Regular Article

Analysis of Staphylococcal Toxins and Clinical Outcomes of Methicillin-Resistant *Staphylococcus aureus* Bacteremia

Masayuki Maeda,^{*,a} Hisashi Shoji,^b Tetsuro Shirakura,^c Takahiro Takuma,^b Kazuhisa Ugajin,^d Kunihiro Fukuchi,^e Yoshihito Niki,^b and Keiko Ishino^a

^aDivision of Infection Control Sciences, Department of Pharmacotherapeutics, School of Pharmacy, Showa University; 1–4–5 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan; ^bDivision of Clinical Infectious Diseases, Department of Medicine, School of Medicine, Showa University; 1–4–5 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan; ^cDepartment of Microbiology, School of Medicine, Showa University; 1–4–5 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan; ^dDepartment of Clinical Laboratory, Showa University Hospital; 1–4–5 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan; and ^eDepartment of Pathology, Showa University Hospital; 1–4–5 Hatanodai, Shinagawa-ku, Tokyo 142–8555, Japan.

Received March 14, 2016; accepted April 5, 2016

It is well known that methicillin-resistant *Staphylococcus aureus* (MRSA) produces many virulence factors, such as hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory factors. The aim of study was to identify staphylococcal pathogenicity that may affect the prognosis of patients with MRSA bacteremia. We obtained 149 MRSA strains from blood cultures between January 2009 and December 2014 in our institution. We collected information on patient characteristics, laboratory data, staphylococcal toxin genes, and susceptibility of the strain toward anti-MRSA agent and analyzed them as factors contributing to 30-d mortality. The “survival” and “dead” groups consisted of 103 and 46 patients, respectively. Multiple logistic regression analysis showed a four-fold increase in the risk of mortality in patients exhibiting isolated MRSA with staphylococcal enterotoxins (SEs) genes as well as toxic shock syndrome toxin-1 (TSST-1) genes [odds ratio: 3.89; 95% confidence interval: 1.20–12.60; $p=0.024$]. Kaplan–Meier analysis also showed significantly higher mortality in patient with isolated MRSA with SEs and TSST-1 genes. After adjusting for confounders, the coexistence of SEs and TSST-1 were independently associated with the 30-d mortality compared with treatment and susceptibility. The coexistence of superantigenic toxin genes greatly affects the clinical course and prognosis of patients with MRSA bacteremia.

Key words methicillin-resistant *Staphylococcus aureus* (MRSA); superantigen; septic shock; bacteraemia

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important pathogens responsible for nosocomial infections. The mortality rate owing to MRSA bacteremia is 20–50%.^{1,2)}

Vancomycin (VCM) has been the standard treatment for MRSA infections for over 50 years. The area under the concentration–time curve [AUC /minimum inhibitory concentration (MIC) ratio] is a representative parameter of the pharmacokinetics–pharmacodynamics (PK–PD) of VCM.³⁾ The Infectious Diseases Society of America recommends a target $AUC/MIC > 400$ for the treatment of severe MRSA infections.⁴⁾ However, this is often difficult to achieve for MRSA strains with high VCM MICs, thus increasing the risk of insufficient therapeutic response. Previous meta-analyses have attempted to address the association between MIC and clinical outcomes.^{5–7)} In contrast to these studies, a recent meta-analysis examining 8291 episodes reported no differences in the risk of death between patients with *S. aureus* high and low VCM MICs.⁸⁾ These results highlight the current lack of consensus regarding the relationship between VCM MICs and the prognosis of patients with MRSA infections.

Our previous study measured MICs at 0.25 $\mu\text{g/mL}$ intervals using the broth microdilution method and found that VCM MICs $\geq 1.5 \mu\text{g/mL}$ affected the prognosis for MRSA bacteremia.⁹⁾ However, there were no significant differences in VCM AUC/MIC and clinical prognosis between the groups.

The pathological mechanism of *S. aureus* infections includes infectious pathogenicity such as pneumonia, cel-

lulitis, osteomyelitis, and infective endocarditis as well as toxic pathogenicity such as food poisoning and toxic shock syndrome.^{10,11)} Several virulence factors have been reported to be associated with the clinical outcomes of MRSA infections. The expression of major virulence factors, such as Panton–Valentine leukocidin (PVL), is known to be correlated with higher mortality in community-associated MRSA infections.^{12,13)} We believe that toxic pathogenicity is closely associated with patient prognosis. *S. aureus* is known to produce various virulence factors,¹⁴⁾ such as toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs), which are superantigenic toxins (SAGT) associated with severe infections.^{11,15,16)} A previous study reported that TSST-1 and SEC1 caused shock when intravenously injected into an animal model.¹⁷⁾ In addition, recent experimental study showed that SAGT play a critical role in the development and progression of *S. aureus* infective endocarditis and sepsis.¹⁸⁾ However, the association between SAGT and the prognosis of patients with MRSA bacteremia has not been sufficiently studied. Therefore, here we aimed to examine the effects of staphylococcal virulence factors on the prognosis of patients with MRSA bacteremia.

MATERIALS AND METHODS

Patients and Bacterial Strains This single-center, retrospective, observational study was performed at the Showa University Hospital, which is a 1014-bed teaching hospital

*To whom correspondence should be addressed. e-mail: m-maeda@pharm.showa-u.ac.jp

in Tokyo, Japan. Clinical MRSA strains ($n=149$) from blood cultures were collected from inpatients older than 15 years of age at our hospital between January 2009 and December 2014. They were divided into two groups: “survival” and “dead” ($n=103$ and 46, respectively). Blood culture was performed using the BD BACTEC FX System (Becton, Dickinson and Company, Franklin Lakes, NJ, U.S.A.). All strains were screened using the MicroScan® WalkAway® system and those identified as MRSA were subjected to oxacillin or ceftioxin testing. Patients with a fever of $\geq 38^\circ\text{C}$ who tested positive for at least one strain per blood culture bottle were included in this study. In patients who tested positive more than one strain, only the first one was tested. We collected information regarding the age, gender, underlying diseases, Charlson comorbidity index (CCI),¹⁹⁾ systemic inflammatory response syndrome criteria and sequential organ failure assessment (SOFA) score,²⁰⁾ laboratory data, colonization of MRSA, duration of central venous catheter, source of infection (soft tissue and bone, central nervous system, respiratory tract, intra-abdominal, urinary tract, and intravascular or medical devices), and administration of anti-MRSA agents and analyzed their contribution to the 30-d mortality.

Antimicrobial Susceptibility Tests We performed antimicrobial susceptibility test on all MRSA strains using the Sensititre® microbroth dilution system (Trek Diagnostic Systems Inc., Cleveland, OH, U.S.A.). Susceptibility toward anti-MRSA agents were tested using original MIC plates (Trek Diagnostic Systems Inc.) with the following antibiotics: VCM, teicoplanin (TEIC), arbekacin, daptomycin, linezolid (LZD), and tigecycline (TIGE) as previously described.⁹⁾ Resistance break points published by the Clinical and Laboratory Standards Institute were used.²¹⁾

Molecular Analysis of Isolated Strains We performed a molecular analysis to evaluate the effect of the following staphylococcal virulence factors on patient prognosis: TSST-1 (*tst*), SE (*sea*, *seb*, and *sec*), leukotoxin [*lukSF-PV* (PVL) and *lukED*], hemolysin (*hla*, *hly*, *hld*, *hlg*, and *hly-2*), exfoliative toxin (*eta* and *etb*). The presence of these virulence factors was determined using PCR methods, as previously described.²²⁾ Staphylococcal cassette chromosome *mec* (SCC-*mec*) types were also identified using previously described methods.²³⁾

Statistical Analysis Continuous variables have been presented as the mean and standard deviation (S.D.) or medians and interquartile ranges and were compared between groups using the independent *t*-test or Mann–Whitney *U* tests. Categorical variables have been presented as the number and percentage of patients within each group and compared using the χ^2 or Fisher’s exact test. Survival was analyzed using the Kaplan–Meier method. Plots were compared using the log-rank test. We used forward stepwise logistic regression to identify independent variables associated with the 30-d mortality and included those variables with $p < 0.05$ in the univariate analysis.

All statistical tests were two-sided; a *p* value of < 0.05 was considered statistically significant, and all analyses were performed using SPSS statistical software (version 23; IBM Japan Ltd.).

Ethics This study was approved by the research ethics committee of Showa University (Approval No. 1476). Informed consent was exempted according to the Ethical

Guidelines for Medical and Health Research Involving Human Subjects by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan.

RESULTS

Clinical Characteristics We analyzed potential factors that affected the 30-d mortality in patients with MRSA bacteremia (Table 1). The analysis revealed significantly higher CCI ($p=0.002$), SOFA score ($p=0.002$), insertion of central venous catheter ($p=0.033$), and lower administration of VCM ($p=0.019$) in the “dead” group than in the “survival” group.

Bacterial Virulence Factors We explored the virulence factor profiles of MRSA genotypes according to the 30-d mortality (Table 2); no differences were found between MRSA strains genotypes. Exfoliative toxins (*eta* and *etb*) and PVL genes were absent, whereas hemolysins (*hla*, *hly*, *hld*, *hlg*, and *hly-2*) and *lukE-lukD* were present. Because differences were observed in the presence of SEs (*sea*, *seb*, and *sec*) and *tst*, we explored the combination of these SAgT genes.

Figure 1 shows the combination of SAgT genes (*tst*, *sea*,

Table 1. Patient Characteristics According to 30-d Mortality and Univariate Model^{a)}

Factor	Survival ($N=103$)	Death ($N=46$)	<i>p</i> Value
Age (years)	70 $\pm$ 16	72 $\pm$ 16	0.536
Gender (male sex)	65.0%	58.7%	0.458
Underlying disease			
Malignancy	30.1%	28.3%	0.820
Diabetes mellitus	34.0%	32.6%	0.870
Renal failure (Hemodialysis)	24.3%	28.3%	0.606
Charlson comorbidity index	3 (2–5)	5 (4–7)	0.002
SIRS	79.8%	82.6%	0.690
SOFA score ^{b)}	5 (3–7)	7 (4–10)	0.002
Creatinine clearance (mL/min)	45 (18–72)	36 (14–61)	0.536
Colonization of MRSA	64.1%	58.7%	0.531
Central venous catheter	48.5%	67.4%	0.033
Catheter insertion period (d)	0 (0–13)	2.5 (0–13)	0.234
Source of infection			
Skin, soft tissue, and bone	19.4%	13.0%	0.344
Central nervous system	1.9%	0%	> 0.99
Lower respiratory tract	5.8%	15.2%	0.111
Infective endocarditis	7.8%	0%	0.059
Intra-abdominal	9.7%	15.2%	0.606
Kidney and urinary tract	9.7%	2.2%	0.174
Catheter or other medical devices related	30.1%	45.7%	0.660
Unknown origin	15.5%	8.7%	0.258
First administered agent			
VCM	66.0%	42.2%	0.019
TEIC	6.8%	10.9%	0.515
ABK	1.9%	4.3%	0.587
LZD	7.8%	6.5%	> 0.99
DAP	1.9%	6.5%	0.171
Others	15.5%	26.1%	0.128

SIRS, systemic inflammatory response syndrome; SOFA, sequential organ failure assessment; MRSA, methicillin-resistant *Staphylococcus aureus*; VCM, vancomycin; TEIC, teicoplanin; ABK, arbekacin; LZD, linezolid; DAP, daptomycin. *a)* Values represent the mean $\pm$ S.D. or median (IQR), unless otherwise indicated. *b)* Data from “survival” ($N=79$) and “dead” ($N=43$) groups.

seb, and *sec*) according to the 30-d mortality. Significantly higher mortality was observed in patients infected with co-existent strains of *tst*, *sea*, *seb*, and *sec* (TABC) (10.7 versus 23.9%; $p=0.035$).

Microbiological Data and Susceptibilities The microbiological characteristics and antibiotic susceptibilities of MRSA strains according to the 30-d mortality are shown in Table 3. The analysis revealed significantly higher VCM MICs and the presence of TABC strains in the “dead” group. There were no significant differences in SCCmec types and 30-d mortality between the two groups.

Risk Factors for 30-d Mortality Multiple logistic regression analyses were performed to identify independent risk factors for 30-d mortality (Table 4). The following factors were found to be significantly associated in the univariate analysis: CCI, SOFA score, insertion of central venous catheter, first administration of VCM, VCM MICs, and strains with TABC (Tables 1–3). In the multivariate analysis controlling for several confounders, strains with TABC were independently associated with higher mortality [odds ratio (OR): 3.89; 95% confidence interval (CI): 1.20–12.6; $p=0.024$].

Impact of Superantigenic Toxins on Clinical Status Ka-

Table 2. Staphylococcal Virulence Gene Profiles

Gene name	Survival (N=103)	Death (N=46)
<i>lukSF-PV</i>	0%	0%
<i>lukED</i>	97.1%	95.7%
<i>hla</i>	100%	100%
<i>hlb</i>	96.1%	97.8%
<i>hld</i>	100%	100%
<i>hlg</i>	99.0%	95.7%
<i>hlg-2</i>	100%	100%
<i>eta</i>	0%	0%
<i>etb</i>	0%	0%
<i>tst</i>	84.5%	87.0%
<i>sea</i>	16.5%	28.3%
<i>seb</i>	68.9%	80.4%
<i>sec</i>	86.4%	87.0%

plan–Meier analysis showed significantly higher 30-d mortality in a subset of patients with MRSA exhibiting the presence of SAgT (Fig. 2). The clinical status of patients with MRSA bacteremia and TABC strain is shown in Table 5. The analysis revealed significantly higher catecholamine administration ($p=0.039$), SOFA score ($p=0.012$), and lower platelet count ($p=0.010$) in the MRSA bacteremia with TABC strain. No significant differences in the CCI were observed between the groups.

The susceptibility of the TABC strain toward the anti-MRSA agent is shown in Table 6. The TABC strain had significantly higher VCM and TEIC MICs ($p=0.008$ and 0.001 , respectively), and the common genotypes were SCCmec type

Table 3. Features of Isolated Strain by 30-d Mortality and Univariate Model^{a)}

Factor	Survival (N=103)	Death (N=46)	p Value
MICs of anti-MRSA agent ($\mu\text{g/mL}$)			
VCM	1.0 (0.75–1.25)	1.25 (1.25–1.5)	0.002
TEIC	1.0 (0.5–1.0)	1.0 (0.5–1.5)	0.301
ABK	2.0 (0.5–2.0)	2.0 (0.5–2.0)	0.763
LZD	2.0 (2.0–2.0)	2.0 (2.0–2.0)	0.848
DAP	0.5 (0.5–2.0)	0.5 (0.5–2.0)	0.193
TIGE	0.5 (0.12–1.0)	0.5 (0.25–1.0)	0.199
SCCmec type			
II	77.7%	80.4%	0.704
IV	13.6%	10.9%	0.645
Others	8.7%	8.7%	>0.99
No. of superantigenic toxin gene from <i>tst</i> , <i>sea</i> , <i>seb</i> , and <i>sec</i>			
None	3.9%	0%	0.312
Single	4.9%	8.7%	0.459
Double	33.0%	23.9%	0.264
Triple	47.6%	43.5%	0.643
All (coexistence of <i>tst</i> , <i>sea</i> , <i>seb</i> , and <i>sec</i>)	10.7%	23.9%	0.035

MIC: minimum inhibitory concentration; MRSA: methicillin-resistant *Staphylococcus aureus*; VCM: vancomycin; TEIC: teicoplanin; ABK: arbekacin; LZD: linezolid; DAP: daptomycin; TIGE: tigecycline; SCCmec: staphylococcal cassette chromosome *mec*. a) Values represent the median (IQR) unless otherwise indicated.

Fig. 1. Combination of Superantigenic Toxin Genes According to 30-d Mortality

Significantly higher mortality observed in patients infected with coexistent strains of *tst*, *sea*, *seb*, and *sec* (TABC). A, *sea*; B, *seb*; C, *sec*; T, *tst*.

Fig. 2. Kaplan-Meier Analysis of 30-d Mortality Based on the Presence of Superantigenic Toxin Genes

TABC, coexistence of *tst*, *sea*, *seb*, and *sec*.

Table 4. Factors Related to 30-d Mortality: Logistic Regression with Multivariate Model

Risk factor	Partial regression coefficient	Adjusted odds ratio	95% Confidence interval	<i>p</i> Value
Charlson comorbidity index	0.19	1.21	1.02–1.44	0.028
SOFA score	0.15	1.16	1.04–1.30	0.009
Administration of vancomycin	−1.23	0.28	0.12–0.68	0.005
MRSA bacteremia with TABC strain	1.36	3.89	1.20–12.6	0.024

SOFA: sequential organ failure assessment; MRSA: methicillin-resistant *Staphylococcus aureus*; TABC: coexistence of *tst*, *sea*, *seb*, and *sec*. Hosmer and Lemeshow test=0.303, discrimination rate=71.3%. Variables identified as significant ($p<0.05$) were entered into multiple logistic regression models.

Table 5. Impact of Superantigenic Toxins on Clinical Status ^{a)}

Characteristics	MRSA bacteremia without TABC strain (N=127)	MRSA bacteremia with TABC strain (N=22)	<i>p</i> Value
Administrated of catecholamine	20.6%	40.9%	0.039
Platelet ($10^4/\text{mm}^3$)	19.3 ± 12.0	12.1 ± 10.2	0.010
SOFA score	5 (4–8)	8.5 (5.5–11)	0.012
Charlson comorbidity index	4 (2–6)	5 (2–7)	0.512

MRSA: methicillin-resistant *Staphylococcus aureus*; TABC: coexistence of *tst*, *sea*, *seb*, and *sec*; SOFA: sequential organ failure assessment. ^{a)} Values represent the mean $\pm$ S.D. or median (IQR) unless otherwise indicated.

II (95.5%).

DISCUSSION

This retrospective observational study is the first to examine the contribution of MRSA bacteremia combined with SAgT toward patient prognosis.

Previously, several studies have evaluated factors influencing the prognosis of patients with *S. aureus* bacteremia.²⁴⁾ Host factors, pathogen factors, clinical management, and microbiological factors, such as methicillin resistance and VCM MICs, were found to be associated with mortality.^{5–7,9,24)}

Table 6. Impact of Strains with Superantigenic Toxin Genes on Susceptibility of Anti-MRSA Agents

Agents	MICs ($\mu\text{g/mL}$)		<i>p</i> Value
	Strain without TABC (N=127)	Strain with TABC (N=22)	
VCM MICs median (IQR)	1.0 (0.75–1.25)	1.25 (1.0–1.5)	0.008
MIC ₉₀	1.5	1.5	—
TEIC MICs median (IQR)	1.0 (0.5–1.0)	1.0 (1.0–2.5)	0.001
MIC ₉₀	1.5	4.0	—
ABK MICs median (IQR)	2.0 (0.5–2.0)	2.0 (0.5–2.0)	0.652
MIC ₉₀	2.0	2.0	—
LZD MICs median (IQR)	2.0 (2.0–2.0)	2.0 (2.0–2.0)	0.059
MIC ₉₀	2.0	3.0	—
DAP MICs median (IQR)	0.5 (0.5–2.0)	1.25 (0.5–2.0)	0.196
MIC ₉₀	2.0	2.0	—
TIGE MICs median (IQR)	0.5 (0.12–1.0)	0.5 (0.25–1.0)	0.561
MIC ₉₀	2.0	1.0	—

MRSA: methicillin-resistant *Staphylococcus aureus*; MIC: minimum inhibitory concentration; TABC: coexistence of *tst*, *sea*, *seb*, and *sec*; VCM: vancomycin; TEIC: teicoplanin; ABK: arbekacin; LZD: linezolid; DAP: daptomycin; TIGE: tigecycline.

However, a recent meta-analysis reported no differences in the risk of death in patients with *S. aureus* high and low VCM MICs.⁸⁾ Our previous study showed that there was no significant difference between the survival and dead group in the VCM AUC/MIC.⁹⁾ Although the reason for this inconsistent

finding remains unclear, our data suggest that strain-specific pathogenicity, rather than vancomycin susceptibility, contributes to the outcome of MRSA bacteremia. Furthermore, the present study showed higher VCM and TEIC MICs in strains with TABC, suggesting that virulence factors and susceptibilities contribute to differences in strain-dependent mortality. The association between strain-specific virulence and clinical outcomes of MRSA bacteremia has also been previously reported.^{25–27)} These factors may help explain the contradictory results of the meta-analysis.

This study shows that the combination of SAgT genes is associated with the clinical status and prognosis for MRSA bacteremia. The superantigens bind to class II major histocompatibility complex on the surfaces of antigen-presenting cells and T-cell receptors of cytotoxic lymphocytes. As a result, large amounts of cytokines are released, causing systemic symptoms such as shock, pancytopenia, and multi-organ failure.¹⁶⁾ Our results demonstrated that significantly lower platelet count, higher catecholamine administration, and higher SOFA score were observed in patients infected with TABC, suggesting severe sepsis or septic shock in patients with MRSA bacteremia. Thus, SAgT induced systemic symptoms that potentially caused poor patient prognosis. In a previous study, Japanese women in Tokyo exhibited a lower prevalence of TSST-1 antibody titers than did Caucasians in the United States.²⁸⁾ We believe that this finding was associated with systemic symptoms caused by the overproduction of SAgT in Japanese. Human-origin intravenous immunoglobulin has a neutralizing effect on TSST-1 and SE produced by MRSA.²⁹⁾ It may be useful for passive immunization therapy in patients with MRSA bacteremia. Protein synthesis inhibitors may also be useful for the treatment of severe staphylococcal infections associated with the production of SAgT. Previous studies have shown that the level of TSST-1 produced decreases in cells treated with the protein inhibitors clindamycin, LZD, gentamicin, and TIGE.^{30,31)} In the clinical setting, these protein inhibitors attenuate SAgT production in life-threatening cases of toxic shock. However, these are adjuvant therapeutic measures that require further clinical research.

Our study had a number of limitations. First, this was a small, single-center study conducted in Tokyo, Japan, and our findings cannot be generalized to other countries with different circulating MRSA strains. Moreover, it was a retrospective study with a limited sample size; therefore, potential and unmeasured confounding factors were not controlled for. Because there was potentially good prognosis in patients treated with primary VCM administration, the lower administration of VCM was observed in “dead” group. The “dead” group includes patients with supportive and palliative care (e.g. end-stage cancer). These potential confounding factors were uncontrolled in this study. Second, we were unable to analyze several staphylococcus virulence factors, particularly various SE genotypes. Therefore, it is possible that the differences in mortality are a result of other virulence factors that we did not examine. A previous study reported that SEA play a key role in *sea*-positive *S. aureus* sepsis by triggering overexpression of inflammatory mediators associated with shock.³²⁾ However, the role of each virulence factor in the prognosis of patients with MRSA bacteremia is still unclear. A larger study is required to examine combinations of various staphylococcal virulence factors. Finally, the PCR-based evaluation of viru-

lence determinants should be interpreted with some caution as it may not directly reflect *in vivo* expression. Although our study has several limitations, the results allow us to conclude that combination of SAgT genes is potentially associated with higher risk of mortality associated with MRSA bacteremia.

In conclusion, after adjustment for several confounders, combinations of SAgT, such as TSST-1 and SEs, were independently associated with the 30-d mortality in MRSA bacteremia. Our results suggest that strain-specific virulence factors, rather than treatment and susceptibility, contributes to the outcome of MRSA bacteremia. This reinforces the importance of adjuvant therapy, such as clindamycin, LZD, gentamicin, TIGE, and immunoglobulin, in severe MRSA infections. Further genome-wide studies and clinical trials should be performed to improve the outcome of MRSA bacteremia.

Acknowledgments We thank our students for providing technical support. The authors would like to thank Enago (www.enago.jp) for the English language review. This study was presented at the Interscience Conference of Antimicrobial Agents and Chemotherapy (ICAAC) 2015, San Diego CA (abstract number B-522).

Conflict of Interest Shoji H, Takuma T, and Niki Y, received donations for this research from Takeda Pharmaceutical Co., Ltd., Pfizer Japan Inc., Daiichi Sankyo Co., Ltd., Bayer Yakuhin, Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Dainippon Sumitomo Pharma Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Kyorin Pharmaceutical Co., Ltd., Astellas Pharma Inc., Chugai Pharmaceutical Co., Ltd., and GlaxoSmithKline K.K. Shoji H, Takuma T, and Niki Y are endowed chairs and funded by Shionogi & Co., Ltd., Meiji Seika Pharma Co., Ltd., and Toyama Chemical Co., Ltd., respectively.

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