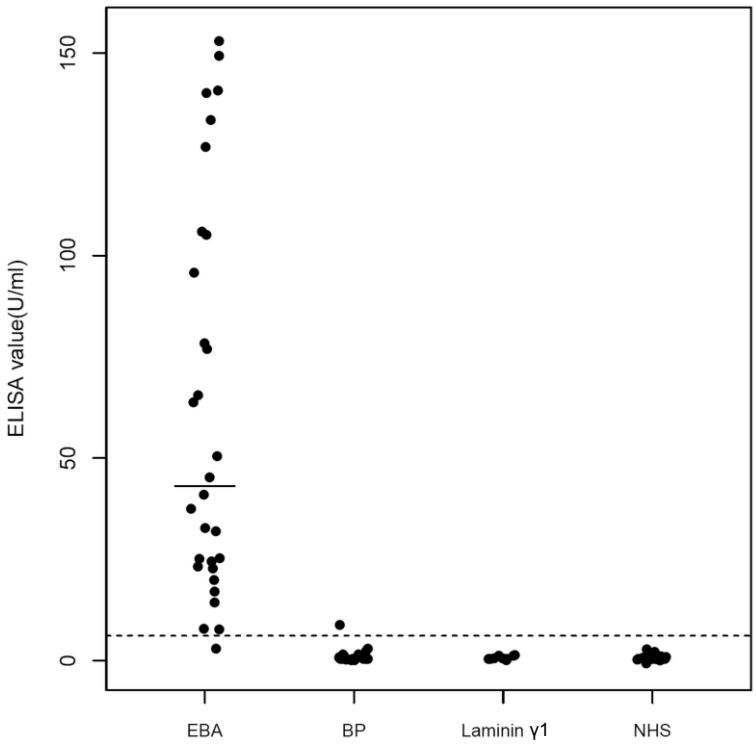

Figure 1. ELISA values in serum samples from patients with epidermolysis bullosa acquisita (EBA), bullous pemphigoid (BP), anti-laminin γ1 pemphigoid (laminin γ1), and normal healthy controls (NHS). The analysis excluded three Brunsting-Perry pemphigoid-like EBA patients. The sensitivity and specificity of the type VII collagen ELISA system were 96.7% and 98.1%, respectively. The dashed line indicates the cut-off value (6.14 U/ml) and the solid line indicates the median value (41.3 U/ml).

2. Sensitivity of IIF using HEK 293 cells transfected with NC1

IIF using HEK 293 cells transfected with NC1 were also 96.7% positive (Figure 2).

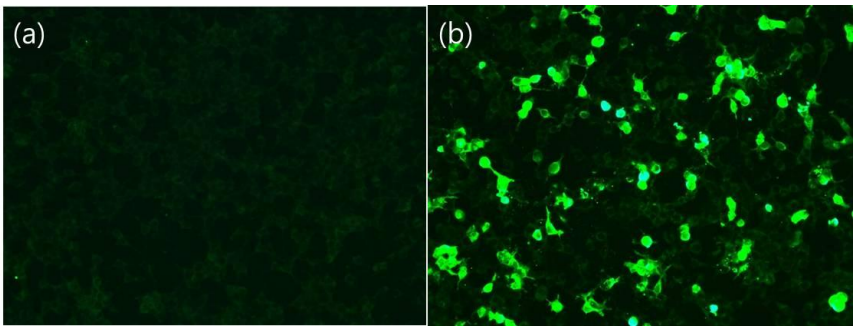

Figure 2. Negative (a) and positive (b) findings of IIF assay using human HEK 293 cells transfected with NC1 domain of type VII collagen.

2. Correlation between ELISA values, IIF titers and disease severity

Disease severity was positively correlated with both ELISA values and IIF titers (r=0.87, p<0.01 and r=0.59, p<0.01). Only ELISA values were correlated with the initial corticosteroid dose (r=0.53, p<0.01). There was a correlation between ELISA values and IIF titers (r=0.82, p<0.01) (Figure 3).

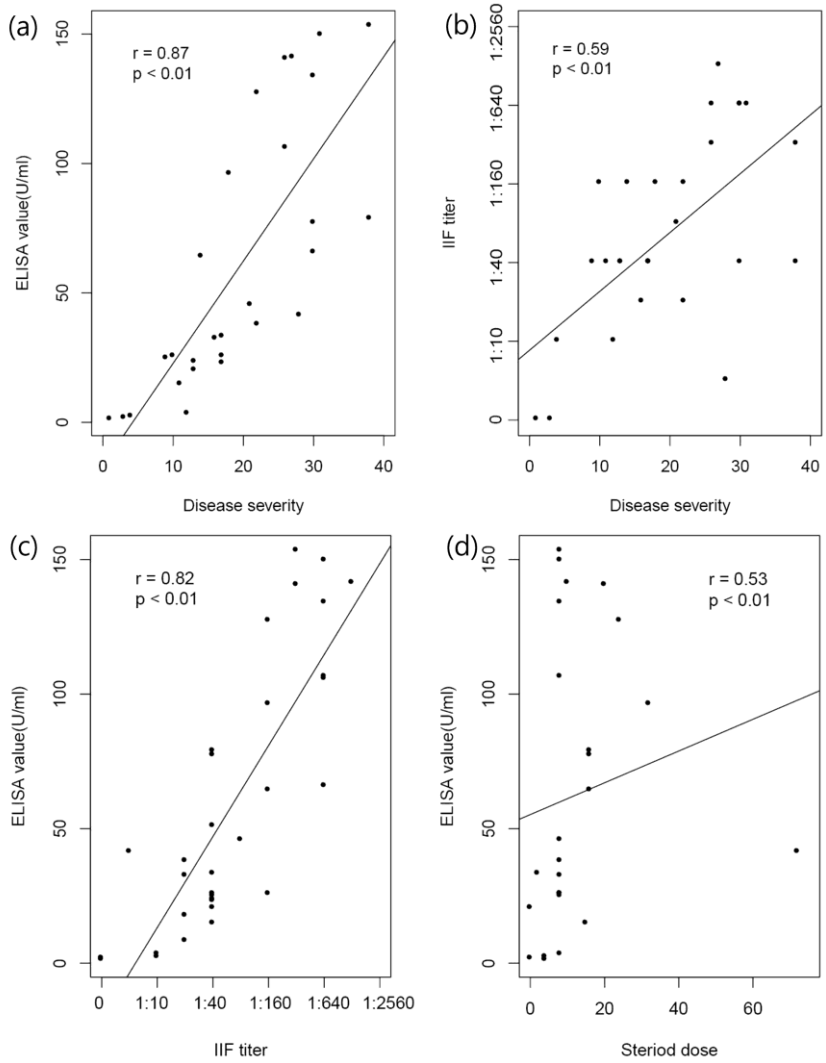

Figure 3. The relationships between ELISA value (a) or indirect immunofluorescence (IIF) titer (b) and disease severity score. ELISA values (r=0.87, $p<0.01$) and IIF titers (r=0.59, $p<0.01$) correlated with disease severity scores. (c) ELISA values was also correlated with IIF titers (r=0.82, $p<0.01$). (d) ELISA values correlated with initial methylprednisolone (MPD) dose (r=0.53, $p<0.01$), but IIF titers did not.

3. ELISA values and IIF titers before treatment and after remission

Disease progression was compared with the IIF titers and ELISA values to investigate which is more closely related to the disease course. First, ELISA values and IIF titers from three patients were measured before treatment and after remission (Figure 4a and b, respectively). ELISA values declined in all patients after remission, while IIF titer increased in one patient after remission.

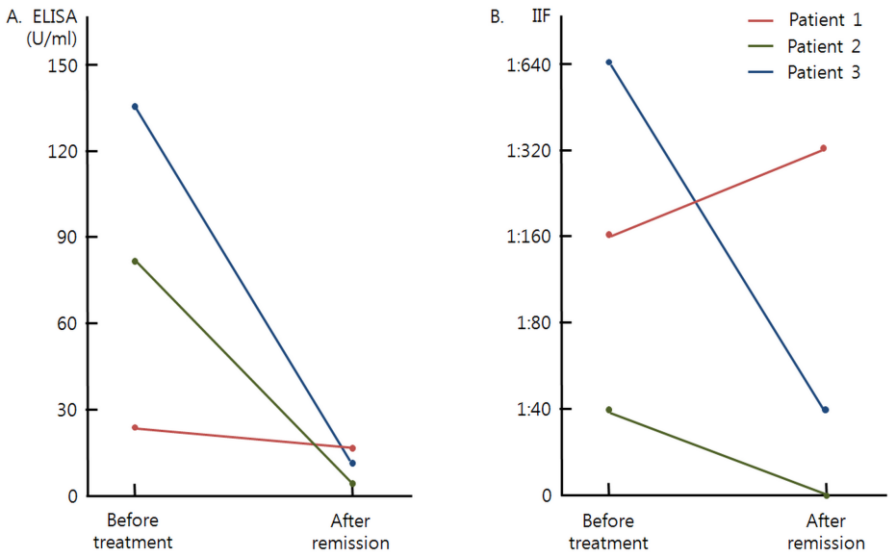

Figure 4. ELISA values (a) and indirect immunofluorescence (IIF) titers (b) of three patients before treatment and after remission.

4. Seven serial ELISA values and IIF titers of one patient with EBA

We performed a retrospective chart review of another patient who had sera collected serially. A 40-year-old man, who had blisters and erosions on the whole body including oral mucosa, has been treated with prednisolone (10-25 mg/d), colchicine (1.2 mg/d), and dapsone (100-150 mg/d) based on his disease severity. When the prednisolone was tapered to 10mg, he experienced an active flare. ELISA and IIF tests were performed seven times during 10 years of follow-up. We found that ELISA values are better correlated with disease severity than IIF titers (Figure 5).

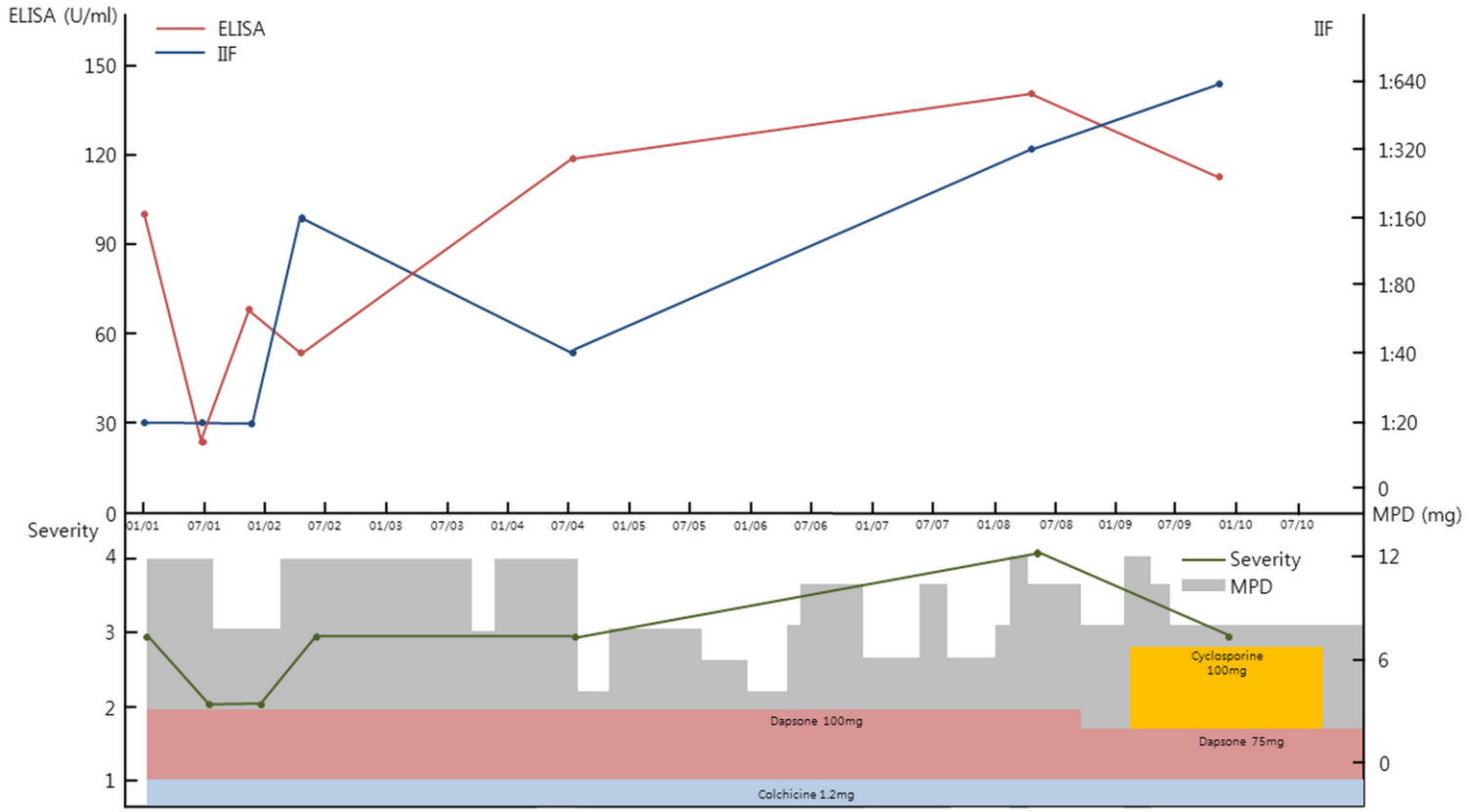
ELISA (U/ml)
ELISA
IIF
IIF
150
120
90
60
30
0
1:640
1:320
1:160
1:80
1:40
1:20
0
01/01
07/01
01/02
07/02
01/03
07/03
01/04
07/04
01/05
07/05
01/06
07/06
01/07
07/07
01/08
07/08
01/09
07/09
01/10
07/10
Severity
MPD (mg)
4
3
2
1
12
6
0
Severity
MPD
Cyclosporine 100mg
Dapsone 100mg
Dapsone 75mg
Colchicine 1.2mg

Figure 5. Clinical course, methylprednisolone dose, indirect immunofluorescence (IIF) titers, and ELISA values from January 2001 to January 2011 of one patient with epidermolysis bullosa acquisita. (MPD; methylprednisolone)

IV. DISCUSSION

Although the pathomechanism of blister formation in EBA remains unclear, several studies have provided evidence that the autoantibodies against type VII collagen are pathogenic. When rabbit and human antibodies specific to the NC1 domain of type VII collagen were passively transferred to mice, they induced subepidermal blister formation and nail dystrophy mimicking EBA[29,30]. The active mouse model of EBA was established by immunizing mice with the recombinant NC1 domain of murine type VII collagen[31,32]. Additionally, a transient neonate EBA resulted from a vertical transfer of maternal IgG autoantibodies against type VII collagen from a mother with EBA, suggesting that these transmitted autoantibodies induced blister formation in the neonate[33]. In addition, serial titers of anti-type VII collagen IgG from each patient with EBA reflect disease severity[22,26,34]. While previously levels of autoantibodies to type VII collagen, detected by ELISA, have been shown to correlate with disease severity at different time points in individual patients, we here demonstrate a correlation of autoantibody serum levels with overall disease severity in a large cohort of EBA patients.

Different ELISAs for the detection of autoantibodies in EBA have been developed using recombinant type VII collagen. Chen et al.[24] reported 100% reactivity by an ELISA using eukaryote-derived recombinant NC1 domain of type VII collagen. In another study of EBA, ELISA using baculovirus-derived recombinant NC1 had 67% sensitivity and 100% specificity[22]. Recently, Komorowski et al.[25] yielded 94.5% sensitivity and 98.7% specificity by an ELISA using recombinant NC1 expressed in human HEK 293 cells. In this study, an ELISA against recombinant NC1 and NC2 domains showed high sensitivity (96.7%) and specificity (98.1%). Our findings are comparable to those of a previous study reporting 91.8%

sensitivity and 98.1% specificity using the same ELISA system[26]. IIF using HEK 293 cells transfected with NC1 showed similar sensitivity, which means that almost all sera in this study have autoantibodies against NC1 domain of type VII collagen. Nevertheless it would be advisable to use type VII collagen ELISA containing recombinant NC1 and NC2 domains rather than using recombinant NC1 domain only, as some EBA patients have autoantibodies against NC2 domain only[7,8]. IIF titer, ELISA value, initial MPD dose and disease severity score were not significantly different between mechanobullous and inflammatory type EBA (data not shown).

Both type VII collagen ELISA and IIF values are positively correlated with disease severity scores, which is consistent with previous reports on pemphigus and bullous pemphigoid[35-37]. In our study, three aspects suggest that ELISA results better correlate with disease severity than IIF. First, we observed that only ELISA, not IIF, correlated with the initial steroid dose which usually reflects EBA severity. Second, we compared ELISA values and IIF titers from 3 patients with EBA before treatment and after remission. The ELISA values for all 3 patients declined after remission, while 1 patient's IIF titer increased after remission (Figure 4). Lastly, sera collected from 1 patient at 7 different times also showed that ELISA values better represent disease severity than IIF titers (Figure 5).

V. CONCLUSION

In conclusion, the ELISA using recombinant NC1 and NC2 proteins is useful for the diagnosis of EBA and evaluation of disease severity.

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ABSTRACT(IN KOREAN)

후천성 수포 박리증 환자에서 type VII collagen의 NC1 과 NC2 recombinant protein을 이용한 ELISA 수치와 임상적 중증도의 비교

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후천성 수포 박리증은 만성적인 자가면역 수포성 질환으로 제 7 교원질을 자가항원으로 인지하여 항체를 만들어낸다. 이 항체들을 찾아내는 방법이 진단에 필수적이라 할 수 있으며, 항체의 양을 측정한다면 질병의 중증도를 예측할 때에도 유용할 수 있다. 최근 후천성 수포 박리증 환자들을 위한 ELISA 가 개발되어 항 제 7 교원질 항체를 측정할 수 있게 되었고, 그에 따라 후천성 수포 박리증을 진단하는 데 사용할 수 있게 되었다. 본 논문은 상기 ELISA 를 사용하여 임상적 중증도와의 비교함으로써 진단적으로 유용하며 질병을 예측할 수 있는 도구인지 알아보고자 한다.

후천성 수포 박리증 환자 30 명, 수포성 유천포창 환자 20 명, 항 라미닌 감마 1 유천포창 환자 9 명, 정상인 24 명을 대상으로 하였다. 제 7 교원질의 NC1 과 NC2 도메인으로 제작한 ELISA 를 사용하였다. 또한 NC1 도메인을 HEK 293 세포 내로 이입시킨 칩을 사용하여 간접면역형광발현을 보았다. 후천성 수포 박리증

환자들의 임상적 특징, 간접면역형광의 역가, ELISA 값을 비교 분석하였다.

그 결과 ELISA 의 민감도와 특이도는 각각 96.7%와 98.1% 였다. NC1 도메인을 HEK 293 세포 내로 이입시킨 칩으로 간접면역형광 발현을 본 결과 96.7%의 양성율을 보였다. 전형적 타입과 염증성 타입간의 ELISA 나 임상적 중증도의 차이는 없었다. 임상적 중증도는 간접면역형광의 역가 ($r=0.59$, $p<0.01$) 및 ELISA 값 ($r=0.87$, $p<0.01$) 에서 양의 비례관계를 보였고, 초기 스테로이드 투여량과 ELISA 값은 양의 비례관계 ($r=0.53$, $p<0.01$)를 보였으나 초기 스테로이드 투여량과 간접면역형광의 역가는 비례관계를 보이지 않았다. 4 명의 환자들은 시간에 따른 임상적 중증도와 간접면역형광의 역가 및 ELISA 값의 관계를 보았다. ELISA 값이 좀 더 임상적 중증도를 잘 반영하는 결과를 관찰하였다.

후천성 수포 박리증에서 수포가 생기는 기전은 잘 알려져 있지 않지만, 수동적 혹은 능동적으로 얻은 항 제 7 교원질 항체로 쥐에게서 수포가 생긴다는 점, 후천성 수포 박리증 어머니에게서 일시적으로 후천성 수포 박리증의 증상을 지닌 신생아가 있었다는 점, 그리고 개개인의 환자에게서 서로 다른 시간에 채취한 혈청 내 항 제 7 교원질 항체가 질병의 중증도를 반영한다는 점을 근거로 항 제 7 교원질 항체가 후천성 수포 박리증의 중요한 기전임을 알 수 있다.

후천성 수포 박리증 환자들의 자가항체를 찾기 위한 ELISA 가 개발되어 왔다. Chen 등은 eukaryote-derived NC1 domain 을 이용하여 100%의 민감도를 관찰하였고, 또 다른 논문은 baculovirus-derived NC1 domain 으로 67%의 민감도와 100%의

특이도를 관찰하였다. 최근 Komorowski 등은 human HEK 293 cell-derived NC1 domain 으로 94.5%의 민감도와 98.7%의 특이도를 관찰하였다. 본 논문은 기존에 91.8%의 민감도와 98.1%의 특이도를 보였던 NC1 와 NC2 domain 을 이용한 ELISA 을 사용하여 96.7%의 민감도와 98.1%의 특이도를 관찰하였다.

ELISA 와 간접면역형광역가는 모두 질병의 중증도와 비례하였지만, 첫째, 3 명의 환자에서 ELISA 역가가 간접면역형광역가보다 치료 후에 더 잘 감소했다는 점, 둘째, 한 환자에게서 여러 번 채취한 혈청에서 ELISA 값이 더 질병의 중증도를 잘 반영했다는 점, 셋째, 초기 스테로이드 용량과 ELISA 와의 양의 비례관계는 간접면역형광역가에서는 안 나타났다는 점으로 ELISA 가 간접면역형광역가보다 질병의 중증도를 더 잘 반영한다는 결론을 내렸다.

결론적으로, NC1 과 NC2 도메인으로 만든 제 7 교원질 ELISA 는 후천성 수포 박리증을 진단하는 데 유용하고, 임상적 중증도를 관찰할 수 있는 도구라고 생각된다.

핵심되는 말: 후천성 수포 박리증, ELISA, 제 7 교원질