Table 1
Comparison of baseline characteristics between patients requiring high-dose and patients with conventional-dose.

	High-dose (n=15)	Conventional-dose (n=27)	P
Age	37.4 (12.8)	36.7 (9.0)	0.85
Men	7/15 (47%)	14/27 (52%)	1.00
Asian	15/15 (100%)	27/27 (100%)	
Substance dependence	2/15 (13%)	3/27 (11%)	1.00
Duration from onset (year)	9.9 (11.6)	8.1 (7.7)	0.56
Antipsychotic-naive	6/15 (40%)	17/27 (63%)	0.20
Haloperidol injection received before enrollment	8/15 (53%)	6/27 (22%)	0.085
CGI-S	5.9 (0.7)	5.8 (0.9)	0.56
PANSS			
Total	120.5 (21.0)	105.2 (24.8)	0.051
Positive scale	32.6 (6.1)	30.5 (6.5)	0.30
Negative scale	28.9 (9.2)	20.9 (8.7)	0.0077
General psychopathology scale	58.9 (11.1)	53.8 (14.1)	0.23
GAF	20.3 (8.3)	23.7 (8.0)	0.20
BMI (kg/m^2)	21.1 (4.0)	21.7 (3.4)	0.66
PANSS total score at the time of starting high-dose	104.5 (21.5)		

Data represent mean (S.D.) or n/N (%), unless otherwise indicated. All substance dependence except one patient with benzodiazepine dependence in the conventional-dose group was alcohol dependence. 'Haloperidol injection received before enrollment': the maximal duration until enrollment was 3 days. CGI-S, Clinical Global Impression Severity rating scale; PANSS, Positive and Negative Syndrome Scale; GAF, Global Assessment of Functioning; BMI, body mass index. Differences in age, duration from onset, CGI-S, PANSS, GAF, and BMI were calculated using the unpaired t-test. Differences in sex, and frequencies of substance dependence, antipsychotic-naïve, and haloperidol injection received before enrollment were calculated using the Fisher's exact test.

3.3. Serum olanzapine concentrations at the time of taking 20 mg/day in patients who subsequently required high-dose olanzapine

Serum olanzapine concentrations at the time of taking 20 mg/day could be obtained from five out of seven patients who subsequently required high-dose olanzapine. The rest two patients refused additional blood samples. The mean time from dosing to sample collection was 14.2 h (S.D. 2.5, range 11–16). Values are shown in Table 2, and the mean value was 47.876 ng/mL (S.D. 21.546). Although Case 2 was a smoker, the serum concentration was not low. The serum olanzapine concentration at the time of taking 20 mg/day in the patient who subsequently discontinued olanzapine due to over-sedation was extremely high (84.856 ng/mL).

4. Discussion

The number of patients allocated to each treatment group did not reach the required number of patients set by power analysis to examine whether olanzapine within 40 mg/day would be superior to risperidone within 12 mg/day in acute schizophrenia patients. Meanwhile, comparison between patients having required high doses and patients having responded to conventional doses revealed a difference in PANSS Negative scale score at baseline, i.e., the score in the former was significantly higher than that in the latter. It suggests that patients with severe negative symptoms do not respond to conventional-dose antipsychotics and require high doses in acute-phase schizophrenia. So far the association between negative symptoms and antipsychotic treatment-resistance has been pointed out (Kinon et al., 1993; Hatta et al., 2003). The association between negative symptoms and gray matter decrease has also been pointed out (Cahn et al., 2006). Severe negative symptoms stood on pharmacological and morphological abnormality, which makes treaters hard to emotionally communicate with such patients, might need additional doses of antipychotics for patients' behavior affected by severe positive and general psychopathology symptoms to be managed.

Although the rates of patients who achieved a ≥50% improvement in PANSS total score by 8 weeks in patients requiring high doses were low (25% for risperidone and 0% for olanzapine), more than half of such patients achieved moderate (≥30%) improvement in PANSS total score (63% for risperidone and 57% for olanzapine). Consequently, monotherapy could be continued in more than half of patients who did not respond to conventional doses. In addition, severe adverse events did not happen as the safety of high-dose olanzapine has been reported (Kinon et al., 2008; Mitchell et al., 2006). When monotherapy is valued more than polypharmacy, olanzapine dosing above the licensed range for non-responders to conventional doses may be acceptable as risperidone up to 12 mg/day is licensed.

Another question was whether patients who require high-dose olanzapine could be predicted by means of pharmacokinetics. In other words, this study examined whether serum olanzapine concentrations for patients who do not respond to conventional doses would be inappropriately low. Olanzapine has little active metabolites (Callaghan et al., 1999), and there is a high correlation between serum and cerebrospinal fluid olanzapine concentrations (Skogh et al., 2011). Therefore, serum olanzapine concentrations reflect most activity of olanzapine. Furthermore, a relationship between clinical outcomes and plasma concentrations has been strongly indicated, and a therapeutic range of 20–50 ng/mL has been found (Mauri et al., 2007). In the present results, serum olanzapine concentrations after 11–16 h from 20 mg/mL dosing to sample collection for patients who subsequently required high doses were above 30 ng/mL. As mean olanzapine plasma concentrations at 24 h after dosing were approximately 70% of those at 12 h after dosing, irrespective of ethnicity (Callaghan et al., 1999), trough plasma concentrations of the five cases that did not respond to 20 mg/day olanzapine must not have fallen below 20 ng/mL (Table 2). Thus, serum olanzapine concentrations for patients who subsequently required high doses were not low, suggesting that the reason for requiring high doses in such patients cannot be explained by pharmacokinetics. Roth (2008) mentioned the possibilities for the efficacy of high-dose olanzapine for treatment-resistant schizophrenia: pharmacodynamics, pharmacokinetics, and pharmacogenetics. So far, Kelly et al. (2006) reported that plasma levels of olanzapine given 50 mg/day were not associated with symptom response, and Citrome et al. (2009) reported no significant correlation between olanzapine concentration and either change in PANSS score or response to treatment. The present study has directly shown evidence that the reason for requiring high-dose olanzapine cannot be explained by pharmacokinetics. To our knowledge, this is the first finding of serum olanzapine concentrations at such timing for patients who did not respond to conventional doses and subsequently required high doses.

In contrast, some side effects might be partly explained by pharmacokinetics because the serum concentration of Case 5 during

Table 2
Characteristics and serum olanzapine concentrations at the time of oral 20 mg/day in patients who did not respond to conventional-dose olanzapine and subsequently required high doses.

	Case 1	Case 2	Case 3	Case 4	Case 5
Age (year)	58	42	28	50	53
Sex	Male	Male	Female	Female	Female
Smoking	Non	One pack of cigarettes/4 weeks	Non	Non	Non
Timing of sample collection after the increase in olanzapine to 20 mg/day (day)	1	11	1	8	1
Time from dosing to sample collection (hour)	16	12	16	11	16
Serum olanzapine concentration (ng/mL)	30.730	36.267	40.103	47.424	84.856
Estimated trough plasma concentrations (ng/mL)[a]	> 21.511	25.387	> 28.072	Slightly low value at 33.197	> 59.399
Discontinuation before 8 week period	No	No	Yes	No	Yes
The reason for discontinuation			NE		SE
The final improvement in PANSS (%)	42.4	31.0	32.3	31.4	24.6

NE, insufficient efficacy; SE, side effects; PANSS, Positive and Negative Syndrome Scale.

[a] Estimated trough plasma concentrations (ng/mL) were determined based on evidence that mean olanzapine plasma concentrations at 24 h after dosing were approximately 70% of those at 12 h after dosing, irrespective of ethnicity (Callaghan et al., 1999).

receiving 20 mg/day that subsequently discontinued olanzapine due to over-sedation was extremely high (84.856 ng/mL, Table 2). This suggests that the patient might have been a slow metabolizer, and that over-sedation might have been associated with the extremely high serum concentration. Similar finding has been observed about olanzapine concentrations and prolactin levels (Citrome et al., 2009).

One strength of this study was that all participants were psychiatric emergency cases requiring admission, mirroring real clinical practice. The absence of support from pharmaceutical companies was also characteristics of the study. One limitation was that sample size was small. Obtaining informed consent in emergency situations is often difficult. In the present study, especially, obtaining consent to use above licensed doses of olanzapine was extremely difficult. Accordingly, the rate of participation in the study among eligible patients was 5%. Second, the present finding may not be applicable to African American, because 89% of them are CYP3A43 genotype AA carriers, and 50% of AA carriers have predicted concentrations less than 20 ng/mL in the range of 15–20 mg/day (Bigos et al., 2011). Third, the study design was single-blinded. Both clinicians and patients may have had expectations about individual antipsychotics in terms of therapeutic potency in acute psychotic episodes, dosage requirements, side-effect profile, and likely need for p.r.n. medication. Such expectations could influence the dosage prescribed, decisions to prescribe p.r.n. medication, and decisions to discontinue the assigned drug. The present findings suggest that conventional doses are hard to take effects irrespective of levels of serum concentrations in Asian acute-phase schizophrenia patients whose negative symptoms clearly exist at the time of admission, and that more than half of such cases show moderate improvement resulted from subsequent treatment with high doses. More studies performed in real clinical practice with minimal bias are required to assist clinicians in making rational treatment decisions.

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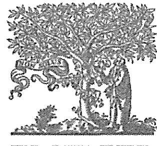

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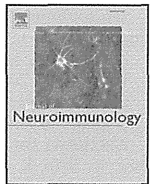

Myasthenia gravis: Predictive factors associated with the synchronized elevation of anti-acetylcholine receptor antibody titer in Kanazawa, Japan

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ABSTRACT

For a brief period, an increased incidence of elevated anti-acetylcholine receptor antibody titer was observed in patients with myasthenia gravis (MG) in Kanazawa, Japan. The purpose of this study was to examine the predictive factors associated with this antibody titer elevation. Decreased odds of titer elevation were seen in patients with early-onset MG than in those with late-onset MG. In patients with non-thymoma-related MG, thymectomy prevented the antibody titer elevation. Our data suggest that late-onset MG may have a different immunogenic response and the thymus might play an immunoregulatory role against extrinsic factors in some types of MG.
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1. Introduction

Myasthenia gravis (MG) is a common autoimmune disorder in which autoantibodies impair the neuromuscular junction, resulting in weakness and fatigability (Meriggioli and Sanders, 2009). In most autoimmune diseases, including MG, the precise mechanism of disease induction has not been clearly established, and it is possible that unknown environmental factors play an important role in these mechanisms (Bach, 2012). For example, homology between proteins from an infectious agent and self-antigens can give rise to molecular mimicry (Bach, 2005). In some cases, cross-reactions may occur between microbial antigens and self-antigens, and this could constitute an initial trigger for the disease (Deitiker et al., 2000; Im et al., 2002; Gammazza et al., 2012). Unfortunately, it is difficult to determine the exact factors responsible for initial triggers and/or exacerbations of MG because etiologic agents could be relatively common and infection may have occurred many years prior to the onset of clinical symptoms (Bach, 2012). Furthermore, MG is an immunologically heterogeneous disorder and disease onset could be due to a wide variety of immune responses (Aarli, 2008; Le Panse et al., 2008).

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In 2012, at Kanazawa University Hospital, a valuable opportunity to investigate the phenomena associated with MG was identified when an elevation of the anti-acetylcholine receptor (anti-AChR) antibody titer was observed in approximately 60% of outpatients with MG. This phenomenon was considered important because it facilitated investigation of factors associated with immune-exacerbation in MG (Somnier, 2005; Le Panse et al., 2008; Meyer and Levy, 2010). Therefore, the purpose of this case–control study was to determine the factors predictive of an elevation in the anti-AChR antibody titer in patients with MG and to determine if these factors indicate different pathogenic backgrounds in MG.

2. Material and methods

2.1. Patients

Diagnosis of seropositive MG was based on the presence of anti-AChR antibodies and one or more of the following criteria, included in the clinical evaluation: positive edrophonium test and decremental responses to repetitive nerve stimulation. In total, 56 patients with MG followed-up at Kanazawa University Hospital from January to August 2012 were included in this study. The inclusion criteria were satisfied by 54 patients, and all included patients had at least 9 months of clinical follow-up. Of the 54 patients with MG, 17 had thymoma and 37 had non-thymoma-related MG. All of the patients with thymoma and 20 with non-thymoma-related MG underwent thymectomy. Clinical data

from September 2010 to August 2012 was retrospectively evaluated. The mean duration of follow-up was 22.6 months (range, 9–24 months). Clinical data recorded for all patients included demographic data, age in August 2012, age at onset, diagnosis of thymoma and thymectomy, administration of prednisolone and immunomodulatory medication during January 2012. None of the patients with MG had received intravenous immunoglobulin or plasma exchange during the follow-up period. Ethics approval was provided by the ethics committee of the Kanazawa University School of Medicine, Kanazawa, Japan.

2.2. Evaluation of anti-AChR antibody titer

The term "outlier" is used to indicate an abnormal value and is applied to any value, within a distribution, that is located at an abnormal distance from the other values in the same distribution. In this study, outliers were identified when the Studentized residual was >2. Cook's distance can also be used to identify outliers. The conventional cut-off for Cook's distance is 4/n, but in this study, Cook's distance >1 was used to identify outliers. A significant elevation of the anti-AChR antibody titer was defined as described below. (1) A regression analysis of antibody titers prior to 2012 was performed and (2) a regression line was calculated. (3) The anti-AChR antibody titers measured in 2012 were defined as significantly elevated when the individual titer value could be considered an outlier. The chances for a titer value being an outlier were calculated using two different methods (Cook's distance and Studentized residual) and matched to the visualized elevation of diachronic change in antibody titers. These methods were selected because these could clearly classify the patients into the following two groups: Group-I showing no elevation of the anti-AChR antibody titer and Group-II showing elevated anti-AChR antibody titer. A graphic representation of each group based on outliers is shown in Fig. 1.

Because antibody titers for each patient varied between 0.3 and 450 nM, the anti-AChR antibody titers measured during the follow-up period were adjusted such that the minimum follow-up adjusted antibody titer was 0 and the maximum follow-up adjusted antibody titer was 1, by using the following formula: adjusted antibody titer = (raw antibody titer − minimum antibody titer) / (maximum antibody titer − minimum antibody titer).

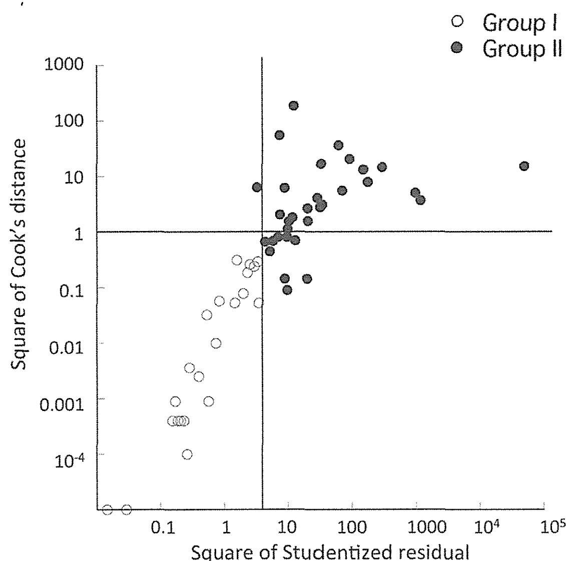

Fig. 1. Graphic representation of Groups I and II based on outliers. Significant elevation in the anti-AChR antibody titer was defined using outliers. Outliers, determined using two different methods, were used for defining Groups I and II. The square of Cook's distance and Studentized residual are shown. The cut-off value for the squared Cook's distance was 1 and for the squared Studentized residual was 4.

2.3. Statistical analysis

SPSS statistical software (version 19.0, IBM, Armonk, NY, USA) was used for statistical analyses. Age, age at onset, and follow-up interval are presented as mean ± standard deviation and were compared using the unpaired Student's t-test. The chi-square test (Fisher's exact test) was used to test for differences between Group-I and Group-II, with respect to thymoma, thymectomy, administration of prednisolone, clinical worsening, and proportion of patients whose age at onset was either <40 years or >40 years.

The adjusted antibody titers were averaged every 3 months, and the obtained data were analyzed using Friedman's two-way ANOVA by ranks because some of the data did not conclusively fit a normal distribution model. Significant peaks were considered for every 3-month comparison.

Odds ratios (ORs) and 95% confidence intervals (CIs) for elevation of antibody titers associated with age, age at onset, thymoma, thymectomy, and administration of prednisolone were estimated using unadjusted and adjusted logistic regressions. All probability values were two tailed and a p-value <0.05 was considered statistically significant.

3. Results

3.1. Elevation of anti-AChR antibody titer during the first 6 months of 2012

The diachronic changes in the anti-AChR antibody titers of four representative patients from each group are shown in Fig. 2. Four patients from Group-II had elevations of antibody titers during the same period of 2012 (Fig. 2A). In contrast, patients from Group-I did not have elevations of antibody titers during this period (Fig. 2B), and it was considered that technical troubles in the AChR antibody assay were excluded. A total of 32 (59.3%) out of the 54 patients with MG showed an elevation in anti-AChR antibody titers during the first 6 months of 2012. Ten out of the 54 patients with MG showed clinical worsening during this period, with symptoms including eyelid ptosis, diplopia, dysphagia, nasal voice, and increased generalized muscle fatigability. The adjusted antibody titers, averaged every 3 months, are also shown in Fig. 2C. In Group-II, the period that included March, April, and May 2012 was significantly different when compared to the other periods (Fig. 2D) ($p < 0.001$).

3.2. Demographics of patients with MG

Demographic data for patients with MG are presented in Table 1. There were significant differences between Group-I and Group-II with respect to age at study enrollment ($p = 0.007$), age at onset ($p = 0.002$), and clinical worsening ($p = 0.036$). No significant differences between the two groups were identified with respect to sex, period of follow-up, thymoma, thymectomy, prednisolone administration, and immunomodulatory medication.

3.3. Association of age and age at onset with anti-AChR antibody titer elevation

Table 2 indicates the unadjusted logistic regression analysis for the elevation of antibody titer with categories for age at study enrollment and age at onset and adjusted analysis for thymectomy and prednisolone administration. On unadjusted analysis, patients aged <40 years had a lower risk of antibody titer elevation compared to patients aged >60 years. However, although age at study enrollment was a predictive factor for antibody titer elevation, another factor, age at onset was a more important predictive factor. In the age category of 40 to 60 years, age at onset, rather than age at study enrollment, had a tendency to be associated with lower risk of antibody titer elevation.

Based on unadjusted logistic regression analysis for elevation of antibody titers with the categories of age at onset and the adjusted

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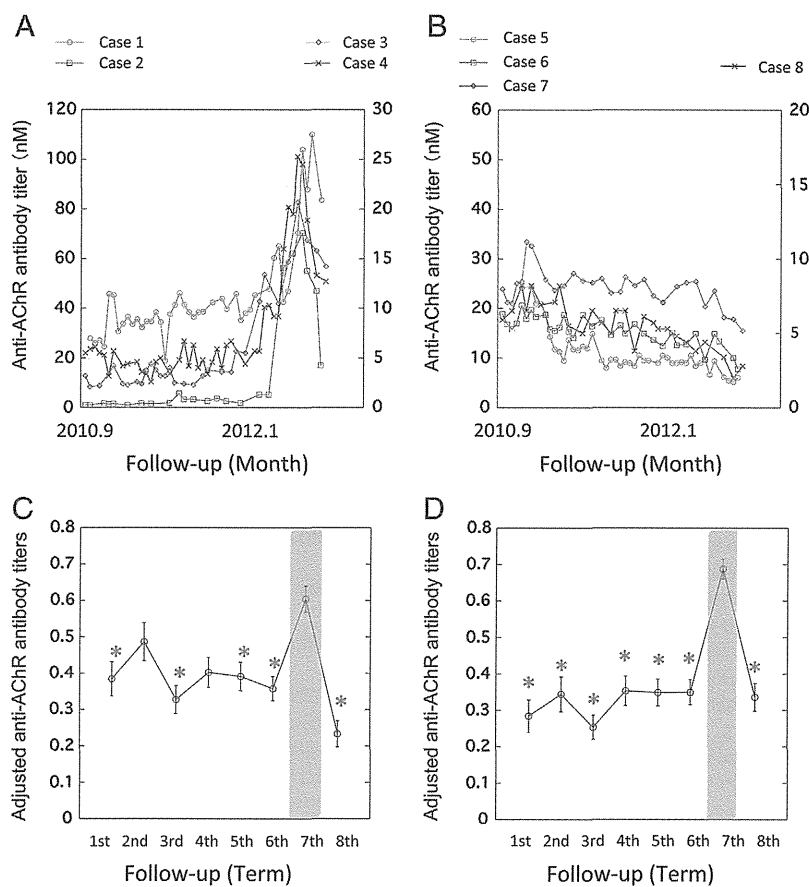

Fig. 2. Elevation of anti-AChR antibody titers. The diachronic changes in anti-AChR antibody titers for four representative patients in (A) Group-II (the elevated antibody group) and in (B) Group-I (the non-elevated group). C: The adjusted antibody titers averaged every 3 months in (C) all patients with MG and in (D) Group-II. A: The anti-AChR antibody titers for Cases 1 and 2 are shown on the left, and those for Cases 3 and 4 on the right. B: The anti-AChR antibody titers for Cases 5, 6, and 7 are shown on the left and those for Case 8 on the right. AChR = acetylcholine receptor. 1st = term from September to November 2010. 2nd = term from December 2010 to February 2011. 3rd = term from March to May 2011. 4th = term from June to August 2011. 5th = term from September to November 2011. 6th = term from December 2011 to February 2012. 7th = term from March to May 2012. 8th = term from June to August 2012. *p < 0.001, based on Friedman two-way ANOVA by ranks, compared to the 7th term.

analysis for thymectomy and prednisolone administration, patients with onset at age <40 years had an approximately 1/12 risk of antibody elevation compared to patients with disease onset at ages >60 years (adjusted analysis OR, 0.080; 95% CI, 0.008–0.810).

Patients were distributed into groups according to age at disease onset (Fig. 3). Of all patients with MG in this study, the proportion in Group-I decreased with advancing age (age <20 years, proportion 1.0; 21–40 years, 0.58; 41–60 years, 0.32; >60 years, 0.10). The majority of patients (63.6%) in Group-I had disease onset at age ≤40 years, compared to those with late-onset MG (age >40 years) ($p = 0.006$).

3.4. Predictive analysis of secondary outcome in patients with thymoma, thymectomy, and prednisolone administration

Although thymoma, thymectomy, and prednisolone administration were not associated with outcomes on unadjusted analysis, thymectomy

Table 1
Demographic characteristics of patients with myasthenia gravis.

Characteristic	Overall (n = 54)	Group-I[a] (n = 22)	Group-II[b] (n = 32)	*p*-Value
Sex (male/female, n)	21/33	8/14	13/19	0.784
Age at study enrollment (mean ± SD, y)	55.7 ± 17.3	48.2 ± 18.2	60.8 ± 14.8	0.007
Onset age (mean ± SD, y)	45.6 ± 17.8	36.7 ± 16.4	51.8 ± 16.3	0.002
Follow-up interval (mean ± SD, months)	120.5 ± 92.8	137.9 ± 81.8	108.6 ± 99.1	0.259
Thymoma (%)	17 (31.5%)	6 (27.3%)	11 (34.4%)	0.767
Thymectomy of patients (%)	37 (68.5%)	18 (81.8%)	19 (59.4%)	0.135
PSL administration (%)	28 (51.9%)	15 (68.2%)	13 (40.6%)	0.057
Immunomodulatory medication[c] (%)	24 (44.4%)	8 (36.4%)	16 (50.0%)	0.407
Clinical worsening (%)	10 (18.5%)	1 (4.5%)	9 (28.1%)	0.036

Abbreviations: PSL = prednisolone.
[a] Group-I: no elevation of anti-acetylcholine receptor antibody titer.
[b] Group-II: elevation of anti-acetylcholine receptor antibody titer.
[c] Included tacrolimus, cyclosporine, azathioprine.

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Table 2
Factors predictive of an elevation in the anti-AChR antibody titer in patients with myasthenia gravis.

Variables (n = 54)	Age groups	Adjusted OR (95% CI)[a]	*p*-Value	Unadjusted OR (95% CI)	*p*-Value
Age at study enrollment	<40 years	0.081 (0.010–0.628)	0.016	0.109 (0.018–0.652)	0.015
	41–60 years	0.996 (0.254–3.907)	0.995	0.875 (0.246–3.115)	0.837
	>60 years	1 (reference)	–	1 (reference)	–
Onset age	<40 years	0.080 (0.008–0.810)	0.032	0.063 (0.007–0.597)	0.016
	41–60 years	0.238 (0.023–2.438)	0.227	0.238 (0.025–2.264)	0.212
	>60 years	1 (reference)	–	1 (reference)	–

Abbreviations: OR = odds ratio; CI = confidence interval.
[a] Multivariate analysis adjusted for thymectomy and prednisolone administration.

and prednisolone administration showed an association with antibody elevation in the adjusted analysis (thymectomy: OR, 0.182; 95% CI, 0.040–0.832; prednisolone administration: OR, 0.271; 95% CI, 0.078–0.943; Table 3). In patients with non-thymoma-associated MG, thymectomy was related to outcome on the adjusted logistic regressions (OR, 0.189; 95% CI, 0.042–0.849). Finally, prednisolone treatment was related to outcome in the patients with MG post-thymectomy, including those with thymoma (OR, 0.167; 95% CI, 0.035–0.785; Table 3).

4. Discussion

This study presents the results based on a valuable opportunity to evaluate patients with MG from Kanazawa University Hospital in 2012. Kanazawa is the prefectural capital of Ishikawa Prefecture, is located in the central portion of the mainland of Japan, and has a population of 450,000. The southeast area of the city faces the mountains and the northwest area faces the Sea of Japan. In this series, 32 out of the 54 patients with MG had elevated anti-AChR antibody titers during this brief period. This increased incidence of titer elevation was thought-provoking, because it facilitated investigation of a potential environmental trigger and the immunological backgrounds of patients with MG.

Molecular mimicry that gives rise to autoimmune disorders, for example Guillain–Barre Syndrome, is well known (Bach, 2005). In MG, molecular mimicry between infectious antigens and epitopes located at the neuromuscular junction has been reported (Deitiker et al., 2000; Im et al., 2002; Gammazza et al., 2012). In addition, Epstein–Barr virus, cytomegalovirus, poliovirus, and mycoplasma are thought to be important triggers for the onset of MG (Deitiker et al., 2000; Cavalcante et al., 2010).

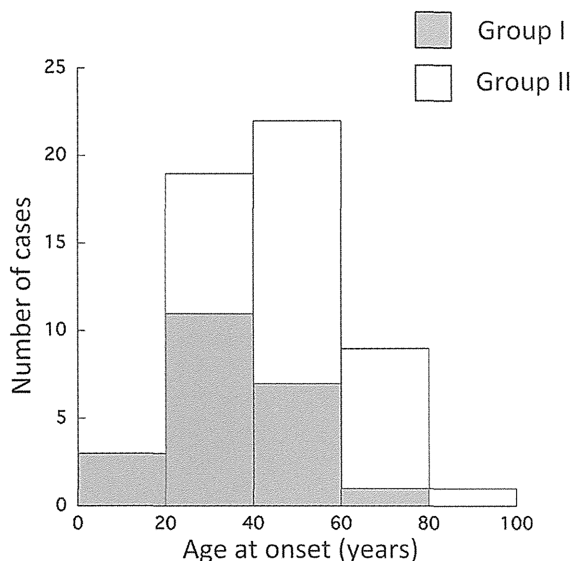

Fig. 3. Distribution of patients in each group according to age at disease onset. The peak numbers of patients in each group were different. The majority of patients in Group-I had disease onset at age <40 years, while the majority of patients in Group-II had disease onset at age >40 years.

The increased incidence of anti-AChR antibody titer elevation seen during this brief period and in a single local area suggested that some extrinsic or environmental factors might have influenced the loss of immunological tolerance in patients with MG. Although exposure to viruses, bacteria, and other infections may be an important extrinsic factor, it is not likely that these infections are extremely contagious diseases or transmitted by close contact. Mild contagious infections, e.g., respiratory infections that may have occurred in this area of Japan during a brief period, might be possible candidates. In post-infectious disease, the precise infection cannot be discerned from the medical history, and such diseases often remain resolved until the development of neurologic symptoms. Viral or bacterial cultures are usually negative, and serologic tests may lack sufficient sensitivity and specificity (Hughes and Rees, 1997). Future studies may allow detailed analysis of such incidences and may help determine the causative agents.

Furthermore, this phenomenon suggested that some patients with MG were susceptible to these factors. This study demonstrated the existence of two groups of patients with MG. One group (Group-I) with early-onset MG was less susceptible to the extrinsic and environmental factors that triggered anti-AChR antibody titer elevation, while the other group (Group-II) with late-onset MG was more susceptible to these factors.

Despite the potential for confounding caused by the presence of medication for MG, genetic predispositions, and aging of the immune systems, this study suggests that patients with late-onset MG might have an immunological susceptibility to extrinsic triggers. Similar to the results of the current study, Somnier proposed that seropositive, late-onset, non-thymoma-associated MG may be provoked by environmental factors (Somnier, 2005). Recently, the epidemiology of MG has

Table 3
Predictive analysis of secondary outcome in patients with thymoma, thymectomy, and prednisolone administration.

Variables	Adjusted OR (95% CI)	*p*-Value	Unadjusted OR (95% CI)	*p*-Value
All patients (n = 54)				
Thymectomy	0.182 (0.040–0.832)	0.028	0.325 (0.089–1.183)	0.088
PSL administration	0.271 (0.078–0.943)	0.040	0.319 (0.102–0.999)	0.050
Thymoma	0.290 (0.069–1.222)	0.092	0.716 (0.218–2.350)	0.582
Patients with non-thymoma-related myasthenia gravis (n = 37)				
Thymectomy	0.189 (0.042–0.849)	0.030	0.205 (0.049–0.861)	0.030
PSL administration	0.329 (0.076–1.427)	0.138	0.369 (0.097–1.412)	0.146
Patients with myasthenia gravis and thymectomy (n = 37)				
Thymoma	0.25 (0.053–1.173)	0.079	0.364 (0.095–1.386)	0.138
PSL administration	0.167 (0.035–0.785)	0.023	0.224 (0.056–0.901)	0.035

Abbreviation: PSL = prednisolone.

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shifted away from early-onset MG toward late-onset MG, thereby resulting in an increase in the number of older patients with MG (Casetta et al., 2010; Murai et al., 2011). It is possible that unknown environmental factors may have driven this change in the epidemiology of MG (Somnier, 2005; Casetta et al., 2010; Meyer and Levy, 2010). These factors might induce the elevation of the anti-AChR antibody titer and may result in overt symptoms of MG in older patients with previously subclinical MG (Vincent et al., 2003).

Thymectomy, prednisolone, and other immunomodulating medications are common treatments for MG. Thymectomy has primarily been proven useful for female patients with early-onset MG and hyperplasia of the thymus (Katzberg et al., 2002; Bachmann et al., 2009), but remains a treatment option for all patients with MG (Jaretzki et al., 2000). In non-thymoma patients in this series, thymectomy was related to the prevention of antibody elevation. It is suggested that the thymus may be an important organ for immunological activation by extrinsic or environmental factors (Le Panse et al., 2008; Cavalcante et al., 2012) and that thymectomy might be effective for the prevention of immunological activation in MG.

In this study, prednisolone administration was also useful for the prevention of antibody elevation. It is well known that prednisolone has an immunosuppressive effect in autoimmune disease and it is likely that prednisolone worked to suppress immunological activation by extrinsic factors in the patients in this study. Therefore, some patients with MG exposed to extrinsic or environmental factors that potentially influence the loss of tolerance in MG would likely benefit from the administration of low-dose prednisolone or immunomodulatory medication (Monsul et al., 2004).

In conclusion, for a brief period, an increased incidence of anti-AChR antibody titer elevation was observed in the Kanazawa area of Japan. With respect to immunological intolerance in MG, patients with late-onset MG had a higher incidence of anti-AChR antibody titer elevation than did patients with early-onset MG. In addition, thymectomy was preventative for the antibody titer elevation in patients with non-thymoma-related MG. Similarly, prednisolone treatment was useful for the inhibition of immune activation against autoantibody in patients with MG post-thymectomy. In this study, it was not possible to unravel the exact extrinsic triggers of antibody elevation because of the relatively short follow-up period. Therefore, additional research is needed to reveal the nature of putative environmental triggers of MG.

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Dual Involvement of Growth Arrest-Specific Gene 6 in the Early Phase of Human IgA Nephropathy

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Abstract

Background: Gas6 is a growth factor that causes proliferation of mesangial cells in the development of glomerulonephritis. Gas6 can bind to three kinds of receptors; Axl, Dtk, and Mer. However, their expression and functions are not entirely clear in the different glomerular cell types. Meanwhile, representative cell cycle regulatory protein p27 has been reported to be expressed in podocytes in normal glomeruli with decreased expression in proliferating glomeruli, which inversely correlated with mesangial proliferation in human IgA nephropathy (IgAN).

Methods: The aim of this study is to clarify Gas6 involvement in the progression of IgAN. Expression of Gas6/Axl/Dtk was examined in 31 biopsy proven IgAN cases. We compared the expression levels with histological severity or clinical data. Moreover, we investigated the expression of Gas6 and its receptors in cultured podocytes.

Results: In 28 of 31 cases, Gas6 was upregulated mainly in podocytes. In the other 3 cases, Gas6 expression was induced in endothelial and mesangial cells, which was similar to animal nephritis models. Among 28 podocyte type cases, the expression level of Gas6 correlated with the mesangial hypercellularity score of IgAN Oxford classification and urine protein excretion. It also inversely correlated with p27 expression in glomeruli. As for the receptors, Axl was mainly expressed in endothelial and mesangial cells, while Dtk was expressed in podocytes. In vitro, Dtk was expressed in cultured murine podocytes, and the expression of p27 was decreased by Gas6 stimulation.

Conclusions: Gas6 was uniquely upregulated in either endothelial/mesangial cells or podocytes in IgAN. The expression pattern can be used as a marker to classify IgAN. Gas6 has a possibility to be involved in not only mesangial proliferation via Axl, but also podocyte injury via Dtk in IgAN.

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Introduction

Gas6 is a growth factor which is post-translationally modified with γ- carboxylation of glutamate residues at its N terminus in the presence of vitamin K and inhibited by warfarin, an optional therapy for human kidney diseases. Gas6 was reported to be involved in the progression of glomerulonephritis and the development of diabetic nephropathy. Gas6 is expressed in the mesangial area in animal kidney disease models, such as rat anti-Thy-1 nephritis [1], anti-GBM nephritis [2] and streptozotocin induced diabetic rat and mouse model [3]. Gas6 induces proliferative and hypertrophic effects on mesangial cells, which lead to worsening of the kidney lesion. However, the expression of Gas6 in human kidney diseases is still unclear and yet to be fully examined.

Gas6 can bind to three kinds of receptors; Axl, Dtk (also called as Tyro3 or Sky), and Mer. Among them, Axl has the highest binding affinity with Gas6 and is expressed in endothelial and mesangial cells in animal kidney disease models [4,5]. On the other hand, Dtk has an intermediate affinity and is expressed mainly in nerves and brain [6,7]. However, a human glomerular SAGE transcriptome database revealed that Dtk does exist in glomeruli [8]. Previously, our group could not detect the

expression of Dtk in cultured mouse mesangial cells [5]. As yet, to our knowledge, no one has examined what kind of cell type expresses Dtk in glomeruli.

Human IgA nephropathy (IgAN) is considered to be the most common form of glomerulonephritis in the world. However, the pathologic manifestations of IgAN are broad and can range from mild mesangial hypercellularity to a rapidly progressive glomerulonephritis with fulminant crescents and endocapillary proliferation. The outcome of IgAN varies greatly [9]. Therefore, it is possible that IgAN is a "syndrome" which can be divided into several subgroups according to etiology, histopathology, or clinical manifestation.

In order to predict the risk of progression of renal disease in IgAN, nephrologists use clinical information such as extent of proteinuria, presence of hypertension, and excretory renal function, which are consistently reported as prognostic factors [10]. Pathologists proposed the Oxford classification for the pathological classification of IgAN to define pathologic variables with acceptable inter-observer reproducibility [11]. Four of these variables: mesangial hypercellularity, segmental sclerosis, endocapillary hypercellularity, tubular atrophy/interstitial fibrosis were shown to have independent value in predicting renal outcome [12]. On one hand, basic researchers reported various molecules which are related with the progression of IgAN. Representative cell cycle regulatory protein p27 is one of the candidates, expressed mainly in podocytes in rat and human normal glomeruli. Expression levels of p27 are decreased in association with mesangial proliferation in experimental mesangial proliferative glomerulonephritis (rat anti-Thy-1 nephritis model) and human IgAN [13,14].

In this study, we investigated whether expression of Gas6 and its receptors is involved in the progression of IgAN through analysis of the relationship between Gas6 expression and clinicopathological characteristics.

Materials and Methods

Ethics Statement

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki and patients' data were analyzed anonymously. All patients gave their informed, written consent. The study was approved by the Research Ethics Committee of Tokushima University or Kitasato University School of Medicine.

Biopsies and Tissues

Forty patients with IgA-dominant immune deposits mainly in the mesangial area diagnosed in Tokushima University Hospital from June, 2010 to September, 2011 were enrolled. Among them, four cases with Henoch-Schönlein purpura nephritis, one case with diabetic nephropathy, three cases with nephrotic syndrome and one case with advanced sclerosing glomerulonephritis were excluded. As a result, 31 human renal biopsies were analyzed for this study. No patients enrolled were treated with warfarin. Six controls consisted of three biopsies from patients with asymptomatic hematuria and three 1-hour biopsies from living donor kidney transplants. They showed minor glomerular abnormality and negative immunofluorescence. The latter three transplant kidney biopsies were collected in Kitasato University hospital.

Human control kidney tissues for isolation of glomeruli were obtained in Tokushima University hospital from the uninvolved portion of tumor nephrectomy specimens, disclosing normal morphology and negative immunofluorescence. Control patient 1 was a 76 year old female, patient 2 a 76 yo male, and patient 3 an 81 yo male. Renal tissues were collected from only patients in whom serum creatinine levels were within normal limits, and in whom diabetic mellitus and proteinuria were absent. All samples were provided from Japanese.

Demographic and Clinical Parameters

Demographics collected included age and gender at the time of biopsy. Clinical parameters collected included systolic and diastolic blood pressures, weight, height, serum creatinine, and 24-hour urine protein and urine protein-to-creatinine ratio within 1 month of the date of biopsy. GFR was estimated using the following new equation [15] applied for Japanese population: Estimated GFR (eGFR) (ml/min/1.73 m^2) = 194×Serum Creatinine $^{-1.094}$×Age $^{-0.287}$ (If female×0.739).

Pathological and Morphometric Analysis of Renal Biopsies

Biopsy adequacy was defined as a minimum of eight glomeruli available for examination by light microscopy. IgAN was confirmed as predominant IgA immunofluorescence in the mesangial area. Renal biopsy tissues were fixed in Dubosque-Brazil's solution and stained with periodic acid-Schiff stain (PAS). Three independent nephrologists independently scored every feature according to the full Oxford score sheet [11,12]. We derived an MEST score based on these results. Discordant scores were observed in a few cases and they were resolved by a meeting between the nephrologists. The mean values were applied to the analysis to see the association with the other variables.

Immunohistochemical Analysis

The immunohistochemical analysis was performed on paraffin-embedded section using indirect immunohistochemistry procedure with the following primary antibodies: rabbit polyclonal antibody against Gas6 (Sigma-Aldrich, St. Louis, MO, USA), goat polyclonal antibody against Axl (R&D Systems, Minneapolis, MN, USA), nephrin (Santa Cruz Biotechnology, Dallas, TX, USA), and mouse monoclonal antibody against CD34 (Dako, Carpinteria, CA, USA) and p27 (BD, Franklin Lakes, NJ, USA), respectively. Frozen sections were stained to detect Dtk using indirect immunofluorescence method with mouse monoclonal antibody against Dtk (R&D Systems). Following the first antibody, sections were incubated with TSA^{TM} Biotin System (PerkinElmer Life Sciences, Boston, MA, USA) and then with DAB (Wako Pure Chemical Industries, Osaka, Japan) or Texas Red Streptavidin (Vector Laboratories, Burlingame, CA, USA) for Gas6, Histofine SAB-PO(Goat) (Nichirei Biosciences, Tokyo, Japan) or biotin SP-conjugated donkey anti-goat antibody (Merck Millipore, Billerica, MA, USA) and then DAB or Texas Red Streptavidin for Axl, Alexa Fluor 488 or 594-conjugated donkey anti-goat antibody (Invitrogen, Grand Island, NY, USA) for nephrin, EnVision+ System- HRP Labelled Polymer (Dako) and then DAB for p27, Alexa Fluor 488-conjugated donkey anti-mouse antibody (Invitrogen) for CD34 and Dtk. The immunohistochemical signal of Gas6 was quantified using an image analyzer with a light microscope (Image Processor of Analytical Pathology; IPAP: Sumitomo Chemical Co., Tokyo, Japan) [3,16]. Gas6 stained area was expressed as a percentage of total glomerular area occupied by Gas6 immunostained area. As for p27 positive cell number, p27 was endogenously expressed mainly in podocytes, but some minimal expression was observed in mesangial cells in normal glomeruli [14]. Therefore, three nephrologists who did not know each sample characteristics blindly examined the number without counting apparent mesangial positive cells. The result was divided

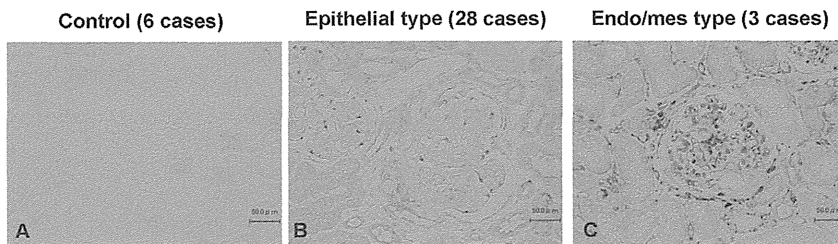

Figure 1. Gas6 expression in human IgA nephropathy. Biopsy samples were immunostained using indirect immunohistochemistry procedure with anti-Gas6 antibody. Representative images are shown in (A) from control patients, (B) from IgA nephropathy cases immunostained in epithelial cells, and (C) from IgA nephropathy cases immunostained in endothelial and mesangial cells. The original magnification was X200. Endo/mes, Endothelial and mesangial.
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by glomeruli number. The mean values of their data were shown. For each sample, at least eight glomerular profiles per patient were measured.

Immunohistochemistry with Antigen Absorbed Primary Antibodies

No less than 10 molar excess of recombinant human Gas6, Axl-Fc or Dtk-Fc (R&D Systems) were mixed with the primary antibody, respectively, and rotated for 8 hours at 4°C. After centrifugation of the primary antibody only or pre-absorbed antibody by antigen at 3000 rpm for 10 min, sections were incubated with the supernatants, respectively. Human Fc portion (Bethyl Laboratories, Montgomery, TX, USA) was used for confirming the antigen specificity.

Isolation of Glomeruli

The glomeruli isolated by sieving method were sonicated in cell extraction buffer (Mammalian cell extraction kit, Biovision Inc. Milpitas, CA, USA) and rotated for 1 hour at 4°C. After centrifugation of the samples, the supernatants were used as total lysates.

Cell Culture Experiment

Conditionally immortalized murine podocytes were provided by Dr. Peter Mundel (Massachusetts General Hospital) [17]. Podocytes were maintained as previously described [18]. Podocytes between passage 20 to 25 were differentiated and stimulated with recombinant murine Gas6 (R&D Systems), or human TGFβ1 (PeproTech, Inc., Rocky Hill, NJ, USA) for 24 hours. Harvested cell lysates were suspended in cell extraction buffer and rotated for 1 hour at 4°C. After centrifugation of the samples, the supernatants were used as total cell lysates.

Western Blotting

Lysates of glomeruli or harvested podocytes were applied to SDS-PAGE and immunoblotted with the primary antibodies indicated.

Urine Gas6 Concentration

Urine samples were centrifuged at 3000rpm for 5 min. Supernatants stored at −80°C were rapidly thawed and centrifuged at 15000rpm for 15 min to remove any urates or phosphates before use in assays. Three times diluted samples were then applied on the ELIZA plate (human Gas6 Duoset ELIZA Development kit, R&D Systems). Data shown were the values collected by urine creatinine concentration.

Statistical Analysis

All values are expressed as mean ± SD. Statistical analysis was performed using SPSS for Windows version 13.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using paired t tests or Man-Whitney test. Correlation was evaluated by Spearman's correlation coefficient by rank test. Significance was defined by P less than 0.05.

Results

IgAN was Divided into Two Subgroups According to Gas6 Expression Pattern

In order to examine the involvement of Gas6 in the progression of human IgAN, immunohistochemical analysis of Gas6 was performed in 6 controls and 31 IgAN patients enrolled. Then, unexpectedly, we found that Gas6 was expressed in podocytes (epithelial type) in 28 of 31 patients and in endothelial and mesangial cells (endo/mes type) in the other 3 cases (Figures 1, S1). We also confirmed the specificity of Gas6 staining by using antigen-absorbed primary antibody (Figure S2).

Gas6 Expression Correlated with Clinical and Pathological Risk Factors

Next, we hypothesized that Gas6 is involved in the progression of human IgAN. Therefore, we compared the expression levels with risk factors for IgAN. Demographic, clinical parameters and pathological findings of controls and IgAN patients were shown in Table 1. Because only a few IgAN biopsies were endo/mes type, we could not compare the characterisitics of endo/mes type patients with those of controls or epithelial type patients. For that reason, we focused on epithelial type biopsies. Then, Gas6 stained area in human IgAN was significantly bigger than that in controls (Figure 2). Moreover, it correlated with a pathological risk factor, Oxford mesansigal hypercellularity score, and a clinical risk factor, urine protein excretion (Figure 3). On the other hand, it did not correlate with sex, age, presence of hypertension, Oxford segmental glomerulosclerosis, endocapillary hypercellularity, or tubular atrophy/interstitial fibrosis scores.

Gas6 Expression Inversely Correlated with p27 Positive Cell Number

To investigate the mechanism of Gas6 involvement in podocytes, we looked for the molecule which is expressed in podocytes and correlated with disease progression. One of the cell cycle regulatory proteins, p27 was reported to be expressed mainly in podocytes in normal glomeruli with decreased expression in proliferating glomeruli, which inversely correlated with mesangial proliferation in human IgAN [14]. Therefore, at first, we

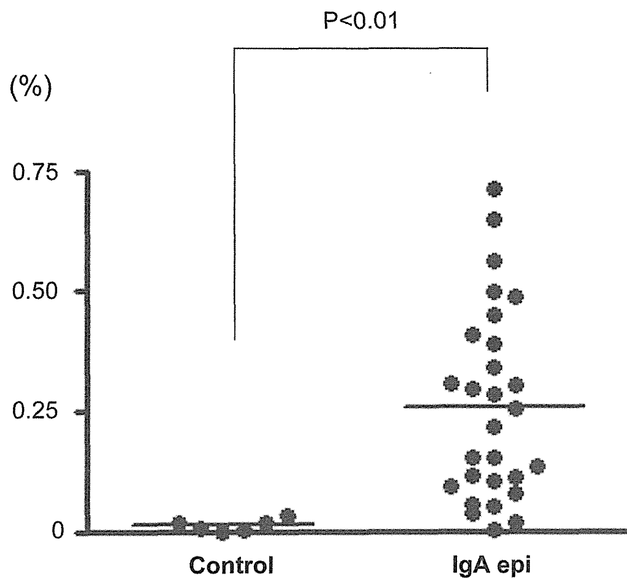

Figure 2. Gas6 stained area in human IgA nephropathy. Gas6 stained area is expressed as a percentage of total glomerular area occupied by Gas6 immunostained area. For each sample, at least eight glomerular profiles per patient were measured. IgA epi, IgA epithelial type.
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confirmed the relationship between p27 positive cell number and Oxford mesangial hypercellularity score (Figure 4A). Next, we found out that Gas6 stained area inversely correlated with p27 positive cell number (Figure 4B). This result suggests that Gas6 mediates progressive glomerular injury in human IgAN.

Expression of Gas6 Receptors in Glomeruli

To further understand the role of Gas6 in podocytes and endothelial/mesangial cells, we investigated whether Gas6 receptors are expressed in normal glomeruli. Expression of Dtk and Axl was detected in human normal glomeruli lysates (Figure 5). Immunohistochemistrical analysis revealed that Dtk and Axl were expressed in podocytes and endothelial/mesangial cells, respectively, in normal and IgA glomeruli (Figures 6A,C, S3). Moreover, in IgAN, Gas6 was colocalized with Dtk/Axl (Figure 6B,D).

Gas6 Downregulated p27 and Upregulated Dtk Expression in Podocytes in vitro

To understand the function of Gas6 in podocytes, we stimulated murine cultured podocytes expressing Dtk with Gas6. Gas6 could reduce the expression of p27, as well as TGFβ1, the representative cytokine involved in the progression of IgAN (Figure 7A,C). These results are compatible with in vivo findings shown in Figure 4 and suggest that Gas6 is involved in podocyte injury. In addition, Gas6 also could increase Dtk receptor (Figure 7A,B).

Discussion

Here we show the expression of Gas6 and its receptors in human IgAN. Gas6 was upregulated in either podocytes or endothelial/mesangial cells. In IgAN, Dtk was the receptor for Gas6 in podocytes, while Axl was in endothelial/mesangial cells. Gas6 expression in podocytes correlated with several prognostic factors, such as mesangial proliferation and urine protein excretion and was inversely associated with p27 expression. Gas6 was involved in human IgAN either via Dtk and Axl in podocytes and endothelial/mesangial cells, respectively.

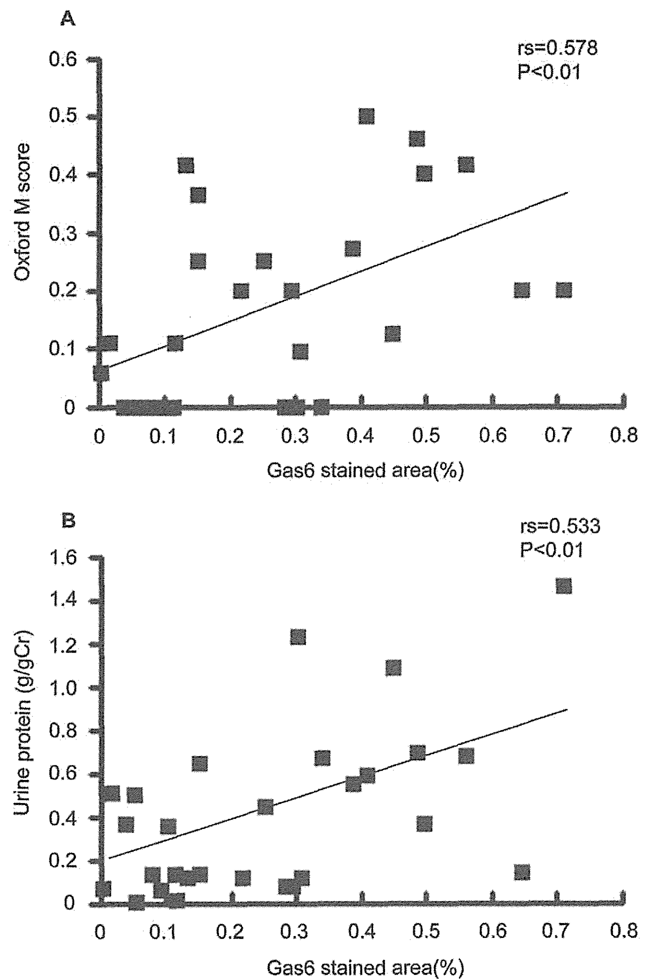

Figure 3. Correlation of Gas6 stained area with prognostic factors. Gas6 stained area in human IgA nephropathy correlated with (A) Oxford mesangial hypercellularity score and (B) urine protein excretion. N = 28. Oxford M score, Oxford mesangial hypercellularity score.
doi:10.1371/journal.pone.0066759.g003

Gas6 is a growth factor expressed in endothelial/mesangial cells with its receptor, Axl, which causes proliferation of mesangial cells in rat glomerulonephritis model [1]. However, in human IgAN, Gas6 upregulation was observed mainly in podocytes, while endothelial/mesangial dominant expression was seen in a few patients. The expression pattern can be a marker to classify IgAN, and we should re-evaluate the therapeutic effects and its prognosis according to the pattern.

In the cases with endo/mes type, according to a previous report [1], the role of Gas6 could be an exacerbation factor for glomerulonephritis through mesangial proliferation, and Axl was the receptor for Gas6. Though there is no statistic evidence, the pathology of endo/mes type tended to be more active than that of epithelial type because of high mesangial and endocapillary hypercellularity score (Table 1), which is consistent with the findings in rat anti-Thy-1 nephritis model which has endo/mes type Gas6 expression and shows an acute and fulminant mesangial proliferation. On the other hand, we have never determined the role of Gas6 in IgAN with epithelial type. As well as TGFβ1, Gas6 could be involved in podocyte injury by decreasing p27 expression,

Table 1. Demographic, clinical parameters and pathological findings.

	Control (n = 6)	IgA epi (n = 28)	IgA e/m (n = 3)
Age	47.8±16.4	36.6±15.1	29.7±6.4
Women	4(66.7%)	14(50.0%)	2(66.7%)
eGFR(ml/min/1.73 m^2)	–	82.2±26.3	93.2±8.8
Urine protein(g/gCr)	–	0.405±0.381	0.371±0.188
Urine protein(g/day)	–	0.467±0.460	0.439±0.068
Oxford classification			
M score	–	0.165±0.167	0.321±0.197
S score 1	–	16(57.1%)	2(66.7%)
E score 1	–	6(21.4%)	3(100.0%)
T score 1 or 2	–	9(32.1%)	1(33.3%)
Hypertension	0(0.0%)	10(35.7%)	0(0.0%)

IgA epi, IgA epithelial type. IgA e/m, IgA endothelial and mesangial type.
M score, Mesangial hyprecellularity score. S score, Segmantal glomeruloscrelosis score. E score, Endocapillary hypercellularity score.
T score, Tubular atrophy/interstitial fibrosis score.
doi:10.1371/journal.pone.0066759.t001

and Dtk was the receptor for Gas6. However, Gas6 was also reported to be a protective factor to prevent apoptosis in another cells [19,20]. Because warfarin therapy is sometimes applied to IgAN patients, the role of Gas6/Dtk in podocytes should be clarified in the further study by finding and analyzing an animal kidney disease model in which Gas6/Dtk pathway is involved.

Dtk is expressed in brain and neuron cells [6,7]. Dtk was also reported to be expressed in kidney abundantly [7], but no one has never determined the expression site in glomeruli. Unfortunately, we could not confirm Dtk receptor upregulation in IgAN patients, as well as the results in vitro (Figure 7A,B). Dtk immunostaining was only successful when we used frozen samples. Therefore, we could not quantify the Dtk staining intensity in enough number of glomeruli per patient. However, to our knowledge, this study is the first report to confirm the cell type in which Dtk is expressed and suggest the role of Dtk in kidney.

In previous reports, Mer mRNA is detected in peripheral blood mononuclear cells, bone marrow mononuclear cells and monocytes, but not in granulocytes. Despite the fact that Mer mRNA is expressed in neoplastic B and T cell lines, it is not detected in normal B or T lymphocytes. In normal human tissues, Mer mRNA is expressed at highest levels in ovary, prostate, lung and kidney [21,22]. We tried to detect Mer protein in glomeruli by using two commercially available antibodies against human Mer. In normal human glomeruli, we could not detect consistent positive bands by westernblotting analysis. In kidney biopsy samples from control and IgAN patients, we could not get positive immunostaining in frozen samples and paraffin-embedded sections (data not shown). Therefore, we have not known the significance of Mer receptor in kidney, but it is still possible that Mer receptor plays some roles in human kidney diseases.

So far, many biomarkers related to IgAN histology have been reported. Concerning podocyte injury, urinary podocalyxin correlated with acute extracapillary abnormalities. The number of urinary podocytes was associated with glomerulosclerosis [23]. With regard to tubular damage, urinary kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were markers for the detection of early tubular damage [24–26]. Relating to pathogensis and histolgical change of IgAN, the level

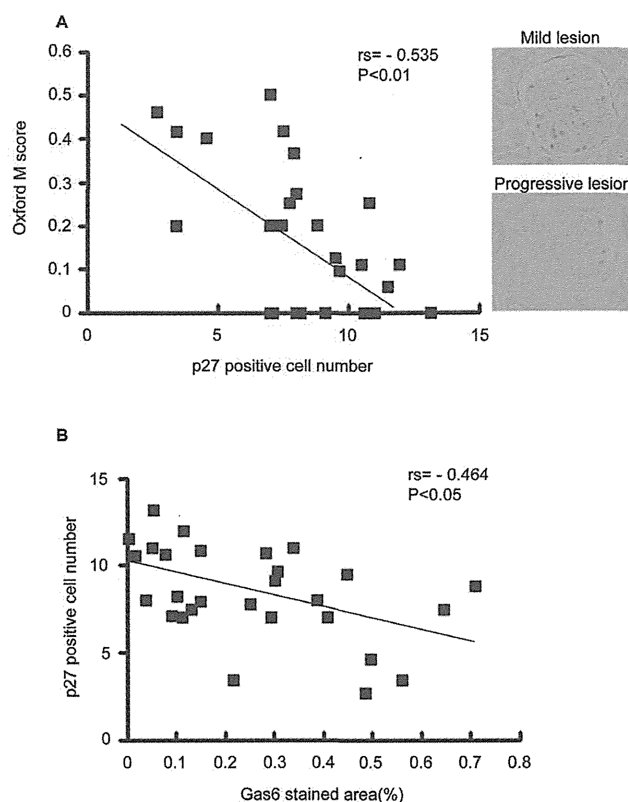

Figure 4. Inverse correlation of Gas6 stained area with p27 positive cell number. (A) Biopsy samples were immunostained using indirect immunohistochemistry procedure with anti-p27 antibody. p27 positive cell number was counted. The result was divided by glomeruli number. For each sample, at least eight glomerular profiles per patient were measured. p27 positive cell number correlated with Oxford mesangial hypercellularity score. (B) Gas6 stained area inversely correlated with p27 positive cell number. N = 28. Oxford M score, Oxford mesangial hypercellularity score.
doi:10.1371/journal.pone.0066759.g004

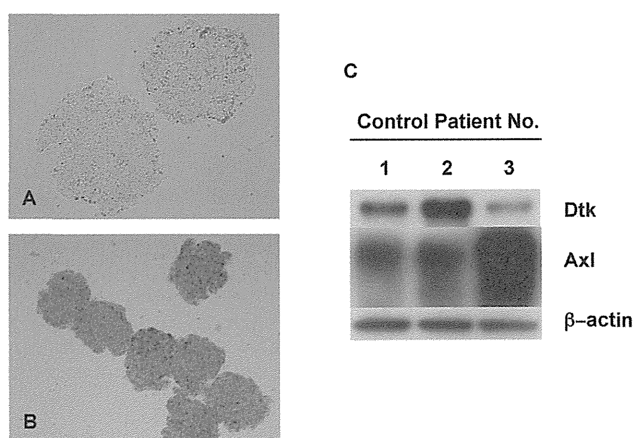

Figure 5. Expression of Dtk and Axl in control glomeruli. (A,B) Photographs of isolated glomeruli. The glomeruli were isolated from the uninvolved portion of tumor nephrectomy specimens by sieving method from three patients. The relative purity of isolated glomeruli was shown. The original magnification was (A) X100. (B) X50. (C) Ten µg of each glomerular lysate was analyzed by westernblotting with the antibodies indicated. Dtk and Axl were expressed in normal glomeruli.
doi:10.1371/journal.pone.0066759.g005

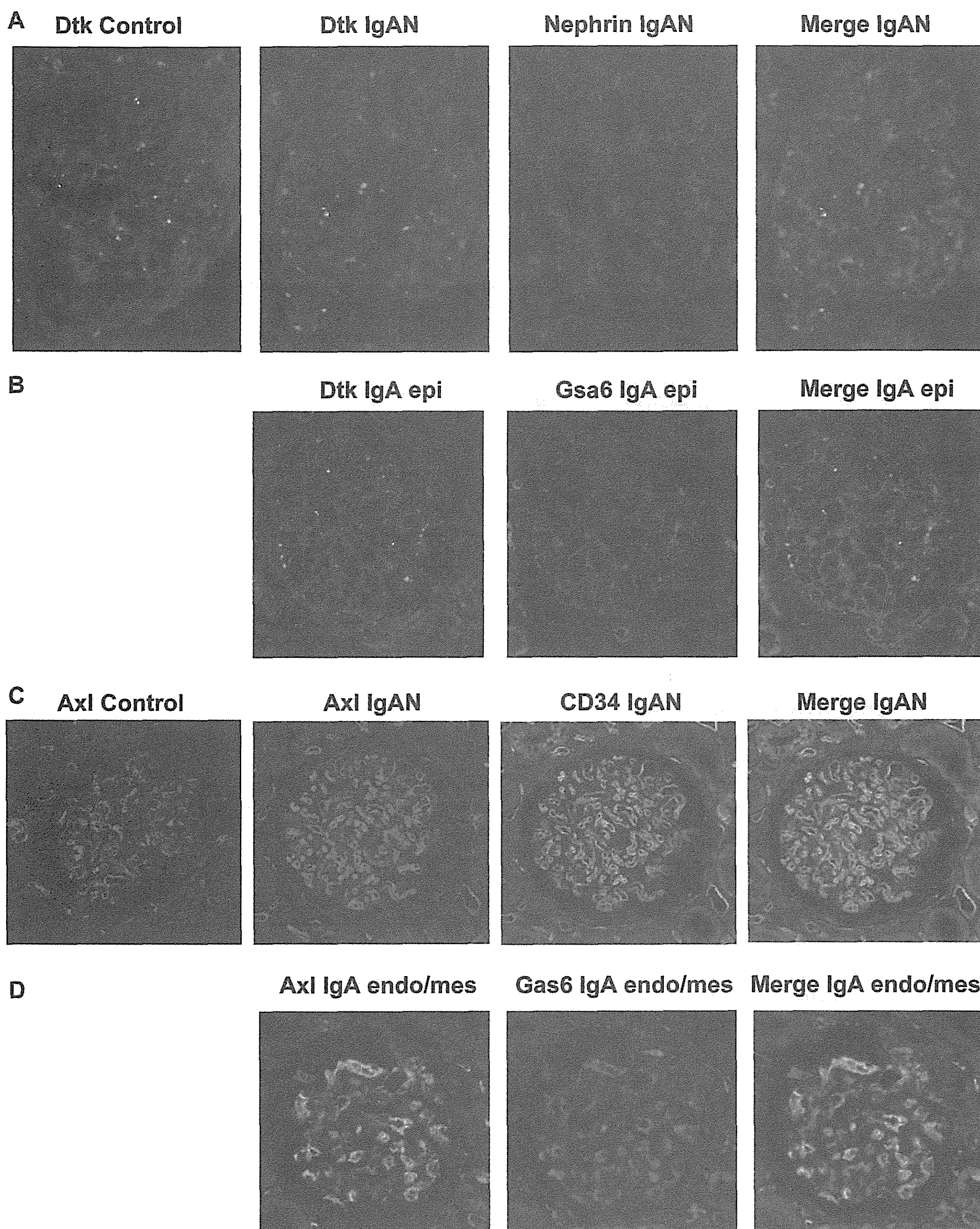

Figure 6. Double immunohistochemistry of Dtk and Axl with cell specific markers or Gas6. Biopsy samples were immunostained using indirect immunohistochemistry procedure with (A) anti-Dtk, anti-Nephrin (a podocyte marker), (B) anti-Dtk, anti-Gas6 or (C) anti-Axl, anti-CD34 (an endothelial cell marker), (D) anti-Axl, anti-Gas6 antibody. (A,B) Dtk immunostaining was detected in both control and IgA nephropathy, mostly merged with Nephrin. It was also merged with Gas6 in epithelial type IgA nephropathy. (C,D) Axl immunostaining was detected in both control and IgA nephropathy, mostly merged with CD34. It was also merged with Gas6 in endothelial and mesangial type IgA nephropathy. X200. IgAN, IgA nephropathy. IgA epi, IgA epithelial type. IgA endo/mes, IgA endothelial and mesangial type.
doi:10.1371/journal.pone.0066759.g006

of galactose-deficient IgA1 in the sera of patients with IgAN was associated with disease progression, but its relationship with histological severity was not analyzed [27]. The alternative complement pathway and the lectin pathway are involved in IgAN. Therefore, excretion of complement proteins, such as membrane attack complex, factor H, and mannose-binding lectin were associated with disease severity including acute and chronic lesions [28,29]. Extracellular matrix is increased with the progression of IgAN. Therefore, the gene expression data of several proteoglycans from the tubulointerstitial compartment correlated with tubular atrophy/interstitial fibrosis [30]. Compared with these biomarkers, Gas6 expression was related to mesangial proliferation, but not to segmental glomerulosclerosis, endocapillary hypercellularity, or tubular atrophy/interstitial fibrosis. We assume that it is because Gas6 can be upregulated in the development of IgAN even without active lesions. It can be

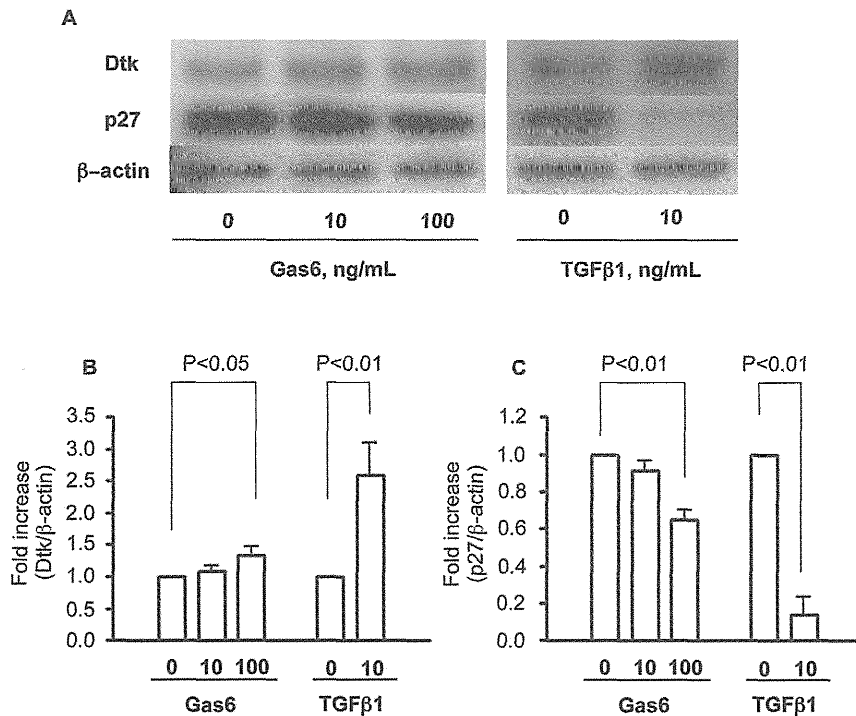

Figure 7. Effect of Gas6 on Dtk and p27 expression in podocytes. Podocytes were differentiated and stimulated with recombinant Gas6 or TGFβ1 for 24 hours. Cell lysates were subjected to immunoblotting with the antibodies indicated. Then, podocytes expressed Dtk. Gas6 could increase the expression of Dtk and reduce p27, as well as TGFβ1. Representative data were shown from four independent experiments. Quantitative examinations of the band density for (B) Dtk and (C) p27 were shown. The columns and error bars are the mean ± SD of four independent experiments.
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unique because most of the other proposed biomarkers were associated with acute or chronic histological injury. Therefore, urinary Gas6 concentration can be promising to detect the early phase of IgAN. We also investigated urine Gas6 concentration. Then, human IgA patients had higher Gas6 excretion than control (IgA epithelial type, IgA endo/mes type vs. Control. 1.01±1.06(N = 28), 2.15±1.95(N = 3) vs. 0.48±0.63(N = 32) μg/gCr, respectively). However, urine Gas6 concentrations of human IgA patients were not associated with prognostic markers such as the Oxford score or proteinuria (data not shown). We postulate that this is due to the relatively low sensitivity of commercially available ELIZA system. The establishment of highly sensitive Gas6 ELIZA system can be one of the promising methods to differentiate between nephritis and the other disease, such as bladder infection or urine stone.

Limitation of this study should be noted. First, this is a single-institution study. Second, most of the cases analyzed were in the early phase of IgAN with low proteinuria. It is partly because an established health check-up system in Japan. If we check advanced IgAN samples, more cases with endo/mes type might be found.

In summary, we could reveal the expression of Gas6 and its receptors, Axl and Dtk in IgAN. Surprisingly, in most patients, Gas6 was expressed in podocytes and Dtk was the receptor for Gas6. Human IgAN can be classified in two different types according to Gas6 expression pattern. We should review the prognosis of IgAN and effect of warfarin by dividing IgAN according to Gas6 expression pattern.

Supporting Information

Figure S1 Double immunohistochemistry of Gas6 with cell specific markers. Biopsy samples were immunostained using indirect immunohistochemistry procedure with (A) anti-Gas6, anti-Nephrin (a podocyte marker) or (B) anti-Gas6, anti-CD34 (an endothelial cell marker) antibody. (A) Gas6 immunostaining was observed outside Nephrin in epithelial type IgA nephropathy. (B) Gas6 immunostaining mostly merged with CD34 in endothelial and mesangial type IgA nephropathy. X200. IgA epi, IgA epithelial type. IgA endo/mes, IgA endothelial and mesangial type.
(TIFF)

Figure S2 Specificity of Gas6 immunohistochemistry. Biopsy samples were immunostained using indirect immunohistochemistry procedure with (A) normal rabbit IgG, (B) anti-Gas6 antibody, and (C) antigen pre-absorbed anti-Gas6 antibody. The staining disappeared by antigen absorption almost completely. X100.
(TIFF)

Figure S3 Specificity of Dtk and Axl immunohistochemistry. Biopsy samples were immunostained using indirect immunohistochemistry procedure with (A) anti-Dtk antibody (X200) and (B) anti-Axl antibody (X100). The staining disappeared by Fc portion conjugated receptor protein absorption almost completely, but not by Fc portion only.
(TIF)

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Author Contributions

Conceived and designed the experiments: KN HA TD. Performed the experiments: KN M. Miyoshi TT. Analyzed the data: KN. Contributed reagents/materials/analysis tools: KN TK NF M. Matsuura ES SY KY HK TF KY HI AM NA TA TM FK SK TM HA. Wrote the paper: KN.

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ORIGINAL ARTICLE

Polymorphism in the human matrix Gla protein gene is associated with the progression of vascular calcification in maintenance hemodialysis patients

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Abstract
Background Matrix Gla protein (MGP) is one of the important proteins inhibiting vascular calcification (VC). Single nucleotide polymorphisms (SNPs) located in the promoter and coding regions of the *MGP* gene affect the transcriptional activity. In this study, we investigated the relationship between the SNPs and progression of VC in patients undergoing maintenance hemodialysis (MHD).
Methods This was a retrospective, longitudinal cohort study of 134 MHD patients whose VC could be followed by multi-detector computed tomography (MDCT) examinations. MGP-SNPs (T-138C, rs1800802 and G-7A, rs1800801) were determined. The progression speed of VC was examined by plotting the abdominal aortic calcium volume scores.
Results The progression speed of VC of patients with the CC genotype of T-138C was significantly slower than that of patients with the CT or TT genotype. Multiple regression analysis showed that CT/TT genotype, greater age at the beginning of MHD, male sex, high levels of calcium × phosphate, low levels of high-density lipoprotein cholesterol, high levels of low-density lipoprotein cholesterol, low levels of ferritin and non-use of angiotensin II receptor blockers were significantly associated with progression of VC.
Conclusions The MGP-138CC genotype may be associated with slower progression of VC in MHD patients. The genotype of the *MGP* gene will be a genomic biomarker that is predictive of VC progression.

Keywords Matrix Gla protein (MGP) · Abdominal aortic calcium volume score (AACVS) · Single nucleotide polymorphisms (SNPs)

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Introduction

Vascular calcification (VC) is a common finding in patients undergoing maintenance hemodialysis (MHD). MHD patients have a 60–80 % prevalence of moderate to severe VC [1–3]. The VC often progresses over a relatively short period of time and is a strong predictor of cardiovascular disease and all-cause mortality in MHD patients [4–6]. Abdominal aortic calcification (AAC) is reported to be a predictor for cardiovascular mortality in the general population, and was also associated with increased risk of congestive heart failure in the Framingham Study. The association between AAC and all-cause and cardiovascular mortality in MHD patients has been shown in several reports. However, the factors contributing to AAC in MHD patients are still not fully understood.

Genetic and biochemical studies have established matrix Gla protein (MGP) as the first protein known to act as a calcification inhibitor in vivo. MGP is a vitamin K-dependent protein of 84 amino acids with a molecular weight of 12 kDa [7, 8]. Although MGP knockout mice are normal at birth, they rapidly develop severe arterial calcifications and subsequent vascular ruptures leading to death within 6–8 weeks [9]. Among three types of arteriosclerosis (i.e., atherosclerosis, Mönkeberg medial calcific

sclerosis, and arteriolosclerosis), arterial medial calcification is the major cause of vascular disease and is rapidly progressive in dialysis patients [2]. Therefore, MGP would be a critical factor in the development of arteriosclerosis in patients with MHD. A few previous reports have investigated serum MGP levels in hemodialysis patients, but the relationship between the serum MGP concentration and VC is controversial [10, 11].

It has been reported that the gene encoding MGP has several single nucleotide polymorphisms (SNPs) in its promoter and coding regions. Many studies have revealed the significance of *MGP* gene polymorphisms at T-138C and G-7A [12–14]. A previous study showed that MHD patients have a different distribution of *MGP* gene polymorphisms as compared with the normal population [14]. However, the influence of MGP polymorphism with respect to the development of AAC in MHD patients is not fully understood [12, 15]. It is a fact that there are no reports which examine the association between MGP polymorphism and AAC. With regard to 'femoral artery' calcification, Herrman et al. [12] reported that it was more prevalent in carriers of the MGP A-7 allele than in MGP GG-7 homozygotes and that T-138C were unrelated to femoral artery calcification in healthy volunteers. In addition, Crosier et al. [15] reported that in males, homozygous carriers of the minor allele of T-138C, G-7A and Ala102Thr were associated with a decreased quantity of 'coronary artery calcification (CAC)', relative to major allele carriers.

To date, the exact mechanisms for accelerated VC have yet to be fully determined. In particular, it is conceivable that the speed of progression of AAC in hemodialysis patients varies widely from patient to patient. Therefore, we examined whether MGP-SNPs affect the progression speed of AAC in MHD patients.

Materials and methods

Study design, setting and participants

This is a retrospective, longitudinal cohort study of MHD patients. As a setting, patients with end-stage kidney disease (ESKD) who started hemodialysis therapy after 2001 at Kawashima Hospital were recruited between August 2009 and November 2010. All of the procedures were performed in accordance with the guidelines of the Helsinki Declaration on Human Experimentation and the Ethical Guidelines on Clinical Research published by the Japanese Health, Labour and Welfare Ministry. This study was approved by the Ethics Committee of Tokushima University and Kawashima Hospital, and written informed consent was obtained from all patients.

The exclusion criteria were (1) past operation for abdominal aortic aneurysms and (2) renal transplantation.

Finally 145 participants were recruited and provided samples which we assayed for two SNPs in the *MGP* gene promoter region—T-138C (rs1800802) and G-7A (rs1800801). Routine abdominal computed tomography (CT) examination is performed once a year in each patient, and we used these data. We enrolled 134 of the 145 patients whose VC could be followed in consecutive multidetector CT (MDCT) examinations; 11 patients were excluded from additional analysis because they underwent MDCT examination once or not at all.

Identification of *MGP* gene genotypes

We selected two common SNPs on the *MGP* gene promoter—T-138C (rs1800802) and G-7A (rs1800801). Whole blood samples were obtained via vascular access at the start of routine hemodialysis treatment, and were used for the extraction of genomic DNA with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). First, T-138C (rs1800802) polymorphism was genotyped using a mismatch polymerase chain reaction (PCR) fragment amplified with the primers for 142 bp region as a pilot study—5′-AAGCATACGATGGCCAAAACTTCTGCA-3′ and 5′-GAACTAGCATTGGAACTTTTCCCAACC-3′ [13]. These PCR products were purified with DNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA, USA) and were digested with the restriction enzyme *Bsr*I, and analyzed in polyacrylamide gel (Fig. 1).

The following primers were designed for a 408 bp region that included T-138C (rs1800802) and G-7A (rs1800801)—5′-TCTGTCCCCAAGCATACGAT-3′ and 5′-ACACAGAGAAATGGGAGAAAAG-3′. These primers were verified by sequencing and PCR was carried out. Purified PCR products were subjected to direct sequencing by using 3730xl DNA Analyzer (Applied Biosystems).

Serum MGP assay

Serum MGP concentrations were quantified with a kit from Biomedica (Vienna, Austria) as described previously [16].

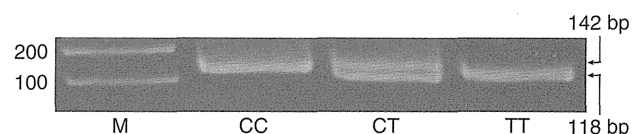

Fig. 1 Genotyping of the T-138C polymorphism using mismatch PCR followed by digestion with the restriction enzyme *Bsr*I. The presence of a T nucleotide at position −138 produced a *Bsr*I restriction endonuclease site giving fragments of 118 and 24 bp. The presence of a C nucleotide at position −138 did not produce a restriction endonuclease site for *Bsr*I. M: 100 bp DNA Ladder

Detection and measurement of VC

In order to evaluate the VC of each patient, we gathered data from the past abdominal CT examinations of each patient and calculated the abdominal aortic calcium volume score (AACVS). The plain abdominal MDCT imaging was performed using an 8-slice Aquarion scanner (Toshiba, Japan). The images from the bifurcation at the beginning of the common iliac artery to the 70-mm cranial interval were transferred to a workstation. Quantification of aortic calcification was carried out with ZIO Workstation software (ZIO, Japan). VC was defined as >130 Hounsfield units of CT value on this workstation, and counted as pixel data. The AACVS was defined according to the following formula—(pixel) × (pixel) × (slice thickness) × (quantity of voxel) [mm^3]. In this formula, (1 pixel) × (1 pixel) × (slice thickness) expresses (1 voxel). The volumetric scoring method named the calcium volume score was referred to in previous articles [17–19].

Statistical methods

We considered two-tailed *p* values <0.05 as statistically significant. All of the statistical analyses were performed using JMP 9.02 (SAS Institute, Cary, NC, USA). Statistical analysis of continuous variables was performed with Kruskal–Wallis analysis because assumptions of normality of the distribution were not verified. Post hoc multiple comparisons were made using the Steel–Dwass method. In addition, statistical analysis of nominal variables was performed with the chi-squared test.

Results

This study was carried out to examine the effects of *MGP* gene promoter polymorphisms (T-138C and G-7A) on the progression of VC in patients undergoing MHD. The T-138C and G-7A polymorphisms are located in the promoter region of the *MGP* gene (Fig. 2). Sequencing results of these polymorphisms are also shown in Fig. 2. The distribution of the T-138C genotype in this study was TT (35.1 %, $n = 47$), CT (52.2 %, $n = 70$) and CC (12.7 %, $n = 17$) (Fig. 3a). Similarly, the frequency of the G-7A genotype was GG (85.1 %, $n = 114$), GA (12.7 %, $n = 17$) and AA (2.2 %, $n = 3$) (Fig. 3b). We then compared the T-138C allele frequency of this study with that from the database of the genome-wide association study (GWAS); a chi-squared test showed no significant differences between them ($p = 0.73$, data not shown). In contrast, we could get no information on the G-7A allele frequency in GWAS. For that reason, we decided to place the primary focus on the analyses of the T-138C genotype.

Clinical characteristics of all patients of each genotype of T-138C are presented in Table 1. We found that the CC genotype was associated with significantly higher concentrations of high-density lipoprotein (HDL) cholesterol ($p = 0.03$).

Figure 4a shows the progression of the AACVS throughout the study (mean $R^2 = 0.87$), and Fig. 4b, c and d show the scores for the CC ($n = 17$), CT ($n = 70$) and TT ($n = 47$) genotypes, respectively. The dashed line shows the mean scores for all patients in each genotype group.

In order to investigate the effect of the T-138C genotype on the serum MGP concentration, we analyzed the MGP concentrations in the sera of MHD patients. There were no

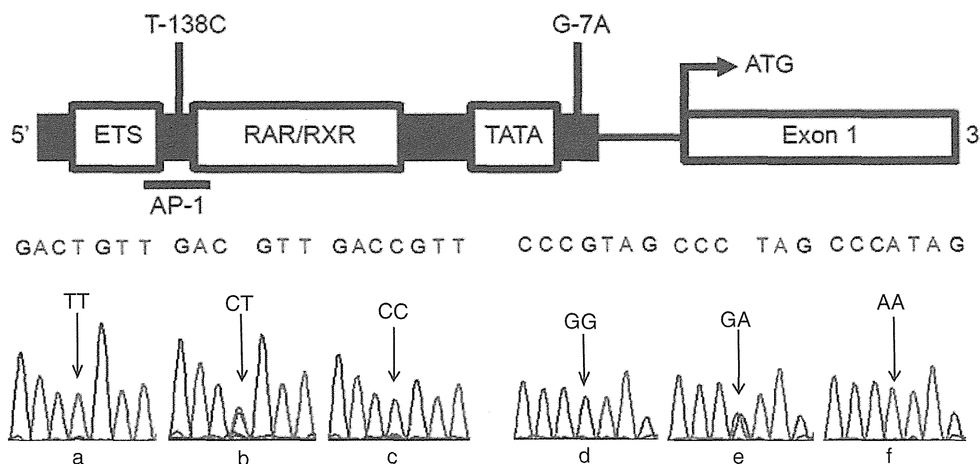

Fig. 2 DNA sequences of the polymorphic region in the MGP (T-138C, G-7A). **a** DNA sequence from individual homozygous for the TT genotype of T-138C. **b** heterozygous for the CT genotype of T-138C. **c** Homozygous for the CC genotype of T-138C. **d** DNA sequence from individual homozygous for the GG genotype of G-7A. **e** heterozygous for the GA genotype of G-7A. **f** homozygous for the AA genotype of G-7A. *ETS* Ets transcription factor family, *AP-1* activating protein-1, *RAR/RXR* retinoid A and X receptor, *TATA* TATA box

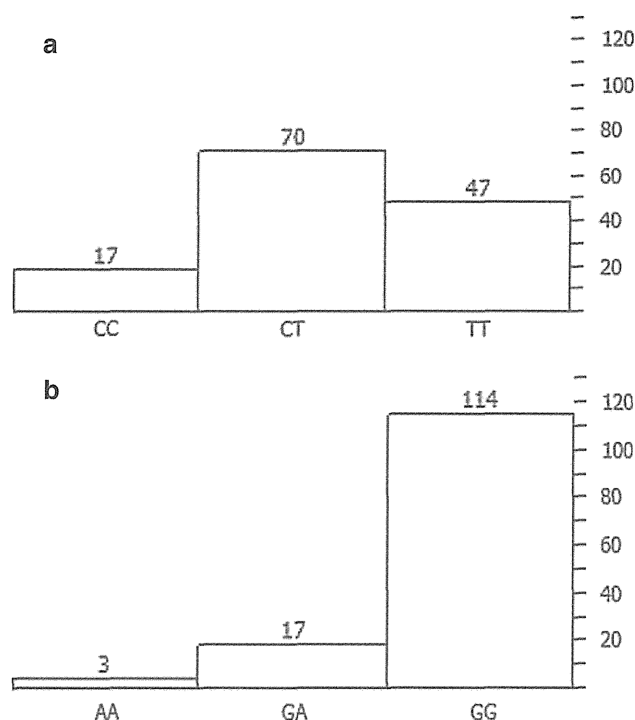

Fig. 3 The distribution of T-138C and G-7A genotype. **a** The distribution of the T-138C genotype ($n = 134$). **b** The distribution of the G-7A genotype ($n = 134$)

significant differences in the serum MGP concentration among the genotypes [CC: 22.57 (21.41, 28.43), CT: 25.10 (21.23, 26.87), TT: 25.01 (23.07, 26.45); unit: nmol/L, $p = 0.72$].

We compared the result of the slope value of the absolute AACVS plots as a linear function among the T-138C genotypes (Fig. 5a). The slope value for the CC genotype [53.00 (12.11, 254.90)] was significantly smaller than that for the CT genotype [319.85 (110.70, 647.80)] and TT genotype [261.00 (85.50, 626.56)] ($p = 0.003$, 0.03). Figure 5b shows the results of the comparison of the *y*-intercepts among the T-138C genotypes; there were no significant differences among them ($p = 0.52$). It is generally believed that the progression of VC at the beginning of MHD would contribute to the acceleration of VC and long-term survival of MHD patients [20]. Interestingly, however, our results indicate that the CC genotype of T-138C significantly contributes to the slowing of VC progression, regardless of differences in the VC volume at the beginning of MHD.

Multiple regression analysis by the best subset regression method between the progression speed of AACVS and related parameters revealed that CT/TT genotypes, greater age at the beginning of MHD, male sex, high levels of calcium × phosphate (Ca × P), low levels of HDL cholesterol, high levels of low-density lipoprotein (LDL) cholesterol, low levels of ferritin and non-use of angiotensin receptor blockers (ARBs) contributed to the progression of VC (Table 2).

Discussion

Although AAC is reported as a predictor for cardiovascular mortality in the general population, it is unknown whether this is also true in MHD patients. In addition, although many studies have focused on coronary calcification, there have been very few studies assessing the progression of AAC in MHD patients. A system for quantification of calcification was described by Kauppila et al. [21] in a subgroup of participants of the Framingham heart study. It relies on lateral lumbar radiographs and the calculation of the AAC score. Its predictive value for cardiovascular events and mortality was validated in the Framingham heart study [22, 23]. Recently, the AAC score was shown to correlate well with electron beam CT scores of the coronary arteries in MHD patients [24]. AAC may also be associated with all-cause and cardiovascular mortality in ESKD [25]. More recently, VC scores determined by MDCT were shown to be useful for evaluating the volume of VC [18]. For that reason, we used MDCT examinations for evaluation of the progression of VC in MHD patients.

The progression speed of VC differed among the MHD patients, and we hypothesized that MGP polymorphisms had some effect on this variation. Our study proved that MHD patients with the MGP T-138C CC genotype exhibited slower progression of VC than those with other genotypes. To our knowledge, this is the first study to reveal that the MGP T-138C polymorphism is closely linked to differences in the progression speed of VC among MHD patients.

MGP T-138C polymorphisms lie in the promoter region of the *MGP* gene, which is critical for the transcriptional activity. Farzaneh-Far et al. [13] previously showed that the −138C variant provides higher levels of MGP transcriptional activity in vascular smooth muscle cells. Therefore, our clinical data imply that the −138C allele increases MGP promoter activity in the arterial vessel and works more protectively against the progression of VC in MHD patients with the CC genotype. Furthermore, a previous study demonstrated that the −138C variant is associated with higher serum MGP levels (+30 %) [13]. On the other hand, the serum MGP level was not correlated with T-138C polymorphisms in another study [26]. Our results in this study also showed no relation between the MGP polymorphisms and serum MGP levels. Several reports have demonstrated that MGP expression was increased in atherosclerotic arteries [27, 28]. From in situ hybridization, it was shown that MGP mRNA transcription takes place in the arterial vessel wall, and is particularly upregulated in atherosclerotic arteries [27]. Thus, local MGP upregulation