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Evaluation of recombinant MGL_1304 produced by *Pichia pastoris* for clinical application to sweat allergy

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M. globosa, Malassezia globosa;
E. coli, *Escherichia coli*; *P. pastoris*, *Pichia pastoris*; AD, atopic dermatitis;
ChU, cholinergic urticaria; HRT, histamine release test; TF, Trigger factor

ABSTRACT

Background: We previously identified MGL_1304 secreted by *Malassezia globosa* as a sweat antigen for patients with atopic dermatitis (AD) and cholinergic urticaria (ChU). However, purifying native MGL_1304 from human sweat or culture supernatant of *M. globosa* (sup-MGL_1304) is costly and time-consuming. Moreover, recombinant MGL_1304 expressed by using *Escherichia coli* (TF-rMGL_1304) needs a large chaperon protein and lacks the original glycosylation of yeasts. Thus, we generated a recombinant MGL_1304 by *Pichia pastoris* (P-rMGL_1304) and investigated its characteristic features.
Methods: Recombinant MGL_1304 proteins expressed by *E. coli* and *P. pastoris* were generated. Properties of these recombinants and native antigens were compared by western blot analysis, histamine release tests (HRT) of patients with AD and ChU, and β-hexosaminidase release tests with RBL-48 cells. P-rMGL_1304-specific IgE in sera of patients with AD were measured by sandwich ELISA.
Results: Western blot analysis revealed that IgE of patients with AD bound to all MGL_1304 recombinants and native antigens. The histamine releasing ability of P-rMGL_1304 was 100 times higher than that of TF-rMGL_1304, and was comparable to that of sup-MGL_1304. Degranulation rates of RBL-48 cells, sensitized with sera of patients with AD in response to the stimulation of P-rMGL_1304, were comparable to those of sup-MGL_1304, whereas those of TF-rMGL_1304 were relatively weak. The levels of P-rMGL_1304-specific IgE in sera of patients with AD were correlated with their disease severities.
Conclusions: P-rMGL_1304 has an antigenicity comparable to the native antigen, and is more useful than TF-rMGL_1304, especially in HRT and degranulation assay of RBL-48 cells.
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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with pruritus and characteristic distribution and morphology of skin lesions.[1,2] Sweat is one of the exacerbation factors of AD,[3,4] and reported as an antigen for type I allergy in 77% patients with AD.[5] Cholinergic urticaria (ChU) is a subtype of urticaria where small reddish wheals develop in response to sweating or the increase of body core temperature. We previously reported that the purified sweat antigen induces degranulation of basophils of patients with AD and those with ChU.[6] Type I antigenicity of the purified sweat antigen was endorsed by the sensitization of a human mast cell line with IgE purified from sera of patients with AD and neutralization of the histamine releasing activity of the purified sweat by sera of the patients.[5] We finally have identified MGL_1304 secreted by *Malassezia globosa* as a sweat antigen for patients with AD and ChU.[7,8] We also reported that levels of MGL_1304-specific IgE in sera of patients with AD were significantly higher than those of healthy subjects and correlated with the severity of AD by ELISAs using purified MGL_1304 and recombinant MGL_1304 expressed by *Escherichia coli* (TF-rMGL_1304).[8] In Japan, a histamine release test (HRT) against sweat antigen, which mainly contains MGL_1304, has been commercially available since 2010 (Allerport® HRT, MANUFACTURER: Shionogi & Co., Ltd., Osaka, Japan, DISTRIBUTOR: Kyowa Medex Co., Ltd., Tokyo, Japan). However, purification of MGL_1304 from human sweat or culture supernatant of *M. globosa* is both time-consuming and costly. Alternatively, TF-rMGL_1304 can be generated at low cost. However, it needs a large chaperon protein to be solubilized and lacks the original glycosylation of yeasts. Thus, another recombinant protein, that is

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similar to the native protein in both glycosylation and conformational structure, is desirable for clinical applications. In this study, we generated recombinant MGL_1304 using a yeast cell line, *Pichia pastoris*, and studied its features and usefulness for clinical applications to patients with sweat allergy.

Methods

Subjects

One-hundred thirty five subjects were included in this study. Nineteen patients with AD and 11 patients with ChU (15 men and 15 women; mean age ± SD: 31.7 ± 16.4 years) were investigated by HRT, and 34 patients with AD (13 men and 21 women; mean age ± SD: 26.2 ± 13.0 years; mean serum IgE ± SD, 4236.8 ± 5472.6 IU/ml) were studied by β-hexosaminidase release of RBL-48 cells. Fifty seven patients with AD (32 men and 25 women; mean age ± SD: 29.8 ± 11.7 years) and 18 healthy individuals (9 men and 9 women; mean age ± SD: 26.6 ± 7.2 years) were subjected to the measurement of the P-rMGL_1304-specific IgE in sera by the sandwich ELISA. Blood samples from patients with AD and ChU were obtained following written informed consent, and the studies were approved by the Ethics Committee of Hiroshima University Institute of Biomedical & Health Sciences.

Generation of recombinant MGL_1304 protein

A recombinant MGL_1304 using *P. pastoris* (P-rMGL_1304) was generated with EasySelect™ *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA). The cDNA of MGL_1304 were subcloned into pPICZα-A *Pichia* expression vector and the plasmid DNA were prepared using One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen). *P. pastoris* (G-115 strain) were transformed using the *Pichia* EasyComp™ procedure. After confirming that the phenotype of the transformants was Mut^+, recombinant *Pichia* strains were cultured for 4 or 5 days. For the secreted recombinant protein (P-rMGL_1304), the culture supernatant was purified by batch method using nickel resin (ProBond™, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instruction. For the intracellular P-rMGL_1304, cell pellets was lysed by the disposable homogenizer (Treff pellet mixers®, Treff AG, Degersheim, Switzerland), and then purified with the same procedure as the secreted recombinant protein. A recombinant human mucin-like 1 (P-rMUCL1), which has not been shown histamine release ability to peripheral blood basophils of patients with AD, was generated as a negative control with the same procedures as P-rMGL_1304. Trigger factor (TF)-fused rMGL_1304 protein (TF-rMGL_1304) was prepared as described previously.[7]

Preparation of native antigen (sup-MGL_1304)

MGL_1304 in the culture supernatant of *M. globosa* (sup-MGL_1304) was isolated as described previously.[7]

Determination of protein concentration

The concentrations of P-rMGL_1304, P-rMUCL1, sup-MGL_1304, and TF-rMGL_1304 were measured by bicinchoninic acid (BCA) protein assay. The immunological amount of MGL_1304 contained in P-rMGL_1304 was determined by the sandwich ELISA as described previously,[8] with a slight modification. Briefly, 10 μg/ml of the mouse monoclonal IgG antibody against purified sweat antigen (Smith-2) was used as capture antibody, and 1:40 diluted the AD standard serum was used as detection antibody. The concentration of sup-MGL_1304 measured by BCA protein assay was used as standard.

Western blot analysis

Samples were loaded into an SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane as reported elsewhere.[7] The membranes were incubated with anti-Penta-His antibody (Qiagen, Hilden, Germany), 1:100 diluted sera of patients with AD or healthy individuals, or 1 μg/ml of Smith-2 at 4 °C overnight. The membrane-bound primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. All images were adjusted by using "auto levels" in Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Histamine release test with peripheral blood basophils of patients with AD or ChU

HRTs with peripheral blood basophils were performed as described previously.[9] Cells were stimulated with 1 μg/ml of goat anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA) and various concentrations of TF-rMGL_1304, P-rMGL_1304, sup-MGL_1304, or P-rMUCL1.

Measurement of β-hexosaminidase release of RBL-48 cells

RBL-48 cells, a rat basophilic leukemia cell line expressing the alpha chain of human high-affinity IgE receptor on the cell surface, were provided by Dr John Hakimi (F. Hoffmann-La Roche, Nutley, NJ, USA).[10] RBL-48 cells (0.3×10^6/ml) were sensitized with 30 times-diluted sera of patients with AD at 37 °C overnight. After the incubation, culture medium was removed and cells were washed for three times with PIPES-buffered saline (25 mM PIPES: pH 7.2, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA) and incubated with the same buffer at 37 °C for 10 min. The cells were then stimulated with 1 μg/ml of TF-rMGL_1304 and various concentrations of P-rMGL_1304 or sup-MGL_1304 for 15 min. The exocytosis of cells was quantified by measuring the hexosaminidase activity of the supernatants as previously described.[11]

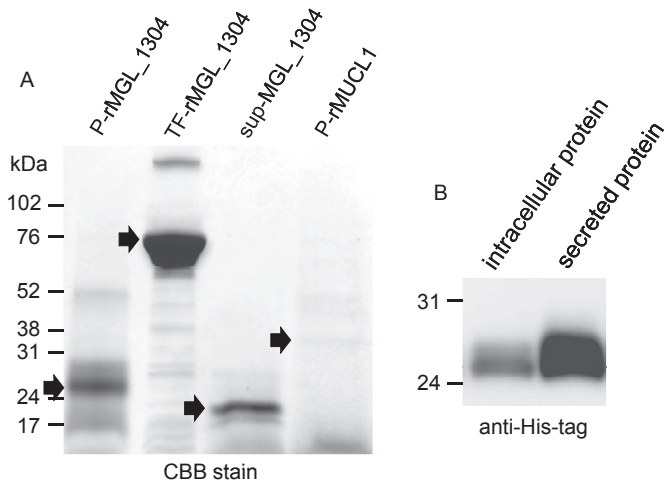

Fig. 1. (A) P-rMGL_1304, TF-rMGL_1304, supMGL_1304 and P-rMUCL1 were electrophoresed and stained with CBB. (B) The secreted and intracellular forms of P-rMGL_1304 were electrophoresed and blotted with anti-His-tag antibody. A representative data of three independent experiments was shown for each panel.

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Measurement P-rMGL_1304-specific IgE of patients with AD and healthy individuals

P-rMGL_1304-specific IgE in sera of patients with AD and healthy individuals were measured by sandwich ELISA as described previously,[8] with a slight modification. In this study, 1 μg/ml of P-rMGL_1304 was used as the antigen, instead of 1 μg/ml of QRX (purified MGL_1304 from human sweat).

Results

Antigenecity of P-rMGL_1304

P-rMGL_1304, TF-rMGL_1304, sup-MGL_1304, and P-rMUCL1 used in the following experiments were stained with Coomassie Brilliant Blue G-250 (Fig. 1A). The molecular mass of P-rMGL_1304 was approximately 26 kDa. The secreted protein and intracellular protein, which were both detected by anti-His-tag antibody, migrated similarly in SDS-PAGE (Fig. 1B). Serum IgE of AD recognized TF-rMGL_1304, P-rMGL_1304, and sup-MGL_1304, but not the negative control, P-rMUCL1, and serum IgE of a healthy individual hardly recognized those proteins (Fig. 2). Monoclonal antibodies against MGL_1304 showed a diversity of affinities to these recombinants and native protein. Smith-2 recognized P-rMGL_1304 and supMGL_1304 stronger than TF-rMGL_1304 (Fig. 3). As expected, P-rMUCL1 was not recognized by Smith-2 (Fig. 3).

P-rMGL_1304 evokes histamine release from basophils of patients with AD or ChU at the concentration equivalent to sup-MGL_1304

Both P-rMGL_1304 and TF-rMGL_1304 showed histamine release from basophils of patients with AD or ChU. However, the concentrations of P-rMGL_1304 and TF-rMGL_1304, for the maximum histamine release were around 0.01 μg/ml and 1 μg/ml, respectively (Fig. 4A). Therefore, the histamine releasing ability of P-rMGL_1304 was 100 times higher than that of TF-rMGL_1304 in weight/ml basis. Taking into account the molecular mass of these protein, 26 kDa for P-rMGL_1304 and 75 kDa for TF-rMGL_1304, the difference is approximately 30 times in terms of molecular basis. P-rMUCL1, a negative control protein produced by *P. pastoris*, did not evoke histamine release from basophils of patients with AD or ChU at any concentration (Fig. 4A). The histamine releasing abilities of P-rMGL_1304 and sup-MGL_1304 were equivalent, when their immunological concentrations were measured by sandwich ELISA (see Methods) (Fig. 4B). In 19 AD patients and 11 ChU patients, the correlation coefficient of percent histamine release between sup-MGL_1304 and P-rMGL_1304, and that between sup-MGL_1304 and TF-rMGL_1304 were 0.985 and 0.982, respectively, when peripheral blood basophils were stimulated with 10 ng/ml of P-rMGL_1304, 10 ng/ml of sup-MGL_1304, or 1 μg/ml of TF-rMGL_1304 (Fig. 4C). There were no significant differences in clinical characteristics, such as disease severity and total serum IgE between the patients who reacted to MGL_1304 and the patients who did not react to MGL_1304.

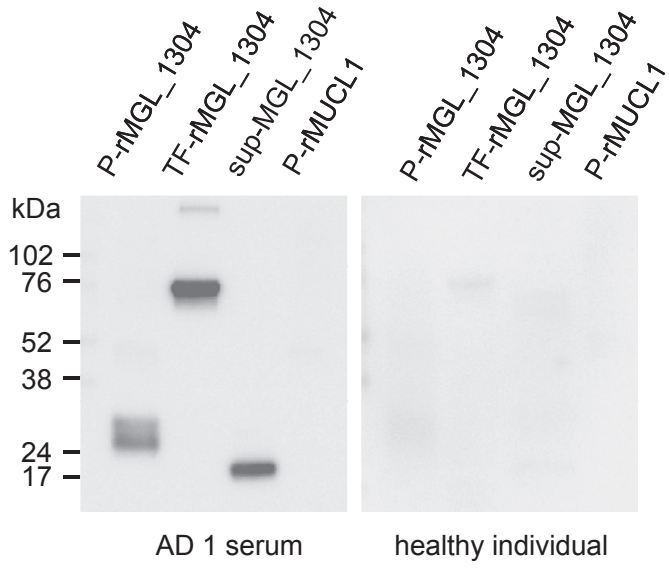

Fig. 2. P-rMGL_1304, TF-rMGL_1304, sup-MGL_1304, and P-rMUCL1 were electrophoresed and blotted with serum of a patient with AD and healthy individual. A representative data of three independent experiments was shown.

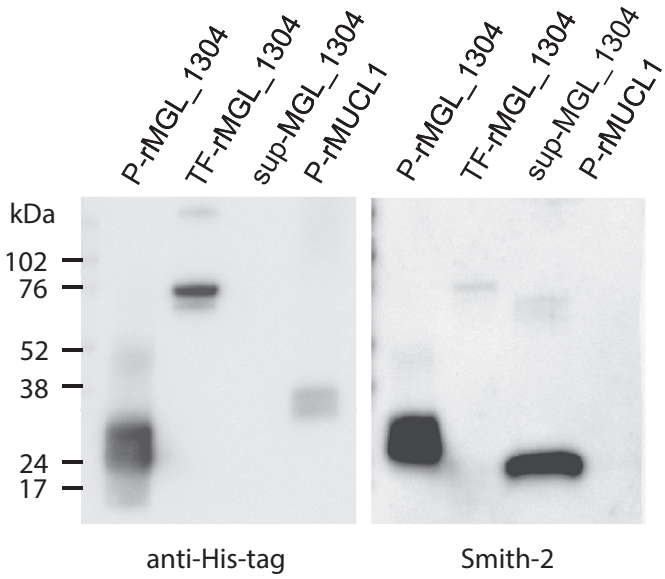

Fig. 3. P-rMGL_1304, TF-rMGL_1304, sup-MGL_1304, and P-rMUCL1 were electrophoresed and blotted with anti-His-Tag antibody and Smith-2. A representative data of three independent experiments was shown for each panel.

P-rMGL_1304 and sup-MGL_1304 similarly cause degranulation of RBL-48 cells

The stimulation of sup-MGL_1304 and P-rMGL_1304 evoked degranulation of RBL-48 cells sensitized with sera of patients with AD. The dose–response curves of these two recombinants were quite similar, when compared by immunological concentrations of MGL_1304 (Fig. 5A). The optimal concentration of both sup-MGL_1304 and P-rMGL_1304 for the degranulation of RBL-48 was approximately 10 ng/ml, which is similar to that for HRT. On the other hand, TF-rMGL1304 induced no or only small amount of degranulation of RBL-48 cells. The stimulation of P-rMUCL1 did not induce degranulation of RBL-48 cells (data not shown). In 34 patients with AD, the correlation coefficient of percent release of β-hexosaminidase between sup-MGL_1304 and P-rMGL_1304, and that between sup-MGL_1304 and TF-rMGL_1304 were 0.885 and 0.627, respectively, when RBL-48 cells were stimulated with 10 ng/ml of P-rMGL_1304, 10 ng/ml of sup-MGL_1304, or 1 μg/ml of TF-rMGL_1304 (Fig. 5B).

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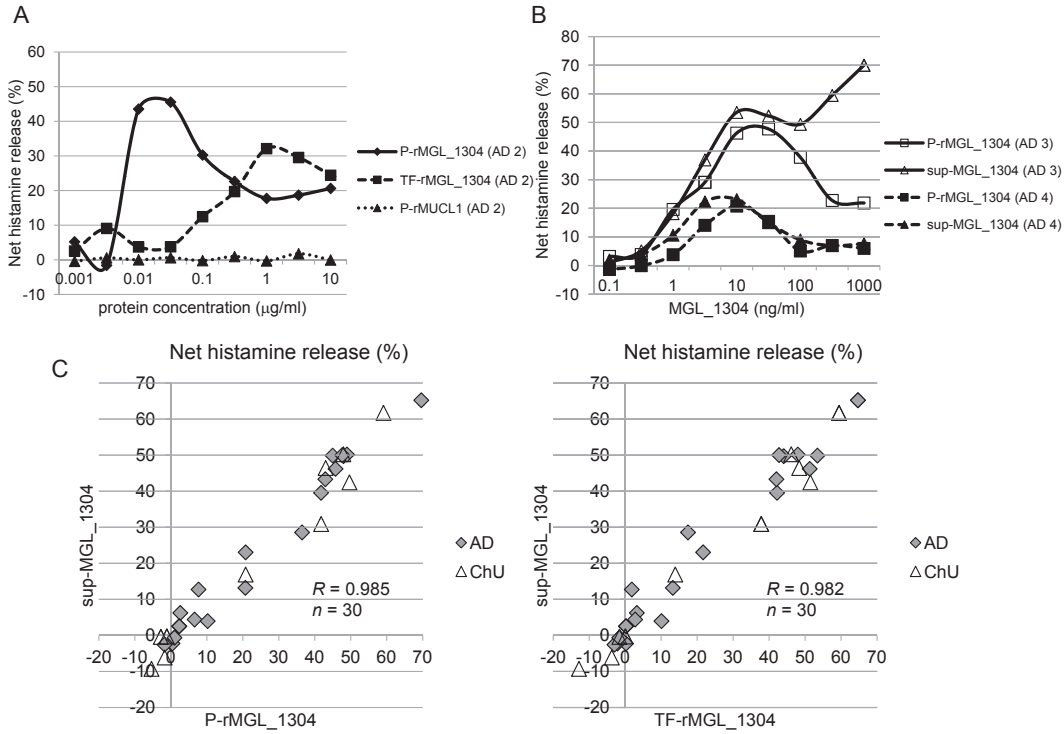

Fig. 4. Peripheral blood basophils of patients with AD or ChU were stimulated with series of concentrations of P-rMGL_1304, TF-rMGL_1304, or P-rMUCL1, and then net histamine release (%) were measured. The concentration of P-rMGL_1304 and TF-rMGL_1304 were normalized by BCA protein assay (A), or by the sandwich ELISA system (B) (see Methods). (C) Peripheral blood basophils were stimulated with 10 ng/ml of P-rMGL_1304, 10 ng/ml of sup-MGL_1304 or 1 μg/ml of TF-rMGL_1304. The correlation coefficient of net histamine release (%) between sup-MGL_1304 and P-rMGL_1304 or sup-MGL_1304 and TF-rMGL1304 were shown.

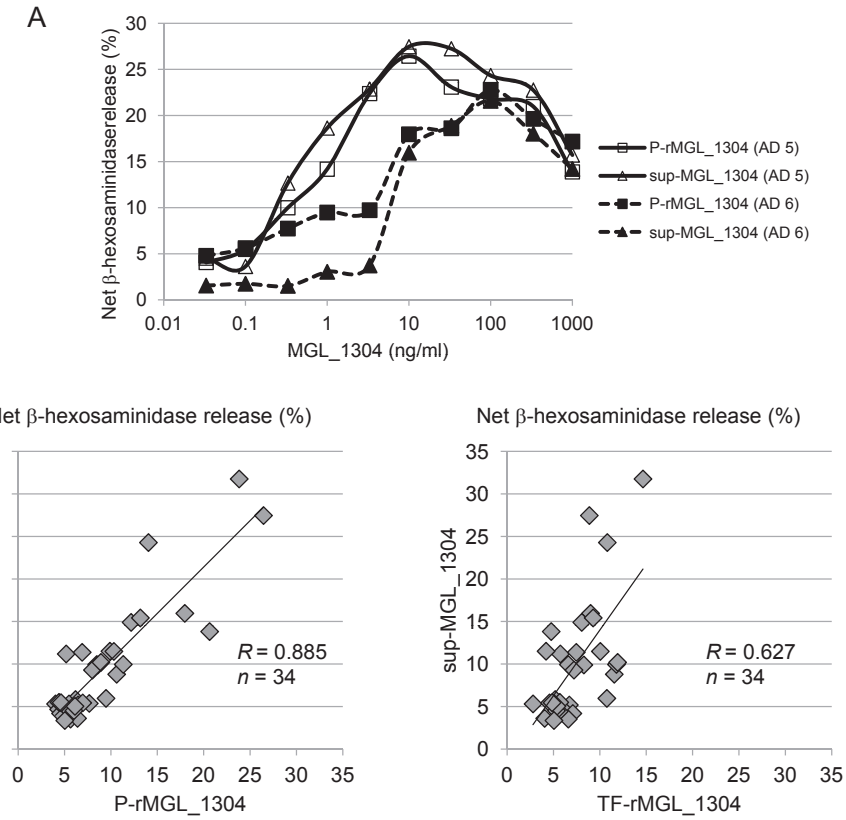

Fig. 5. (A) RBL-48 cells were sensitized with two patients with AD, stimulated with series of concentrations of P-rMGL_1304 and sup-MGL_1304, and net β-hexosaminidase release (%) were measured. The concentrations of MGL_1304 were normalized by the sandwich ELISA. (B) RBL-48 cells were sensitized with sera of patients with AD, stimulated with 10 ng/ml of P-rMGL_1304, 10 ng/ml of sup-MGL_1304, or 1 μg/ml of TF-rMGL_1304, and release of β-hexosaminidase were measured. The correlation coefficient of net β-hexosaminidase release (%) between sup-MGL_1304 and P-rMGL_1304, or sup-MGL_1304 and TF-rMGL1304 were shown.

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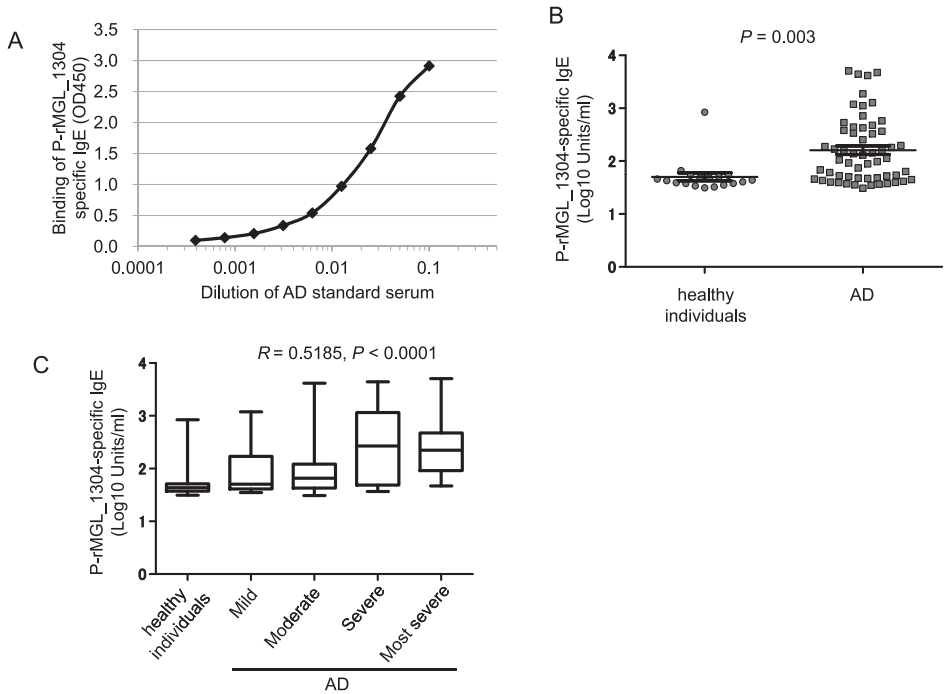

Fig. 6. (A) A standard curve of P-rMGL_1304-specific IgE plotted by using AD standard serum in ELISA. (B) Serum levels of P-rMGL_1304-specific IgE in patients with AD and healthy individuals were plotted and analyzed by Mann–Whitney test. (C) The levels of P-rMGL_1304-specific IgE in sera of patients with various severities of AD and healthy individuals were analyzed by Spearman rank correlation.

P-rMGL_1304-specific IgE of patients with AD is correlated with severity of AD

We successfully measured MGL_1304-specific IgE in sera of patients with AD by the ELISA system using P-rMGL_1304 as the antigen, instead of QRX (Fig. 6A). As the levels of IgE against purified MGL_1304 and those against TF-rMGL_1304 reported previously, the levels of P-rMGL_1304-specific IgE in sera of patients with AD were significantly higher than those of healthy individuals (Fig. 6B) and correlated with the severity of AD (Fig. 6C).

Discussion

In this study, we generated recombinant MGL_1304 by using *P. pastoris* and investigated its antigenicity by comparing with TF-rMGL_1304 produced by *E. coli* and native MGL_1304. In western blot analysis, serum IgE of patients with AD similarly bound to these proteins. However, in degranulation assays using peripheral blood basophils and RBL-48 cells, P-rMGL_1304, but not TF-rMGL_1304, showed an antigenicity comparable to the native protein.

P. pastoris is a methylotrophic yeast which widely used for heterologous protein expression.[12,13] The AOX1 gene of *P. pastoris* metabolizes methanol in the medium as its sole source of carbon. *P. pastoris* is easy to culture, and the recombinant protein is readily purified in large quantities at a low cost, because almost no other exogenous proteins are contained in culture medium for *P. pastoris*. The recombinant protein is produced intracellularly or extracellularly, with many eukaryotic posttranslational modifications such as glycosylation, disulfide bond formation and proteolytic processing.[12,13] In comparison with other protein expression systems such as mammalian cells or insect cells, it is superior in terms of costs and yield of proteins.

The antigenicity of P-rMGL_1304 was similar to the native protein, but it was slightly different from TF-rMGL_1304 analyzed by western blot analysis using the monoclonal antibodies. The affinity for monoclonal antibodies might be altered by the post-translational modification by yeast. Tag and chaperon proteins connected to MGL_1304 may also interfere the antigen–antibody interactions. P-rMGL_1304 possesses a polyhistidine tag in its C-terminus, and TF-rMGL_1304 possesses a chaperon protein, TF, in its N-terminus. Binding studies with various truncated proteins of MGL_1304 revealed that Smith-2 binds to the N-terminal region of MGL_1304,[7] suggesting that N-terminal TF might attenuate the binding of monoclonal Smith-2, but not polyclonal IgE of AD patients, to TF-rMGL_1304.

The advantage of diagnosing sweat allergy by using humanized mast cells is the functional evaluation of IgE against MGL_1304 without requiring fresh basophils from patients. However, TF-rMGL_1304 did not cause degranulation of RBL-48 cells bearing serum IgE of patients with AD which was shown to bind to TF-rMGL_1304 in western blot analysis. On the other hand, P-rMGL_1304 causes equivalent degranulation of RBL-48 cells, suggesting that P-rMGL_1304 is more similar to the native protein than TF-rMGL_1304. The reason for the difference between these recombinants in the assay with RBL-48 is unknown. This assay may not represent activities of basophils and mast cells of patients themselves, but may overcome disadvantages of HRT such as the necessity of discontinuation of drugs and failure of evaluation of the subjects with non-responding basophils.

In conclusion, it is considered that P-rMGL_1304 has the conformational structure and antigenicity quite similar to native MGL_1304. We believe that this recombinant protein not only improves the diagnosis of sweat allergy, but also facilitates the development of new desensitization therapy against MGL_1304 for the future.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

TK, TH, and MiH designed the study and wrote the manuscript. TK, KI, MH, YY and AT contributed to data collection. All authors read and approved the final manuscript.

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