

shows the sensitivity, specificity, positive likelihood ratio, and Youden index for ROC analysis at various cutoff scores for the mFS-J. According to the value of the Youden index, the best cutoff score for the mFS-J was 9/10.

Discussion

This is the first study to validate the mACR 2010-J and mFS-J, which is the quantification scale of the mACR 2010-J. The positive likelihood ratio of 16.3 for the mACR 2010-J is sufficiently high as a positive test. We cannot directly compare the likelihood ratios for the mACR 2010-J and the ACR 2010-J, as the present study group is quite different from that used in the study of the ACR 2010-J [4]. However, the value of mACR 2010-J is sufficiently high compared with that of ACR 2010-J, for which the positive likelihood ratio was 8.8. Therefore, the modification of ACR 2010-J may be superior to the original ACR 2010-J as a positive test.

The best cutoff score for the mFS-J was 10, which is just the same as the FS-J [4]. Furthermore, the positive likelihood ratio for the mFS-J (Table 2) is as high as that for the FS-J at the cutoff score [4]. As the mFS-J is simpler than the FS-J, mFS-J may be superior to FS-J based on the original ACR 2010-J as a quantification scale. Meanwhile, the best cutoff score of 10 in the present study is smaller than that of the original study on the FS performed in the USA (cutoff score 13) [5]. One explanation for this difference is in patient characteristics. In the present study, comorbid psychiatric disorders were excluded, while in the previous study they were not. Patients with major depressive disorder, panic disorder, or anxiety disorder usually have somatic symptoms similar to those of ACR 2010, and comorbidity of major depressive disorder, panic disorder, or anxiety disorder is not rare [1]. Therefore, the population in the previous study may have been modified by comorbid psychiatric disorders. Thus, the cutoff score of 10 in the present study might reflect fibromyalgia itself more than that of 13 in the previous study. Another possible explanation is cross-cultural differences in expression or rating of symptoms.

The internal consistency with a Cronbach's α coefficient for the mFS-J (WPI + the modified SS) of 0.603 is lower than that for the FS-J (WPI + the original SS) of 0.747 [4]. The modified SS consists of fatigue, waking unrefreshed, cognitive symptoms, plus having pain/cramps in the abdomen, depression, and headache, resulting from 38 somatic symptoms in general having been deleted from the original SS. Therefore, the modified SS values neuropsychiatric symptoms more than the original SS. In contrast, WPI is the number of pain areas, which is simply somatic. Accordingly, the internal consistency for the mFS-J (WPI + the modified SS) might have been lower than that

for the FS-J (WPI + the original SS). As FM shows a variety of symptoms and is suspected of involving not only musculoskeletal but also central nervous system [14], the not so high value of internal consistency for the mFS-J may not necessarily be a shortcoming.

Thus, mACR 2010-J as a positive test and mFS-J as a quantification scale might be suitable for assessing fibromyalgia among Japanese chronic pain populations. A strength of the present study is that the findings represent real clinical practice in Japan, since the study was performed in a clinic specialized in FM which is visited by the largest number of FM patients in Japan. A limitation of this study is that the findings may not be applicable to all patients, since FM patients with other musculoskeletal diseases, such as spondylitis, were not included in it. Further studies with patients of other countries or ethnicities will be needed to determine cross-cultural or ethnic differences in expression or rating of symptoms.

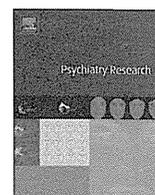
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Conflict of interest None.

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The possibility that requiring high-dose olanzapine cannot be explained by pharmacokinetics in the treatment of acute-phase schizophrenia



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ABSTRACT

We examined clinical characteristics including serum olanzapine concentrations for acute schizophrenia patients who required above conventional doses. We performed a rater-blinded, randomized clinical trial in 12 psychiatric emergency sites. Eligible patients were 18–64 years old and met diagnostic criteria for schizophrenia, acute schizophrenia-like psychotic disorder, or schizoaffective disorder. A total of 42 patients were randomly assigned by means of sealed envelopes to receive risperidone (3–12 mg/day; $n=20$) and olanzapine (10–40 mg/day; $n=22$), with follow-up at 8 weeks. The Negative score of the Positive and Negative Syndrome Scale was significantly higher in patients who required high doses than in patients who responded to conventional doses. Serum olanzapine concentrations at the time of oral 20 mg/day could be obtained from 5 out of 7 patients who subsequently required high-dose olanzapine. All values were more than 30 ng/mL after 11–16 h from dosing to sample collection, and the mean value was 47.876 (S.D. 21.546) ng/mL. Such concentrations are appropriate with respect to a therapeutic range of 20–50 ng/mL. The present study has shown evidence that the reason for requiring high-dose olanzapine cannot be explained by pharmacokinetics in the treatment of acute-phase schizophrenia.

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1. Introduction

Olanzapine and risperidone are most frequently used among second generation antipsychotics for the acute treatment of psychosis

in hospitalized patients (Choi et al., 2011), as the superiority of them has been reported in several randomized clinical trials (RCTs) (Kraus et al., 2005; McCue et al., 2006; Hatta et al., 2009). However, no difference in effectiveness between the two antipsychotics was reported in the previous RCTs, in which both antipsychotics were given within the licensed doses, i.e. 20 mg/day for olanzapine and 12 mg/day for risperidone. Remarkably, the upper dose of olanzapine is equivalent to half of the upper dose of risperidone (Gardner et al., 2010). Therefore, a clinical question has been raised whether

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olanzapine would be superior to risperidone when olanzapine is allowed to be given above the licensed dose as needed.

In clinical practice, it has been reported that nearly 50% of olanzapine prescription were above 20 mg/day in the U.S. (Citrome et al., 2007) and that the median for olanzapine recommendation dose by U.S. experts was 30 mg/day (Gardner et al., 2010). The upper limit of olanzapine dose in the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study, in which olanzapine was the most effective in terms of the rates of discontinuation, was designed to be 30 mg/day (Lieberman et al., 2005).

In chronic schizophrenia inpatients that showed suboptimal response to treatment, there is a RCT that the use of high doses of olanzapine were allowed (Volavka et al., 2002). In the study, it has been reported that clozapine and olanzapine were superior to haloperidol, but that the superiority of risperidone over haloperidol was not obvious. In treatment-resistant schizophrenia patients, there were reports that olanzapine at higher than customary doses demonstrated similar efficacy to clozapine (Meltzer et al., 2008) or less effective than clozapine (Kumra et al., 2008). Meanwhile, such a RCT has not been conducted in acute-phase schizophrenia patients.

Another clinical question was who needs high-dose risperidone or olanzapine. Especially, olanzapine has little active metabolites (Callaghan et al., 1999), in contrast to risperidone. Furthermore, there is a high correlation between serum and cerebrospinal fluid olanzapine concentrations (Skogh et al., 2011). Therefore, serum olanzapine concentrations would reflect most activity of olanzapine. Accordingly, serum olanzapine concentrations for patients who require high doses are worth measuring to investigate pharmacokinetic characteristics of such patients. However, serum olanzapine concentrations at 20 mg/day for patients who do not respond to conventional doses have not been well investigated.

We therefore prospectively examined whether olanzapine within 40 mg/day would be superior to risperidone within 12 mg/day in acute schizophrenia patients. In addition, we examined clinical characteristics for patients who required above conventional doses. Especially, in order to investigate whether serum olanzapine concentrations for patients who did not respond to conventional doses would be inappropriately low, serum olanzapine concentrations were measured. The present study was performed with emergency-based, newly admitted patients without support from pharmaceutical companies, reflecting real-world practice.

2. Methods

2.1. Setting and participants

Of the 80 psychiatric emergency wards authorized by the Japanese government, 12 (15%) participated in the present study. These wards were located all over Japan, and were responsible for local emergency cases. Most admissions to these hospitals represented behavioral emergencies and approximately 60% were brought in by the police. All were involuntary admissions as an immediate danger to themselves or others, according to the 1995 Law Concerning Mental Health and Welfare for the Mentally Disabled. Details of the clinical setting are described elsewhere (Hatta et al., 1998). According to government policies, psychiatric emergency services have been expanded in both metropolitan and local areas over the last 17 years. The quality of sites and patients in the present study was therefore homogenous. This activity was conducted by the Japan Acute-phase Schizophrenia Trial (JAST) study group (Hatta et al., 2009, 2011, 2012).

During the study period, between June 1, 2011 and January 31, 2012, a total of 1746 patients were admitted and assessed for eligibility. Eligible patients were 18–64 years old, newly admitted as emergency cases, and meeting the criteria of the DSM-IV-TR for schizophrenia, schizophreniform disorder, or schizoaffective disorder. Patients with obvious complications such as liver dysfunction, renal dysfunction, heart failure, respiratory failure, or diabetes mellitus were excluded, as were patients who were pregnant or who wanted to become pregnant.

2.2. Study design

All study protocols were approved by the institutional review board at each site, and written informed consent was obtained from patients or their legally

authorized representatives. Patients who refused oral medication were initially treated with injections. Times of injections before enrollment were not limited. After resolution of agitation, the investigators informed patients orally and in writing about the trial, and invited them to participate.

Patients were randomized using the sealed envelope method in a rater-blind manner to either risperidone or olanzapine for 8 weeks. For randomization, we referred to a random number table, with sequentially numbered, opaque, sealed envelopes used to conceal the allocation sequence.

The initial doses of risperidone and olanzapine were 3 mg/day and 10 mg/day, respectively. Doses were subsequently increased or decreased at the discretion of the treating psychiatrist. The maximum of licensed dose for olanzapine is 20 mg/day, which is equivalent to 6 mg/day for risperidone (Gardner et al., 2010). Therefore, the definition of higher dose in the present study was more than 20 mg/day for olanzapine and more than 6 mg/day for risperidone. The maximum doses of risperidone and olanzapine were 12 mg/day and 40 mg/day, respectively, considering dose equivalency (Gardner et al., 2010). Use of benzodiazepines was allowed and documented. Use of valproate as a mood stabilizer was also allowed and documented. However, use of other mood stabilizers and antidepressants was not permitted. Use of anticholinergic drugs was also not allowed unless acute extrapyramidal side effects appeared.

2.3. Procedures

Before starting the trial, site-coordinators were trained to assess outcomes as raters. All site-coordinators were experienced psychiatrists. A training video was used to train raters in assessment of the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1991). The primary outcome measure was all-cause discontinuation by 8 weeks.

Efficacy outcomes consisted of PANSS, the Clinical Global Impressions-Improvement Scale (CGI-I: 1, very much improved; 2, much improved; 3, minimally improved; 4, no change; 5, minimally worse; 6, much worse; and 7, very much worse) (Guy, 1976), and the Global Assessment of Functioning (GAF) (Jones et al., 1995). Safety and tolerability outcomes were determined based on vital signs, weight, laboratory data, electrocardiography (ECG), and the Drug-induced Extrapyramidal Symptom Scale (DIEPSS), which includes parkinsonism, akathisia, dystonia, and dyskinesia (Inada, 1996). Data including PANSS, CGI, GAF, vital signs, weight, laboratory data, ECG, and DIEPSS were collected on admission and every 2 weeks thereafter. Data were also collected at the time of discontinuation of the allocated treatment. Sexual side effects were recorded when reported by patients, and sedation was recorded when described by patients as an aversive subjective experience or when observed. Raters did not work on the wards involved in the study, were not involved with treatment, and were blinded to the drug assignments. The tested drug was discontinued when the treating psychiatrist judged the efficacy of the drug to be insufficient, when the treating psychiatrist judged side-effects of the drug to be intolerable, or when the patient reported non-adherence. Before a judgment of insufficient efficacy could be made, the drug dosage was increased to the maximum. The definition of drug non-adherence in the study performed in emergency and acute wards, where drug administration was managed by nurses, was that a patient declines the continuation of assigned medication.

2.4. Determination of olanzapine in human serum

Fasting blood samples were collected in the morning at least 10 h after the last evening dose of olanzapine for analyses of olanzapine. Serum for concentration analysis was separated and stored frozen at -20°C until analysis.

A liquid chromatography/tandem mass spectrometry method was used for analysis of olanzapine. Olanzapine and internal standard (IS: LY170222) were extracted from serum (100 μL) by liquid-liquid extraction using tert-butyl methyl ether. The processed sample (10 μL) was injected into high-performance liquid chromatography/tandem mass spectrometer that was equipped with a C_{18} column, using 10 mmol/L ammonium acetate/acetonitrile and acetonitrile as the mobile phase under gradient conditions. Olanzapine and IS were detected using a multiple reaction monitoring mode with positive ion (olanzapine: m/z 313 \rightarrow 256; IS: m/z 327 \rightarrow 270). Calibration for olanzapine was linear within the concentration range of 0.25–100 ng/mL.

2.5. Statistical analysis

Differences between categorical variables in patient demographics and clinical characteristics were calculated using Fisher's exact test. Differences between sequential variables were calculated using the unpaired t test (with Welch correction if applicable). If data were not sampled from Gaussian distributions, a non-parametric test (Mann-Whitney test) was used. Mean improvement in the PANSS total score was calculated as $100 \times (\text{baseline score} - \text{week} \times \text{score}) / (\text{baseline score} - 30)$ (Leucht et al., 2009). Kaplan-Meier curves were used to estimate the probability of treatment discontinuation at 8 weeks. Statistical analyses were performed using SPSS version 17.0J software (SPSS, Tokyo, Japan). All statistical tests were two-tailed. Values of $P < 0.05$ were regarded as statistically significant.

In our previous randomized clinical study, 25% of patients allocated to risperidone, of which the maximum dose was allowed up to 12 mg/day, discontinued risperidone by 8 weeks, while 12% of patients allocated to olanzapine, of which the maximum dose was allowed up to 20 mg/day, discontinued olanzapine by 8 weeks (Hatta et al., 2009). In the present study, the maximum dose of olanzapine was allowed up to 40 mg/day. As no previous data are available regarding the discontinuation rate for patients allocated to olanzapine with such conditions, we assumed 10% decrease in discontinuation rate by 8 weeks from our clinical experiences. The statistical power was set as $\text{power}=1-\beta=80\%$, and sensitivity as $\alpha=5\%$ to enable detection of differences in the effects of the augmentation strategy. Power analysis consequently set the required number of patients at 34 patients per group.

This study is registered in the UMIN Clinical Trials Registry (number: UMIN000 005526; <http://www.umin.ac.jp/ctr/>).

3. Results

3.1. Comparison between patients allocated to risperidone and patients allocated to olanzapine

Fig. 1 shows the trial profile. Forty-two patients were randomly assigned to two treatment groups. Baseline characteristics of randomized patients were much the same between groups, and mean (\pm S.D.) maximum doses of risperidone and olanzapine were 6.9 ± 2.7 mg/day and 23.0 ± 10.2 mg/day, respectively, suggesting relative dose equivalency. However, the number of patients allocated to each treatment group did not reach the required number of patients set by power analysis. Therefore, it is not conclusive about the primary outcome measure although time to treatment discontinuation for any cause did not differ between treatment groups (47.0 days [95%CI 39.9–54.0] for risperidone vs. 47.0 days [40.0–54.0] for olanzapine, $P=0.93$).

With respect to safety and tolerability outcomes, the rate of extrapyramidal symptoms was significantly higher in patients taking risperidone than in patients taking olanzapine ($P=0.0080$), corresponding to the significant difference in the rate of adjunctive anticholinergic drug use between the groups ($P=0.013$). No significant differences between treatment groups were identified in mean change from baseline for fasting glucose, cholesterol, triglycerides, or weight. Over-sedation was observed in one patient taking

olanzapine (max. dose, 30 mg/day). Sexual side effects were not observed.

No significant difference in the rate of patients who required high doses was seen between the risperidone group and the olanzapine group (40% [8/20] vs. 32% [7/22], $P=0.75$). The rates of patients who achieved a $\geq 50\%$ improvement in PANSS total score by 8 weeks in patients requiring high-dose risperidone and in patients requiring high-dose olanzapine were 25% [2/8] and 0% [0/7], respectively. Meanwhile, the rates of patients who achieved moderate ($\geq 30\%$) improvement in PANSS total score in patients requiring high-dose risperidone and in patients requiring high-dose olanzapine were 63% [5/8] and 57% [4/7], respectively.

3.2. Comparison between patients having required high doses and patients having responded to conventional doses

Fifteen patients required high doses. Of these patients, six patients were drug-naïve. Among the rest nine patients, only one patient that was allocated to risperidone met the definition of treatment-resistant schizophrenia at the time of study entry (Suzuki et al., 2011). The high-dose group was in a greater trend in the mean PANSS total score at baseline than the conventional-dose group ($P=0.051$, Table 1). In line with it, the high-dose group was in a greater trend in the rate of patients who received haloperidol injections at the time of admission than the conventional-dose group ($P=0.085$). Also, the high-dose group was in a greater trend in the times of haloperidol injections at the time of admission than the conventional-dose group (median 1 vs. 0, $P=0.098$). All subscale scores of PANSS were very high in both groups. Although there were no significant differences in scores of PANSS Positive scale and General psychopathology scale between groups, PANSS Negative scale score was significantly higher in the high-dose group than in the conventional-dose group ($P=0.0077$).

The mean PANSS total score at the time of starting high doses in the high-dose group was 104.5 (S.D. 21.5), which is as high as that at baseline in the conventional-dose group (105.2 [S.D. 24.8], Table 1).

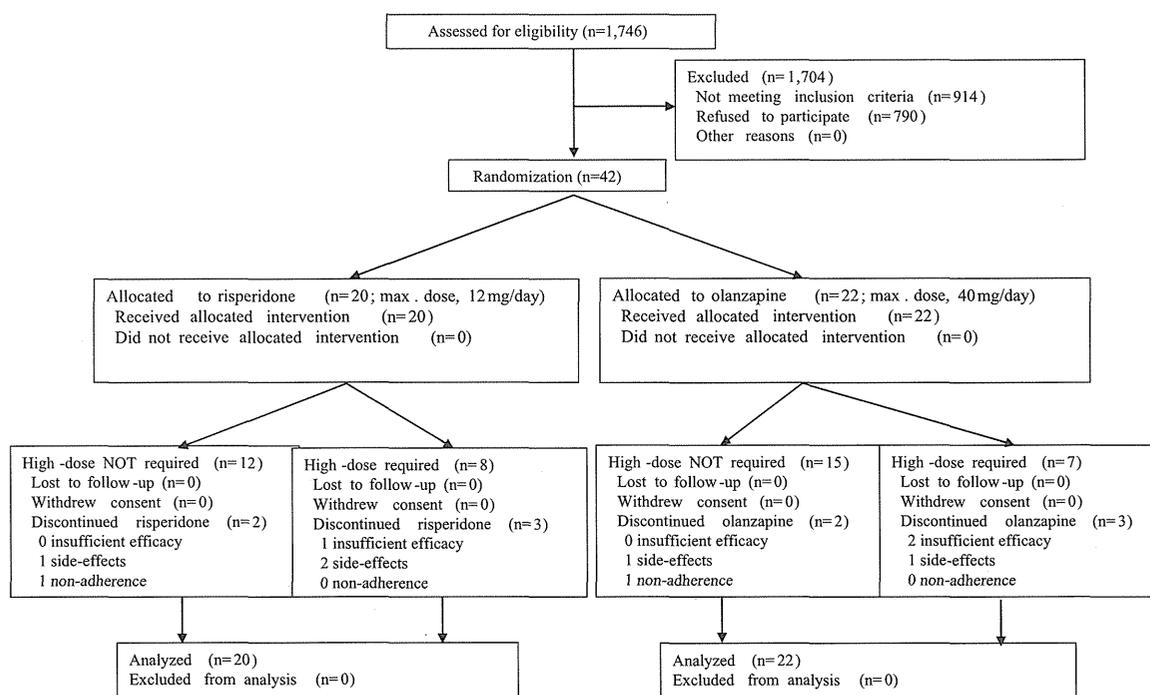


Fig. 1. Trial profile.

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-naïve | 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 antipsychotics for patients' behavior affected by severe positive and general psychopathology symptoms to be managed.

Although the rates of patients who achieved a $\geq 50\%$ 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 ($\geq 30\%$) 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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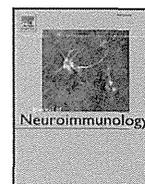
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Myasthenia gravis: Predictive factors associated with the synchronized elevation of anti-acetylcholine receptor antibody titer in Kanazawa, Japan

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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).

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

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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).

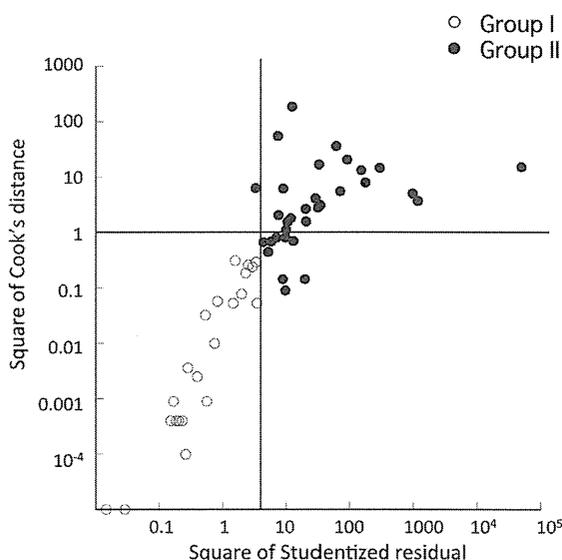


Fig. 1. Graphic representation of Groups I and II based on outliers. Significant elevation in the anti-AChR antibody titer was defined using outliers. Outliers, determined using two different methods, were used for defining Groups I and II. The square of Cook's distance and Studentized residual are shown.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

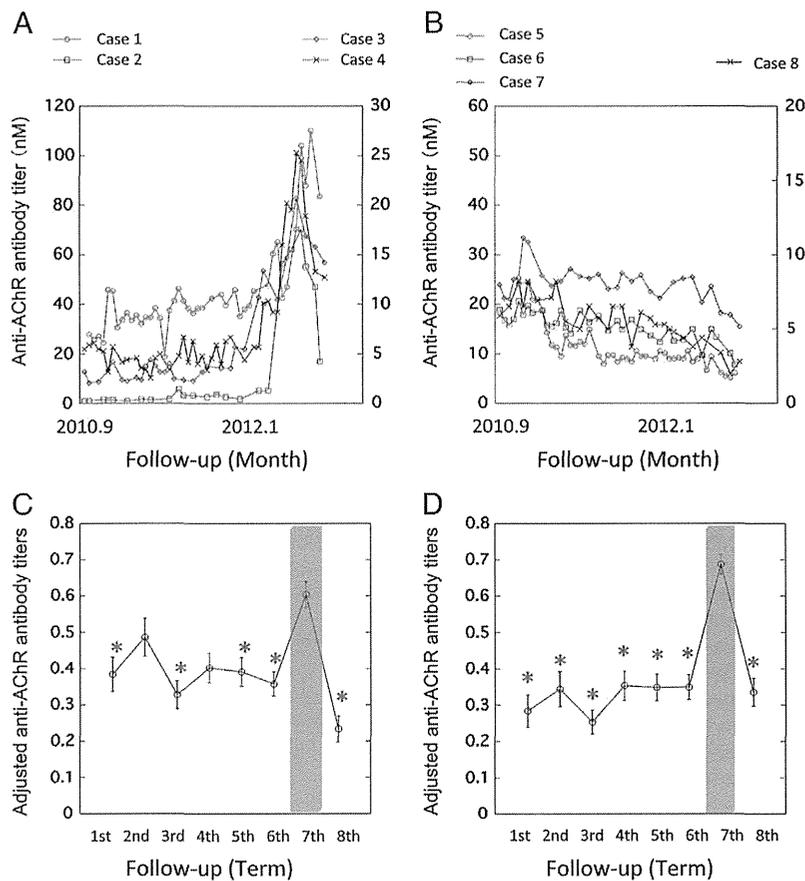


Fig. 2. Elevation of anti-AChR antibody titers. The diachronic changes in anti-AChR antibody titers for four representative patients in (A) Group-II (the elevated antibody group) and in (B) Group-I (the non-elevated group). C: The adjusted antibody titers averaged every 3 months in (C) all patients with 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) | <i>p</i> -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).

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

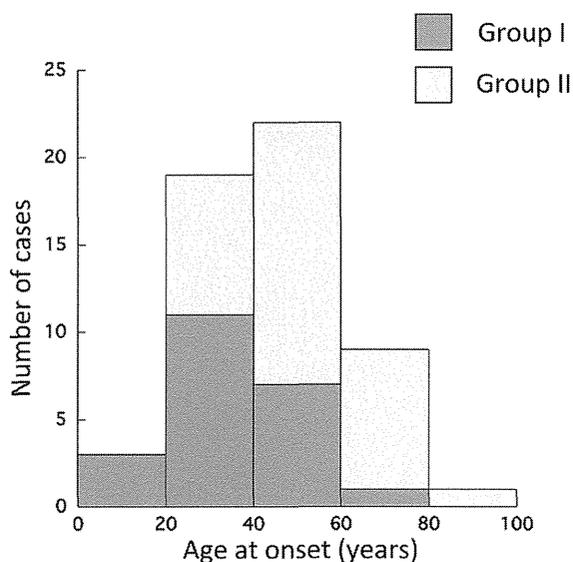


Fig. 3. Distribution of patients in each group according to age at disease onset. The peak numbers of patients in each group were different. The majority of patients in Group-I had disease onset at age <40 years, while the majority of patients in Group-II had disease onset at age >40 years.

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 |
|---------------------------------------------------------------------|----------------------|---------|------------------------|---------|
| <i>All patients (n = 54)</i> | | | | |
| 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 |
| <i>Patients with non-thymoma-related myasthenia gravis (n = 37)</i> | | | | |
| 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 |
| <i>Patients with myasthenia gravis and thymectomy (n = 37)</i> | | | | |
| 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.

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 m²) = 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 a 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 TSATM 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

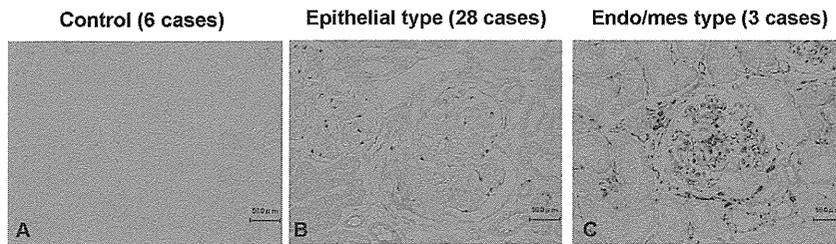


Figure 1. Gas6 expression in human IgA nephropathy. Biopsy samples were immunostained using indirect immunohistochemistry procedure with anti-Gas6 antibody. Representative images are shown in (A) from control patients, (B) from IgA nephropathy cases immunostained in epithelial cells, and (C) from IgA 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 \pm 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 characteristics 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 mesangial 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

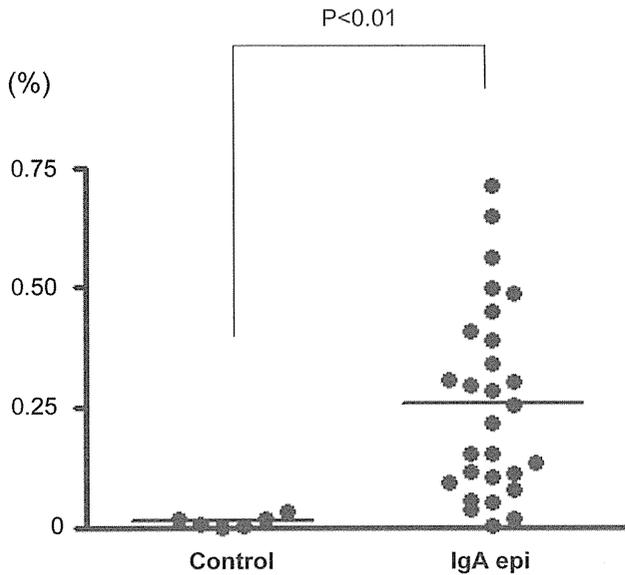


Figure 2. Gas6 stained area in human IgA nephropathy. Gas6 stained area is expressed as a percentage of total glomerular area occupied by Gas6 immunostained area. For each sample, at least eight glomerular profiles per patient were measured. IgA epi, IgA epithelial type.
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confirmed 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). Immunohistochemical 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 TGF β 1, 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

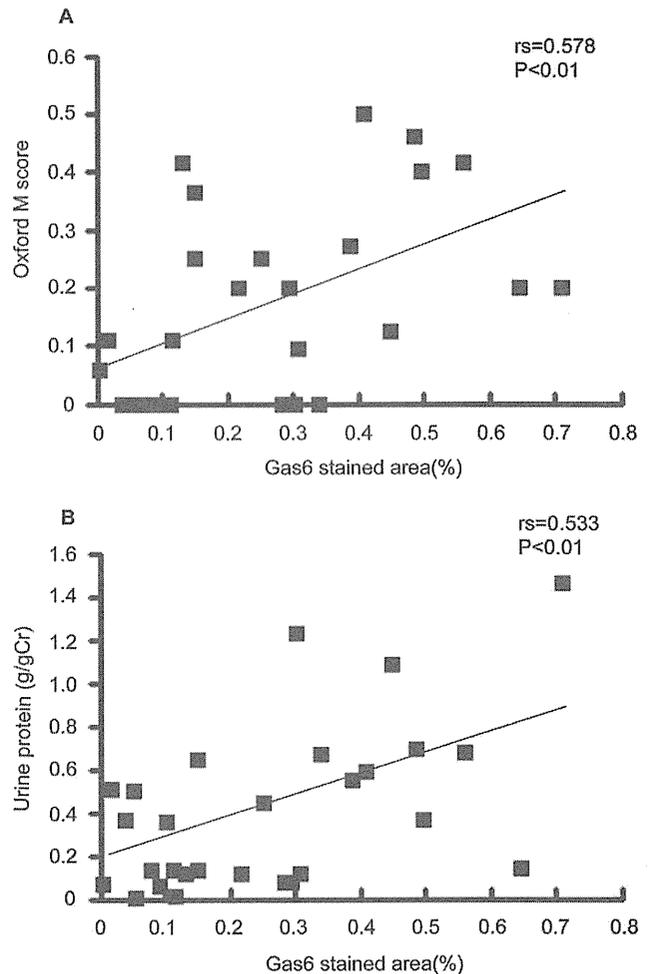


Figure 3. Correlation of Gas6 stained area with prognostic factors. Gas6 stained area in human IgA nephropathy correlated with (A) Oxford mesangial hypercellularity score and (B) urine protein excretion. N=28. Oxford M score, Oxford mesangial hypercellularity score.
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involved in human IgAN either via Dtk and Axl in podocytes and endothelial/mesangial cells, respectively.

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 TGF β 1, 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 hypercellularity score. S score, Segmental glomerulosclerosis 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 western blotting 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 pathogenesis and histological change of IgAN, the level

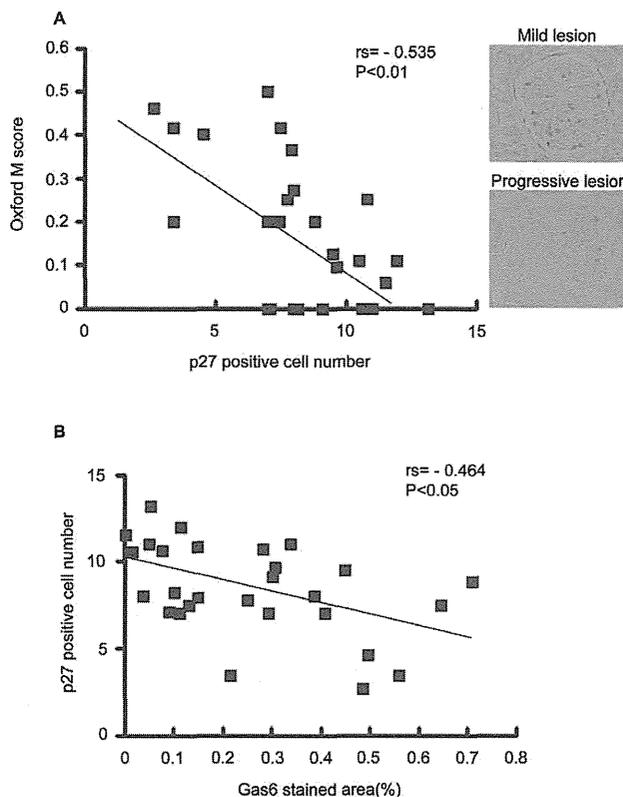


Figure 4. Inverse correlation of Gas6 stained area with p27 positive cell number. (A) Biopsy samples were immunostained using indirect immunohistochemistry procedure with anti-p27 antibody. p27 positive cell number was counted. The result was divided by glomeruli number. For each sample, at least eight 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

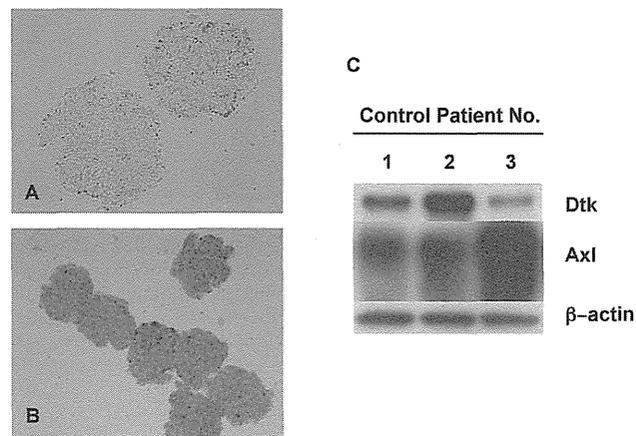


Figure 5. Expression of Dtk and AxI in control glomeruli. (A,B) Photographs of isolated glomeruli. The glomeruli were isolated from the uninvolved portion of tumor nephrectomy specimens by sieving method from three patients. The relative purity of isolated glomeruli was shown. The original magnification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

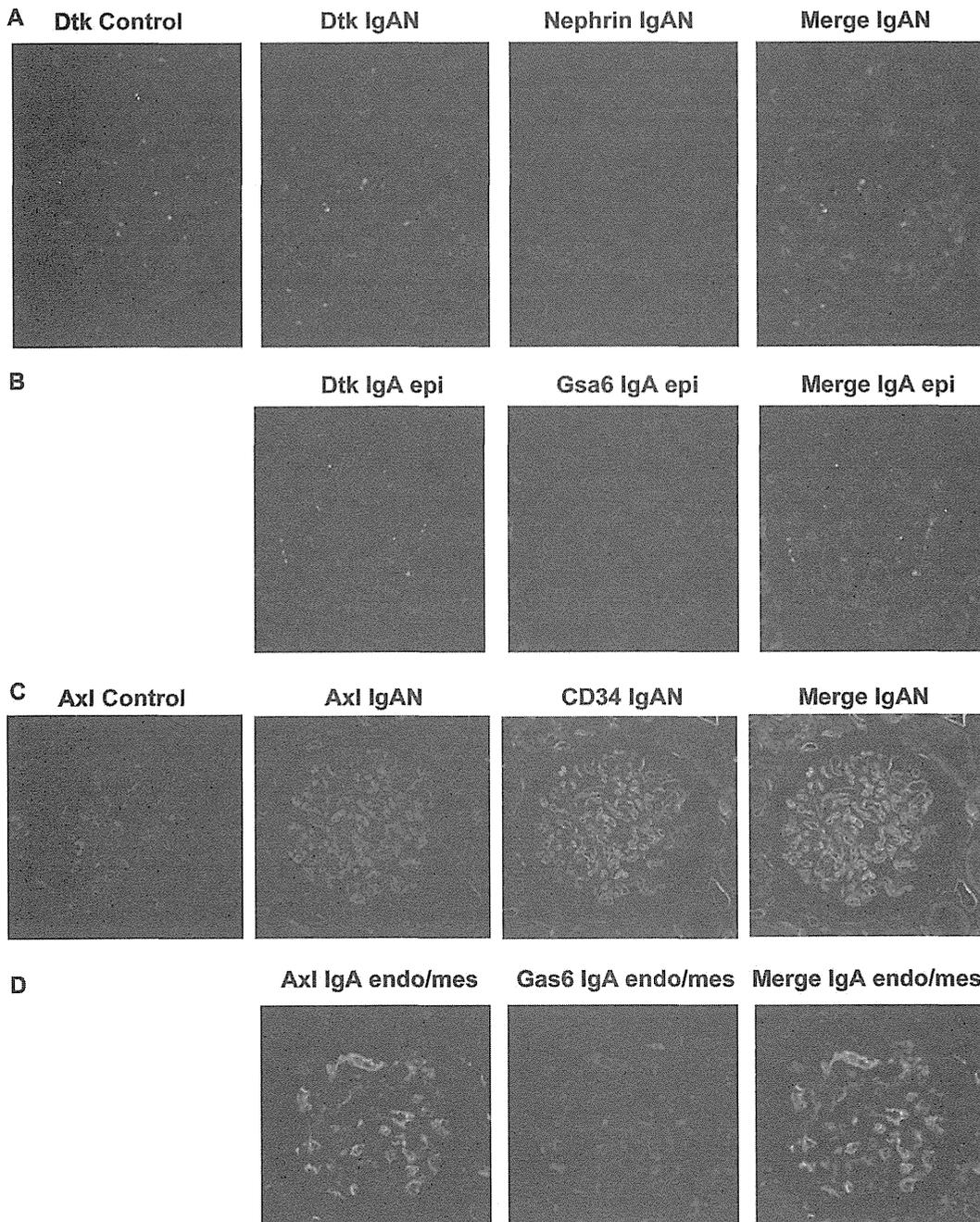


Figure 6. Double immunohistochemistry of Dtk and Axl with cell specific markers or Gas6. Biopsy samples were immunostained using indirect immunohistochemistry procedure with (A) anti-Dtk, anti-Nephrin (a podocyte marker), (B) anti-Dtk, anti-Gas6 or (C) anti-Axl, anti-CD34 (an endothelial cell marker), (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

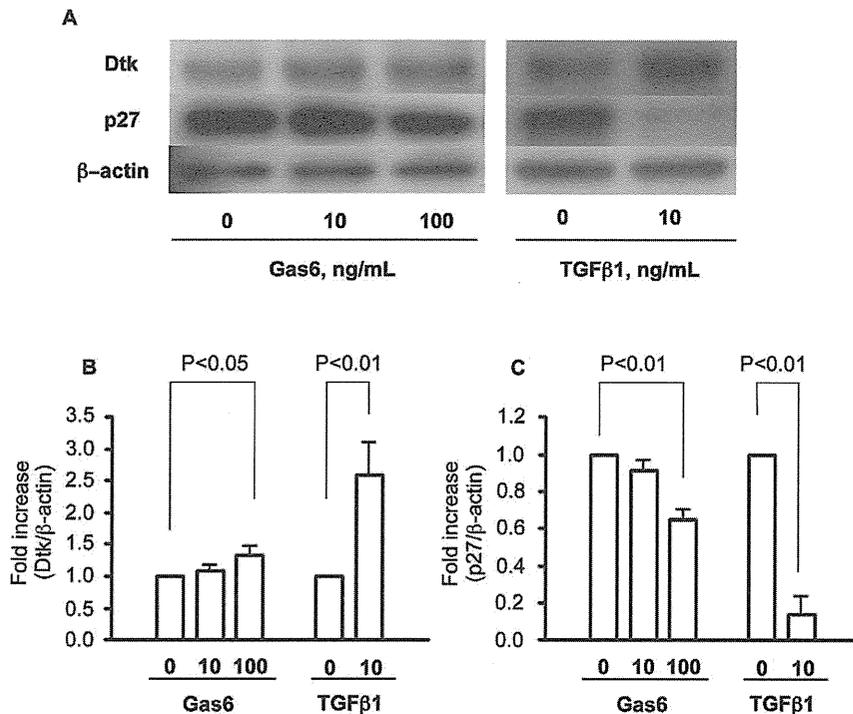


Figure 7. Effect of Gas6 on Dtk and p27 expression in podocytes. Podocytes were differentiated and stimulated with recombinant Gas6 or TGF β 1 for 24 hours. Cell lysates were subjected to immunoblotting with the antibodies indicated. Then, podocytes expressed Dtk. Gas6 could increase the expression of Dtk and reduce p27, as well as TGF β 1. 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 \pm 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) μ g/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. (TIFF)