

# Correlation of serum levels of complement C4a desArg with pathologically estimated severity of glomerular lesions and mesangial hypercellularity scores in patients with IgA nephropathy

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**Abstract.** The aim of the present study was to explore serum biomarkers for the pathology of IgA nephropathy using serum proteomics. The subjects were 57 patients with IgA nephropathy who were divided into two groups (group 1, n=25; group 2, n=32) and 14 healthy controls. Serum protein profiles were analyzed using the ProteinChip surface-enhanced laser desorption ionization (SELDI) system. Associations between signal intensities of proteins and histological findings in patients with IgA nephropathy were studied in group 1. Serum levels of a candidate biomarker protein (complement component C4a desArg) for IgA nephropathy were determined by enzyme linked-immunosorbent assay (ELISA) in group 2 and the relationships of these levels with histological findings were evaluated. There were significant differences in 93 protein signals between patients in group 1 and controls. Among these signals, 3 proteins at 8592, 8757 and 8806 m/z were significantly correlated with the severity of glomerular lesions. The protein at 8592 m/z was identified as C4a desArg and the signal intensity of 8592 m/z was strongly correlated with serum C4a levels, including C4a desArg, determined by ELISA. In addition, the serum levels of C4a (mainly C4a desArg) were significantly higher in patients in group 2 compared to controls and were correlated with the severity of glomerular lesions and with mesangial hypercellularity scores. In conclusion, the serum

levels of complement C4a desArg are significantly higher in patients with IgA nephropathy compared to healthy controls and are significantly correlated with the severity of glomerular lesions and mesangial hypercellularity scores. Thus, serum C4a desArg is a potential biomarker for the severity of histological findings in patients with IgA nephropathy.

## Introduction

IgA nephropathy is the most common type of primary glomerulonephritis (1). Renal biopsy is required for definite diagnosis of primary glomerulonephritis and IgA nephropathy accounts for 40% of cases detected using this approach in East Asia and 20% in Europe. In Japan, 47% of cases of primary glomerulonephritis are diagnosed as IgA nephropathy. In addition, 20-30% of patients with IgA nephropathy progress to end-stage renal disease (ESRD) within 20 years after onset (2,3) and require hemodialysis or renal transplantation.

The pathology of IgA nephropathy is not fully understood, but involves marked mesangial IgA deposits that are often associated with complement component deposits. Furthermore, recurrence of mesangial IgA deposits is seen in approximately 50% of IgA nephropathy patients undergoing renal transplantation (4), while these deposits disappear in most patients without IgA nephropathy after renal transplantation from a patient with IgA nephropathy (5,6). In addition, patients with IgA nephropathy often develop gross hematuria following upper respiratory infection, and tonsil stimulation by an ultra short wave may cause deteriorated urinary findings at 3 h after the mechanical stimulation (7,8). These findings suggest that extrarenal factors and humoral factors are involved in IgA nephropathy.

Since the pathophysiology of IgA nephropathy is unclear, most current treatments aim to reduce immune reactions and inflammation in glomeruli and tubulointerstitium, which result in renal fibrosis. Corticosteroid-induced immune regulation delays progression to ESRD (9), but is only recom-

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mended for patients with increased pathological activity due to possible adverse reactions (10). The Oxford classification of IgA nephropathy was proposed in July 2009 as standard criteria for evaluation of the pathological activity of IgA nephropathy. The pathological findings are related to the clinical features (11-13). Therefore, histological evaluation in patients with IgA nephropathy is required. However, multiple renal biopsies performed for pathological evaluation may cause severe tissue damage and complications. Thus, it would be of value to find a clinically useful biomarker that is correlated with pathological findings in IgA nephropathy.

Biomarkers for various diseases have recently been detected by mass spectrometry (14). Mass spectrometry is generally limited for quantitative analysis, but a semiquantitative assessment can be achieved using the ProteinChip surface-enhanced laser desorption ionization (SELDI) system (15). In this study, we used this system to explore the serum biomarkers which correlate with the pathological activity evaluated histologically in accordance with the Oxford classification in patients with IgA nephropathy.

## Materials and methods

### *Clinical characteristics in patients with IgA nephropathy.*

The first group of subjects (group 1) consisted of 25 patients with IgA nephropathy confirmed by renal biopsy from 2006 to 2007 and 14 healthy controls without renal dysfunction used as controls. To verify the utility of serum biomarkers identified in group 1, serum biomarker candidates were determined by enzyme linked-immunosorbent assay (ELISA) in serum from 32 patients with IgA nephropathy (group 2) diagnosed by renal biopsy from 2008 to 2011 (Table I). The backgrounds of the patients and controls were the same, except for urinary protein excretion and age (Table I). Patients with eGFR <30 ml/min, patients who did not provide appropriate serum at the time of diagnosis, and those with <10 glomeruli in a renal biopsy specimen were excluded in each group. The study was approved by the Ethics Committee of Kagoshima University Hospital and Nanpuh Hospital. Written informed consent was obtained from all subjects.

*Pathological evaluation.* Renal tissues obtained by needle biopsy under ultrasound guidance were evaluated by light microscopy, immunofluorescence microscopy and electron microscopy. Subjects diagnosed with nephritis without IgA nephropathy, such as nephritis related to hepatic dysfunction, lupus nephritis, and poststreptococcal glomerulonephritis, based on clinical course and pathological characteristics were excluded from the study. Subjects with dominant IgA deposits found in the mesangial region by immunofluorescence microscopy and with mesangial proliferative glomerulonephritis confirmed by light microscopy were diagnosed with IgA nephropathy.

Paraffin-embedded specimens were stained with hematoxylin and eosin, periodic acid-Schiff reaction, Masson's trichrome, and periodic acid silver-methenamine, and were evaluated according to the Oxford classification of IgA nephropathy (12,13).

Glomerular lesions were evaluated as follows; for mesangial proliferation, all glomeruli without global sclerosis were

classified as normal, mild, moderate and severe based on <4, 4-5, 6-7 and  $\geq 8$  mesangial cells/mesangial area, respectively. The mesangial hypercellularity score was calculated as the mean number of glomeruli for scoring mesangial proliferation (normal, 0; mild, 1; moderate, 2; severe, 3). Active glomerular lesions were defined as glomeruli with a cellular crescent, fibrocellular crescent, or endocapillary proliferation. The severity of glomerular lesions was defined as the ratio of active glomerular lesions relative to the total number of glomeruli. Chronic lesions of glomeruli were defined based on the number of glomeruli with global sclerosis, segmental sclerosis, or a fibrous crescent. The severity of chronic lesions was defined as the ratio of chronic lesions relative to the total number of glomeruli.

*ProteinChip SELDI system.* A comprehensive protein analysis using the ProteinChip SELDI system was performed as previously described (16,17) under the following conditions, in which several protein signals can be obtained in a stable manner. Native serum was applied to a hydrophobic cation-exchange chip (CM10, Bio-Rad Laboratories, Hercules, CA, USA). Sinapinic acid was used as the matrix and 50 mM sodium acetate (pH 4.5) as the binding/washing buffer. Protein signal intensities from 2,000 to 11,000 m/z were compared between the patient group 1 and control groups.

Binding/washing buffers of pH 5.0-11.0 were prepared in increments of pH 1.0 for use in experiments to predict the isoelectric point of the target protein. These buffers were sodium acetate (pH 5.0), phosphate (pH 6.0 and 7.0), Tris-HCl (pH 8.0 and 9.0), and sodium carbonate (pH 10.0 and 11.0). Each was used to wash 7 spots on the CM10 chip and then the spectrum was obtained by time-of-flight mass spectrometry. The pH at which the peak disappeared was estimated as the approximate pI value. Peptides were predicted from the pI value and the molecular weight based on a published database (TagIdent: <http://web.expasy.org/tagident/>).

### *Crude fractionation, demineralization and concentration of serum samples.*

Crude fractionation of serum samples was performed using a weak cation-exchange absorbent column (HiTrap CM FF 5 ml column, GE Healthcare Bio-Sciences KK, Tokyo, Japan) to remove proteins that inhibit reactions between biomarker candidate proteins and specific antibodies. Serum containing biomarker candidates was diluted 5 times with phosphate buffer (pH 7.5) and applied to the column. Proteins and peptides bound to the column were then eluted using 0.5 M NaCl in phosphate buffer. The eluent was desalted and concentrated using an ultrafiltration column (Vivaspin 500 Polyethersulfone 5,000 MWCO, Sartorius Stedim Biotech, Goettingen, Germany) and an ultrafilter (5,000 MWCO). Protein levels were determined by Bradford's method (Quick Start Protein Assay, Bio-Rad Laboratories).

*Immunoprecipitation.* A combination of 50% protein A (Protein A Sepharose CL-4B, GE Healthcare) suspension and 1:5 diluted rabbit anti-human C4a polyclonal antibody (#A206, Complement Technology, Inc., Tyler, TX, USA) was incubated with shaking overnight at 4°C. The rabbit anti-human C4a antibody-bound protein A and serum samples containing biomarker candidate proteins were mixed, incu-

Table I. Characteristics of patients with IgA nephropathy and healthy controls.

Characteristics	Healthy controls (n=14)	Patient group 1 (n=25)	Patient group 2 (n=32)	P-value <sup>a</sup>		
				C vs. G1	C vs. G2	G1 vs. G2
Gender (M/F)	5/9	11/14	16/16	0.614	0.371	0.652
Age	34.4±6.9	34.1±13.2	28.8±10.8	0.792	0.035	0.110
UPE (g/g Cre)	ND	0.80±0.72	0.49±0.55	ND	ND	0.081
Serum Cre (mg/dl)	0.76±0.17	0.83±0.27	0.74±0.15	0.682	0.830	0.505
eGFR (ml/min)	81.9±15.8	82.0±26.2	95.8±29.1	0.942	0.133	0.085
Serum IgA (mg/dl)	261.1±68.5	327.8±115.4	330.3±150.2	0.083	0.126	0.676
Serum C3 (mg/dl)	93.8±11.9	103.3±17.2	99.2±15.3	0.098	0.252	0.426
Serum C4 (mg/dl)	21.2±4.0	25.4±7.4	24.2±5.7	0.119	0.108	0.635

<sup>a</sup>Comparison of data by Fisher's exact test or Mann-Whitney U test, as appropriate. ND, not done; UPE, urinary protein excretion; Cre, creatinine; eGFR, estimated glomerular filtration rate; C, control; G1, group 1; G2, group 2.

bated, and then centrifuged to separate supernatant from precipitates of the C4a-anti C4a antibody-protein A complex. Normal rabbit IgG (sc-2027, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as a control antibody. The supernatant was divided into two parts; one was diluted with sodium acetate buffer (pH 4.5) and used for ProteinChip SELDI system, while the other was incubated with sample buffer with  $\beta$ -mercaptoethanol at 95°C and then used in tricine-SDS-PAGE. The collected precipitates were washed with PBS several times and incubated with sample buffer with  $\beta$ -mercaptoethanol to release C4a including C4a desArg from the C4a-anti C4a antibody-protein A complex. The resulting mixture was centrifuged to remove protein A and the remaining supernatant was used for tricine-SDS-PAGE.

*Tricine-SDS-PAGE and western blot analysis.* Tricine-SDS-PAGE was performed as previously described (18), using 0.1 M Tris/0.0225 M HCl and 0.1 M Tris/0.1M Tricine/0.1% SDS as the anode and cathode buffers, respectively. For the western blotting, the rabbit anti-human C4a antibody (#A206, Complement Technology, Inc.) was used as the primary antibody and goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology, Inc.) as the secondary antibody for visualization of the target proteins with a chemiluminescent reagent (ECL Plus, GE Healthcare UK Ltd., Buckinghamshire, UK).

*Purified C4a desArg using the ProteinChip SELDI system.* Purified C4a desArg (#A107, Complement Technology, Inc.) was prepared as solutions of 1 and 5  $\mu$ g/ml. These solutions were diluted 10 times with sodium acetate buffer (pH 4.5) and diluted solutions (100  $\mu$ l) were applied to the ProteinChip SELDI system with the CM10 chip. C4a desArg was also added to serum from healthy controls to give a concentration of 50  $\mu$ g/ml. The mixture was diluted 10 times with PBS and 10 times with sodium acetate buffer (pH 4.5) and then diluted solutions (100  $\mu$ l) were applied to the ProteinChip SELDI system.

*Determination of serum levels of complement C4a/C4a desArg.* The level of serum C4a including C4a desArg was

Table II. Representative discriminatory signals and mean values for patients with IgA nephropathy (group 1) and healthy controls.

Number	m/z	Patient group 1 (n=25)	Healthy controls (n=14)	P-value
1	3191	12.68±4.68	21.32±5.11	<0.0001
2	8592	7.85±8.38	2.89±2.58	0.0008
3	4465	9.67±3.20	5.66±3.00	0.0009
4	6005	4.08±1.53	5.53±1.22	0.0012
5	4611	3.60±3.86	1.37±1.07	0.0013
6	6149	1.63±0.85	2.46±0.78	0.0013
7	8924	23.96±9.31	13.22±8.25	0.0013
8	4641	15.60±3.74	12.20±2.09	0.0014
·	·	·	·	·
·	·	·	·	·
27	8757	2.24±1.00	1.39±0.74	0.0084
·	·	·	·	·
·	·	·	·	·
38	8806	3.18±1.16	2.34±0.80	0.0118
·	·	·	·	·
·	·	·	·	·
93	10256	4.61±2.52	7.29±4.03	<0.050

determined using an ELISA kit (Human C4a ELISA kit, BD Biosciences, Franklin Lakes, NJ, USA).

*Statistical analysis.* Data are expressed as the means  $\pm$  standard deviation (SD). Differences in laboratory data and signal intensities obtained by SELDI were evaluated by Mann-Whitney U test. Regression analysis was performed to examine the correlation of continuous variables. A P-value <0.05 was considered to indicate a statistically significant

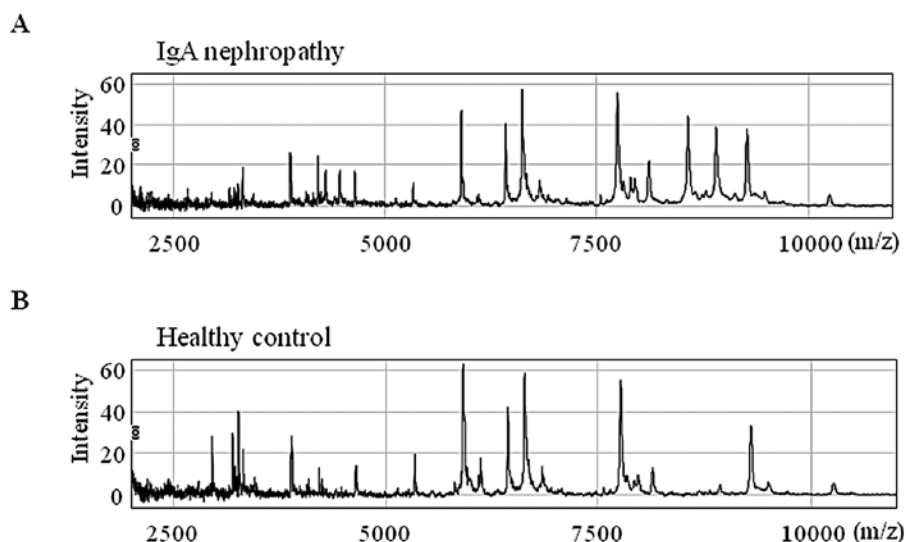


Figure 1. Representative SELDI spectra (2,000–11,000 m/z) of serum from patients with IgA nephropathy and healthy controls. (A) IgA nephropathy; (B) healthy controls.

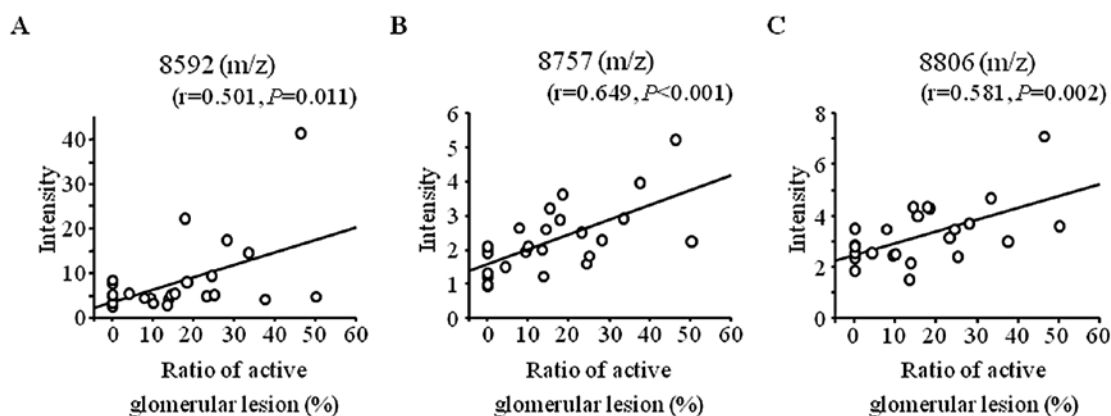


Figure 2. Correlation between three protein peaks and pathological severity estimated by rate of active glomerular lesion. (A) 8592; (B) 8757; (C) 8806 m/z.

difference. Analyses were performed with StatView 4.5 software (Abacus Concepts, Berkeley, CA, USA) or ProteinChip Software, version 3.2.1 (Bio-Rad Laboratories).

## Results

**Exploration of biomarker candidates in patients with IgA nephropathy.** Serum spectra were obtained from 25 patients with IgA nephropathy and 14 healthy controls using the ProteinChip SELDI system (Fig. 1). A total of 558 signal clusters were detected from 2,000 to 11,000 m/z, and 93 of these signals differed significantly between the patients and controls (Table II). Simple regression analysis was performed between the intensity of these 93 signals and the severity of glomerular lesion evaluated histologically, with the goal of finding biomarker candidate proteins correlated with the pathological activity of IgA nephropathy. In this process, 3 signals (8592, 8757, 8806 m/z) were identified as potential biomarkers (Table II, Fig. 2A–C). A protein at 8592 m/z was also increased in the patients with IgA nephropathy compared to the controls, with a mean intensity >5 in the patients (Table II).

**Identification of a biomarker candidate protein (8592 m/z).** The protein signal at 8592 m/z was not detected using the ProteinChip SELDI system (CM10 chip) in serum diluted with buffer at pH 10.0, which suggested that the isoelectric point of the protein at 8592 m/z was between pH 9.0–10.0. Based on the TagIdent database of isoelectric point and molecular weight, the signal at 8592 m/z was expected an inactivated peptide of C4a anaphylatoxin (C4a desArg; 8590 Da, pI=9.6).

The protein signal at 8592 m/z detected in serum of patients with IgA nephropathy (Fig. 3A) was not eliminated by immunoprecipitation using the control antibody (Fig. 3B), but disappeared with immunoprecipitation using anti-C4a antibody (Fig. 3C). Western blot analysis confirmed the presence of C4a or C4a desArg by the immunoprecipitation assay (Fig. 3D). Purified C4a desArg diluted 10 times with sodium acetate buffer (pH 4.5) also gave a signal at 8592 m/z using the ProteinChip SELDI system (CM10 chip) and the signal intensity was dependent on the concentration (Fig. 4B and C). Similarly, a signal at 8592 m/z appeared when purified C4a desArg was added to serum from healthy controls (Fig. 4E), at the same position as the protein signal in serum from patients

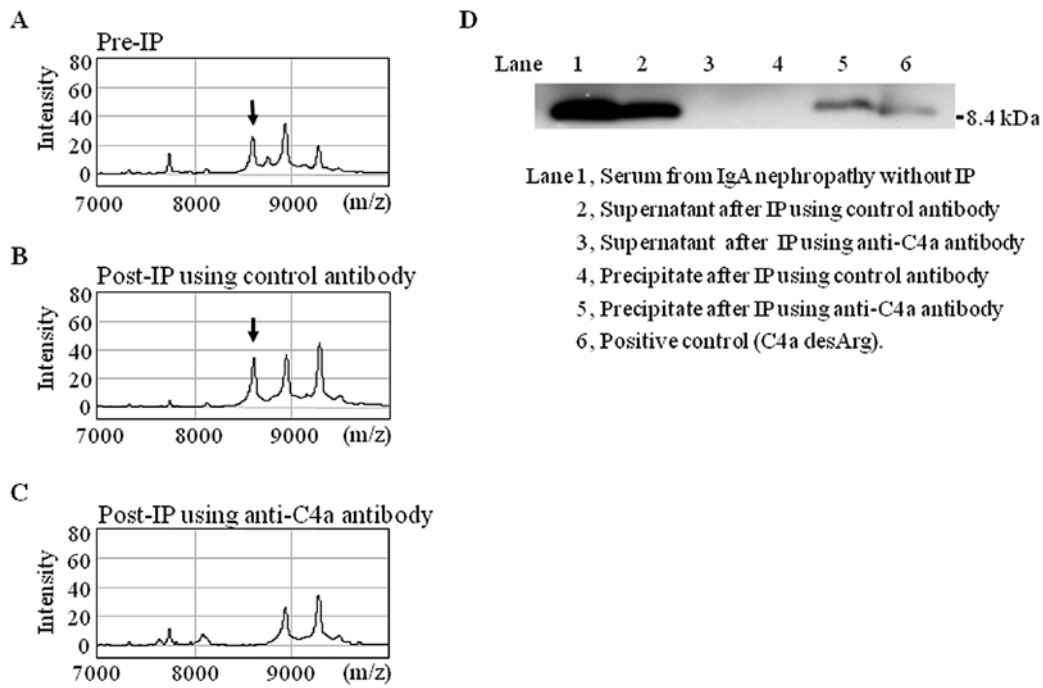


Figure 3. Immunoprecipitation (IP) and analysis with the ProteinChip SELDI system or western blotting using anti-C4a antibody. A protein signal at 8592 m/z (arrow) was detected in serum from patients with IgA nephropathy prior to IP (A) and following IP using control antibody (B). By contrast, this signal was not detected in serum from patients with IgA nephropathy following treatment of the serum with anti-C4a antibody (C). C4a or C4a desArg was also detected in serum from patients with IgA nephropathy without IP (lane 1) and in supernatant after IP using control antibody (lane 2), but not in supernatant after IP using anti-C4a antibody (lane 3). C4a or C4a desArg was not detected in precipitate after IP using control antibody (lane 4), but was detected in precipitate after IP using anti-C4a antibody (lane 5). A positive control using C4a desArg and anti-C4a antibody is shown in lane 6.

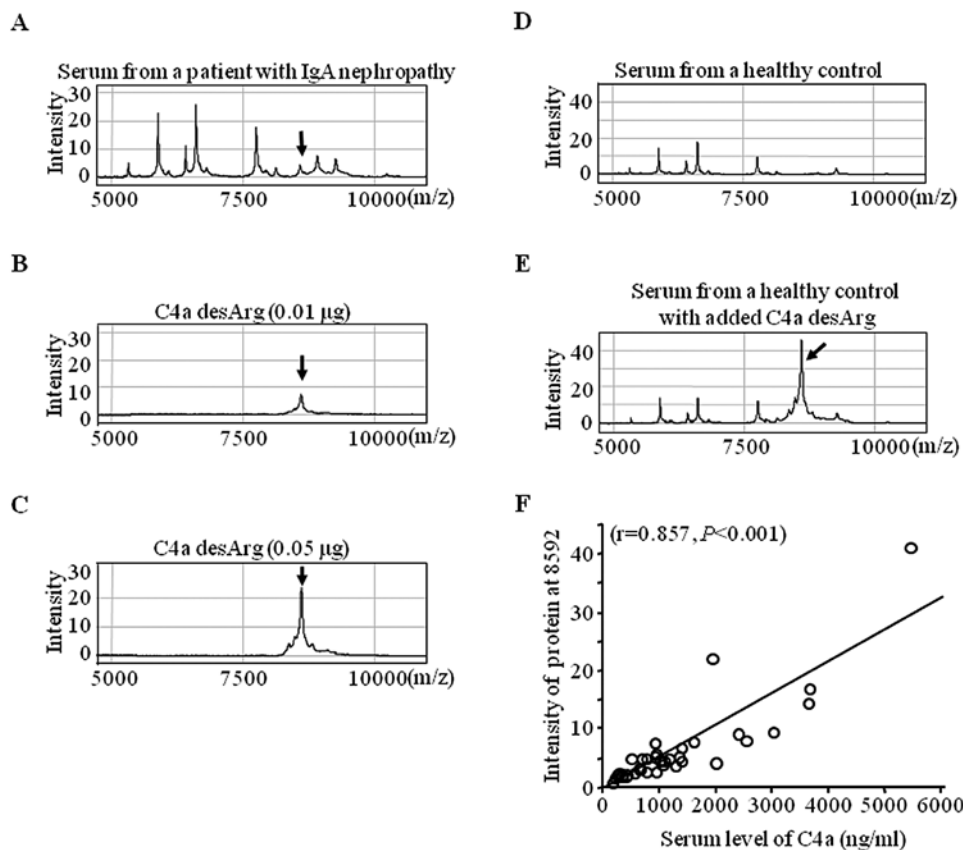


Figure 4. Detection of purified C4a desArg on a SELDI spectrum at 8592 m/z and correlation of the signal intensity at 8592 m/z with C4a (including C4a desArg) levels determined by ELISA. (A) SELDI spectrum of serum from patients with IgA nephropathy. (B and C) SELDI spectra of phosphate-buffered saline mixed with C4a desArg. (B) 0.01 µg; (C) 0.05 µg. (D) SELDI spectrum of serum from healthy controls. (E) SELDI spectrum of serum from healthy controls mixed with C4a desArg (0.05 µg). (F) The signal intensity at 8592 m/z was strongly correlated with the level of C4a (including C4a desArg) determined by ELISA in patients with IgA nephropathy. Arrows indicate C4a desArg detected at 8592 m/z (A, B, C and E).

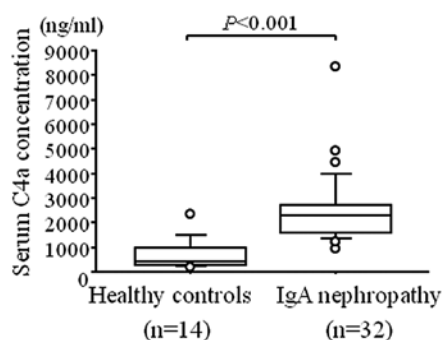


Figure 5. Serum levels of C4a/C4a desArg in patients with IgA nephropathy in group 2 were higher than those in healthy controls.

with IgA nephropathy (Fig. 4A). These results show that the peak at 8592 m/z was due to C4a desArg.

In addition, the levels of C4a, including C4a desArg, determined by ELISA were strongly correlated with the signal intensity at 8592 m/z ( $r=0.86$ ,  $P<0.001$ , Fig. 4F). Thus, our results suggest that most C4a evaluated by ELISA in patients with IgA nephropathy is in the form of inactivated peptide C4a desArg.

*Relationship between pathological data and serum C4a/C4a desArg in patient group 1.* The serum levels of C4a (mainly C4a desArg) in group 1 determined by ELISA were significantly higher in patients with IgA nephropathy compared to healthy controls ( $1564.5\pm 1129.0$  vs.  $708.2\pm 622.1$  ng/ml,  $P=0.002$ ). Correlations of the severity of glomerular lesion with the serum levels of C4a/C4a desArg and other laboratory data (urinary protein excretion, eGFR, IgA) were also examined in group 1. The severity was positively correlated with the serum levels of C4a/C4a desArg ( $r=0.62$ ,  $P<0.001$ ) and urinary protein excretion ( $r=0.55$ ,  $P=0.005$ ). In addition, the mesangial hypercellularity score, which is related to a decrease in renal function (12), was positively correlated with the serum levels of C4a/C4a desArg ( $r=0.54$ ,  $P=0.005$ ). By contrast, the severity of chronic glomerular lesions was not correlated with the serum levels of C4a/C4a desArg.

*Clinical significance of serum C4a/C4a desArg in patient group 2.* To confirm the results from patient group 1, the clinical significance of the serum levels of C4a/C4a desArg were evaluated in a second group of patients with IgA nephropathy (group 2). The C4a/C4a desArg levels determined by ELISA in this group were also significantly higher than those in healthy controls (Fig. 5,  $P<0.001$ ). The serum levels of C4a/C4a desArg also correlated with the severity of glomerular lesion (Fig. 6A,  $P=0.035$ ) and were significantly correlated with mesangial hypercellularity scores (Fig. 6B,  $P=0.014$ ) in group 2.

## Discussion

Three signals (8592, 8757, 8806 m/z) of potential biomarkers related to the severity of glomerular lesions were identified in serum proteomics of patients with IgA nephropathy. The signal at 8592 m/z was confirmed to be due to C4a desArg, which was also shown to be positively correlated with mesangial hypercellularity scores in two groups of patients with IgA nephropathy.

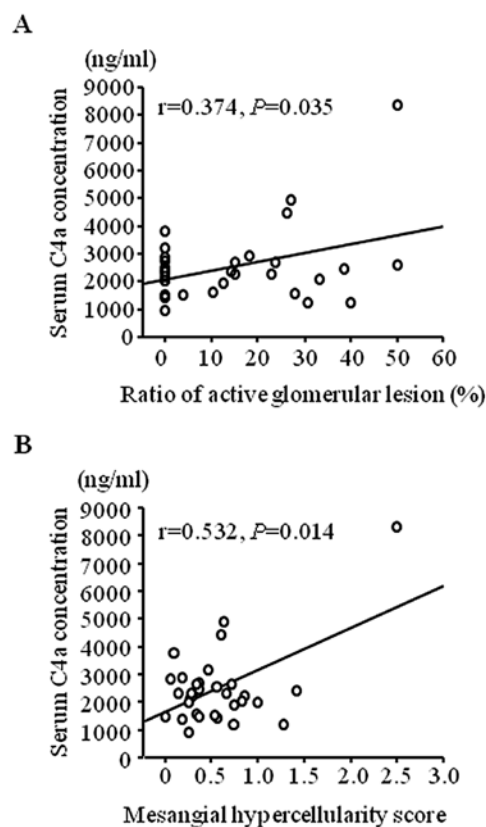


Figure 6. Correlation between serum levels of C4a/C4a desArg and the histological findings in IgA nephropathy patients in group 2. Serum levels of C4a/C4a desArg were significantly correlated with the ratio of active glomerular lesions (A) and mesangial hypercellularity scores (B).

C4a is an anaphylatoxin and strong inflammation-inducing agent derived from complement component C4. C4a desArg, which has low anaphylatoxin activity, is formed from C4a by fast cleavage of arginine by carboxypeptidase N in serum. It is considered that the majority of C4a in serum is C4a desArg, and we confirmed a strong correlation between C4a levels determined by ELISA and the level of C4a desArg detected by SELDI. The complement pathway is activated in patients with IgA nephropathy and, in this study, C4a desArg levels were significantly higher in these patients than in healthy controls. By contrast, there was no significant difference in C4 levels between the patients and healthy controls. These results suggest that the C4a desArg level can serve as an index of complement activation in IgA nephropathy.

Serum levels of C4a (mainly C4a desArg) determined by ELISA were significantly correlated with pathologically estimated mesangial hypercellularity scores and with the severity of glomerular lesions in both groups of patients with IgA nephropathy. The mesangial hypercellularity score is a measure of mesangial cell proliferation and is likely to be a prognostic factor for renal function and to indicate the severity of glomerular lesions. In proposing the Oxford classification, the working group of the International IgA Nephropathy Network and the Renal Pathology Society suggested that the mesangial hypercellularity score was the most reproducible pathological finding among investigators (13). Therefore, our results suggest that the serum level of complement C4a

desArg may serve as a biomarker to estimate the mesangial hypercellularity score, as a prognostic factor, and as an index of the effect of immunoregulatory therapy in patients with IgA nephropathy. A long-term follow-up study is required to confirm these results.

Complement deposition is observed in IgA deposition sites in patients with IgA nephropathy, which suggests that the complement system may be involved in the pathology, and increased plasma levels of the anaphylatoxins C3a and C4a have been reported in these patients (19). However, the mechanism and clinical significance of complement activation in IgA nephropathy is unclear. Activation may occur through an alternative pathway (20), but it has recently been proposed that the lectin pathway is involved in complement activation in IgA nephropathy (21,22). Furthermore, C4d deposition in glomeruli and activation of the lectin pathway influence the prognosis of IgA nephropathy and the severity of renal injury (23,24). The current study also showed that serum C4a desArg levels were correlated with mesangial hypercellularity scores and the severity of glomerular lesions. Complement components other than C4 remain to be investigated; however, our study indicates that complement activation by C4 may be important in IgA nephropathy.

Our results indicated a correlation between serum levels of complement C4a (mainly C4a desArg) and histological activity estimated by severity of glomerular lesions and mesangial hypercellularity scores in both groups of patients with IgA nephropathy. By contrast, serum levels of complement C4a or C4a desArg were not related to the severity of chronic glomerular lesions. Urinary protein excretion is used as a laboratory test of the severity of renal injury and is strongly influenced by chronic glomerular lesions, which are unlikely to be responsive to anti-inflammatory therapy. By contrast, serum levels of complement C4a and C4a desArg are not influenced by urinary protein excretion. The independence of the serum level of C4a desArg with respect to urinary protein excretion may also make it a useful index of the effect of anti-inflammatory therapy in IgA nephropathy.

One limitation of identification of a complement-related molecule, including an anaphylatoxin such as C4a desArg, as a biomarker candidate is that complement may be activated even after blood sample collection (25,26). Pfeifer *et al* showed that C4a/C4a desArg levels increased over time in plasma samples from patients with systemic lupus erythematosus stored at 37°C without addition of futhan (25). The C4a/C4a desArg levels in plasma samples of healthy controls collected in the same manner and stored for 60 min at 37°C were also higher than those measured immediately after collection (26). Thus, the serum levels of complement C4a/C4a desArg measured in the current study were likely to be higher than those in blood *in vivo*. However, the effects *ex vivo* can be minimized by shortening the time from sample collection to centrifugation and freezing to eliminate sample variability. The measured C4a desArg levels might reflect complement activation *ex vivo* as well as that *in vivo*, but these levels still showed a considerable correlation with the severity of glomerular lesion and mesangial hypercellularity scores in two separate groups of patients with IgA nephropathy. Therefore, we conclude that the C4a/C4a desArg levels in these patients are useful as a surrogate marker of disease severity. Verification of these findings

requires measurements in samples with control of complement activation *ex vivo*. A prospective study is also required to confirm the correlation between serum C4a desArg levels and the pathological activity of IgA nephropathy.

In conclusion, the serum level of C4a (mainly C4a desArg) is significantly higher in patients with IgA nephropathy compared to healthy controls and is significantly correlated with the severity of glomerular lesions and mesangial hypercellularity scores evaluated histologically using the Oxford classification of IgA nephropathy. Thus, serum C4a desArg is a potential biomarker for the severity of histological findings in patients with IgA nephropathy.

## References

1. Donadio JV and Grande JP: IgA nephropathy. *N Engl J Med* 347: 738-748, 2002.
2. D'Amico G: Influence of clinical and histological features on actuarial renal survival in adult patients with idiopathic IgA nephropathy, membranous nephropathy, and membranoproliferative glomerulonephritis: survey of the recent literature. *Am J Kidney Dis* 20: 315-323, 1992.
3. Alamartine E, Sabatier JC, Guerin C, Berliet JM and Berthoux F: Prognostic factors in mesangial IgA glomerulonephritis: an extensive study with univariate and multivariate analyses. *Am J Kidney Dis* 18: 12-19, 1991.
4. Odum J, Peh CA, Clarkson AR, *et al*: Recurrent mesangial IgA nephritis following renal transplantation. *Nephrol Dial Transplant* 9: 309-312, 1994.
5. Sanfilippo F, Croker BP and Bollinger RR: Fate of four cadaveric donor renal allografts with mesangial IgA deposits. *Transplantation* 33: 370-376, 1982.
6. Koselj M, Rott T, Vizjak A and Kveder R: IgA nephropathy as a donor-transmitted disease in renal transplant recipients. *Transplant Proc* 23: 2643-2646, 1991.
7. Shiraishi S, Tomoda K, Matsumoto A, Kyomoto R and Yamashita T: Investigation of the local provocation test to PPP and IgA nephritis. *Acta Otolaryngol Suppl* 523: 178-181, 1996.
8. Yamabe H, Osawa H, Inuma H, *et al*: Deterioration of urinary findings after tonsil stimulation in patients with IgA nephropathy. *Acta Otolaryngol Suppl* 523: 169-171, 1996.
9. Pozzi C, Andrulli S, Del Vecchio L, *et al*: Corticosteroid effectiveness in IgA nephropathy: long term results of a randomized, controlled trial. *J Am Soc Nephrol* 15: 157-163, 2004.
10. Barratt J and Feehally J: Treatment of IgA nephropathy. *Kidney Int* 69: 1934-1938, 2006.
11. Feehally J, Barratt J, Coppo R, Cook T and Roberts I: International IgA Nephropathy Network: International IgA nephropathy network clinico-pathological classification of IgA nephropathy. *Contrib Nephrol* 157: 13-18, 2007.
12. Working Group of the International IgA Nephropathy Network and the Renal Pathology Society; Cattran DC, Coppo R, Cook HT, *et al*: The Oxford classification of IgA nephropathy: rationale, clinicopathological correlations, and classification. *Kidney Int* 76: 534-545, 2009.
13. Working Group of the International IgA Nephropathy Network and the Renal Pathology Society, Roberts IS, Cook HT, Troyanov S, *et al*: The Oxford classification of IgA nephropathy: pathology definitions, correlations and reproducibility. *Kidney Int* 76: 546-556, 2009.
14. Yoshida M, Hatano N, Nishiumi S, Irino Y, Izumi Y, Takenawa T and Azuma T: Diagnosis of gastroenterological diseases by metabolome analysis using gas chromatography-mass spectrometry. *J Gastroenterol* 47: 9-20, 2012.
15. Tomosugi N, Kawabata H, Wakatabe R, Higuchi M, Yamaya H, Umehara H and Ishikawa I: Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. *Blood* 108: 1381-1387, 2006.
16. Kanmura S, Uto H, Kusumoto K, *et al*: Early diagnostic potential for hepatocellular carcinoma using the SELDI ProteinChip system. *Hepatology* 45: 948-956, 2007.
17. Kanmura S, Uto H, Sato Y, *et al*: The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma. *J Gastroenterol* 45: 459-467, 2010.
18. Schägger H: Tricine-SDS-PAGE. *Nat Protoc* 1: 16-22, 2006.

19. Abou-Ragheb HH, Williams AJ, Brown CB and Milford-Ward A: Plasma levels of the anaphylatoxins C3a and C4a in patients with IgA nephropathy/Henoch-Schönlein nephritis. *Nephron* 62: 22-26, 1992.
20. Wyatt RJ, Kanayama Y, Julian BA, Negoro N, Sugimoto S, Hudson EC and Curd JG: Complement activation in IgA nephropathy. *Kidney Int* 31: 1019-1023, 1987.
21. Matsuda M, Shikata K, Wada J, Sugimoto H, Shikata Y, Kawasaki T and Makino H: Deposition of mannan binding protein and mannan binding protein-mediated complement activation in the glomeruli of patients with IgA nephropathy. *Nephron* 80: 408-413, 1998.
22. Endo M, Ohi H, Ohsawa I, Fujita T, Matsushita M and Fujita T: Glomerular deposition of mannose-binding lectin (MBL) indicates a novel mechanism of complement activation in IgA nephropathy. *Nephrol Dial Transplant* 13: 1984-1990, 1998.
23. Espinosa M, Ortega R, Gomez-Carrasco JM, Lopez-Rubio F, Lopez-Andreu M, Lopez-Oliva MO and Aljama P: Mesangial C4d deposition: a new prognostic factor in IgA nephropathy. *Nephrol Dial Transplant* 24: 886-891, 2009.
24. Roos A, Rastaldi MP, Calvaresi N, *et al*: Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease. *J Am Soc Nephrol* 17: 1724-1734, 2006.
25. Pfeifer PH, Kawahara MS and Hugli TE: Possible mechanism for in vitro complement activation in blood and plasma samples: futhan/EDTA controls in vitro complement activation. *Clin Chem* 45: 1190-1199, 1999.
26. Morgan E, Varro R, Sepulveda H, *et al*: Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clin Immunol* 110: 252-266, 2004.