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Original Article

Different hypersensitivities against homologous proteins of MGL_1304 in patients with atopic dermatitis

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AD	atopic dermatitis
HRT	histamine release tests
M.	<i>Malassezia</i>
RACE	rapid amplification of cDNA ends
TF	Trigger Factor

ABSTRACT

Background: Atopic dermatitis (AD) is exacerbated by sweating, and the skin of most patients with AD are resided by *Malassezia* (*M.*) fungi. Recently, MGL_1304 produced by *Malassezia globosa* was identified as the major histamine releasing antigen in human sweat.

Methods: The full length cDNA of the counterpart of MGL_1304 in *Malassezia restricta* (Mala r 8), was cloned by degenerate PCR and rapid identification of cDNA ends (RACE). Recombinant MGL_1304, and its counterparts, Mala s 8 (produced by *Malassezia sympodialis*) and Mala r 8 were prepared, and compared in their allergenicities by dot blot analysis and histamine release tests with sera and basophils of patients with AD.

Results: The identities between MGL_1304 and Mala s 8, MGL_1304 and Mala r 8, and Mala s 8 and Mala r 8 were 68%, 78%, and 76%, respectively, in protein sequences. Dot blot analysis revealed that the level of IgE binding to Mala s 8 was higher than that of MGL_1304. However, histamine release tests revealed that MGL_1304 and Mala r 8 possessed higher activity than Mala s 8. In addition, the crude lysate of *M. globosa* showed higher histamine release ability than that of *M. sympodialis*.

Conclusions: Patients with AD showed hypersensitivities against MGL_1304 and its homologs. However, the allergenicities of the homologs are variable and the histamine release activities may be different from the solid-phase binding activities for IgE. Sweat allergy should be carefully evaluated with biological activities of MGL_1304 and its homologs of other *Malassezia* fungi residing on the skin.

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Introduction

Atopic dermatitis (AD) is a chronic relapsing eczematous skin disease characterized by pruritus and inflammation, accompanied by cutaneous physiological dysfunction (dry and barrier-disrupted skin).¹ Most patients possess atopic diathesis which are defined as personal or family history of atopic diseases, and a predisposition to the overproduction of immunoglobulin E (IgE).¹ Sweat is one of the major exacerbation factors for atopic dermatitis across all patient age groups.² Recently, we discovered MGL_1304 secreted from *Malassezia* (*M.*) *globosa* as a major histamine releasing antigen contained in human sweat.³ Recombinant MGL_1304 induced histamine release from basophils of patients with AD. We

established an ELISA system measuring immunoglobulins (IgE, IgG, and IgG4) specific against MGL_1304 and found that the levels of MGL_1304-specific IgE correlated with severity of AD.⁴

Malassezia is a skin colonizing fungi related to pityriasis versicolor, *Malassezia* folliculitis, seborrheic dermatitis, and atopic dermatitis.⁵ Fourteen *Malassezia* species were reported to exist and 3 species (*M. globosa*, *Malassezia restricta*, and *Malassezia sympodialis*) were predominant in human skin in Japanese population.⁶ The ratio of *M. globosa* and *M. restricta* varied according to the severity of AD.⁷

Interestingly, a homologous protein of MGL_1304 is conserved in other *Malassezia* fungi and certain kinds of mushrooms. Mala s 8,⁸ which was reported as an allergen for patients with AD, is a counterpart of MGL_1304 in *M. sympodialis*. The allergenicity of Mala s 8 has only been estimated by binding of IgE, but not by functional ability such as histamine release. The homologous protein in another major *Malassezia*, *M. restricta*, was not registered in any accessible databases.

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Molecular-based allergy diagnostics is an approach used to map the allergen sensitization of a patient at a molecular level, using purified natural or recombinant allergenic molecules (allergen components) instead of allergen extracts, and it allows for an increased accuracy in allergy diagnosis.⁹ However, the measurement of specific IgE against *Malassezia* fungi is performed by mixed crude lysates of *Malassezia* spp. In western blot analysis, MGL_1304 was a minor allergen in the crude lysate of *M. globosa*, although it was a major histamine release allergen in human sweat.³ Therefore, component resolved diagnosis might be needed for the sweat and *Malassezia* allergy.

In this study, we cloned the homologous molecule of MGL_1304 in *M. restricta* and *M. sympodialis*, and investigated their allergenicities to patients with AD.

Methods

Subjects

Sixty-four patients with AD (34 males/30 females; mean age $\pm$ SD: 28.2 ± 11.3 ; mean serum IgE $\pm$ SD: 6775.7 ± 10766.2 IU/ml; mean serum thymus and activation-regulated chemokine $\pm$ SD: 2644.5 ± 3142.6 pg/ml) who were diagnosed by the criteria of Japanese Dermatological Association (JDA),¹ were recruited and investigated by western blot/dot blot analysis and histamine release tests (HRT). These patients were divided into 4 subgroups showing different disease severities (mild: 18; moderate: 23; severe: 12; most severe: 11) by using severity index of Japanese guideline for AD.² These studies were approved by the Ethics

Committee of Hiroshima University Institute of Biomedical & Health Sciences (Eki-630, June 6, 2012).

Extraction of nucleotides from *Malassezia* species

M. globosa (CBS 7966), *M. sympodialis* (CBS 7222), and *M. restricta* (CBS 7877) were obtained from American Type Culture Collections (Manassas, VA, USA). Extraction of genomic DNA from these fungi was performed as described previously.⁶ Extraction of total RNA and generation of cDNA by reverse transcriptase were performed as described previously.³

Degenerate PCR and rapid amplification of cDNA ends (RACE)

Five degenerate primers (F1, F2, R1, R2, and R3) compatible to the amino acids-conserved regions between MGL_1304 (GenBank: XP_001732036) and Mala s 8 (GenBank: CAA09886) were designed as shown in Figure 1 and Table 1. The degenerate PCR was performed with genomic DNA extracted from *M. restricta* and combinations of these primers. The amplified DNA fragment was cloned into *Kpn* I site of pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA) and DNA sequence of the insert was confirmed. The full length sequence of the counterpart of MGL_1304 in *M. restricta* was determined by RACE.¹⁰ Based on the DNA sequence of the fragment, 3'-RACE and 5'-RACE were performed by using 3'-Full RACE Core Set (Takara Bio, Shiga, Japan) and 5'-Full RACE Core Set (Takara Bio), respectively, according to the manufacturer's instructions with specific primers shown in Table 1.

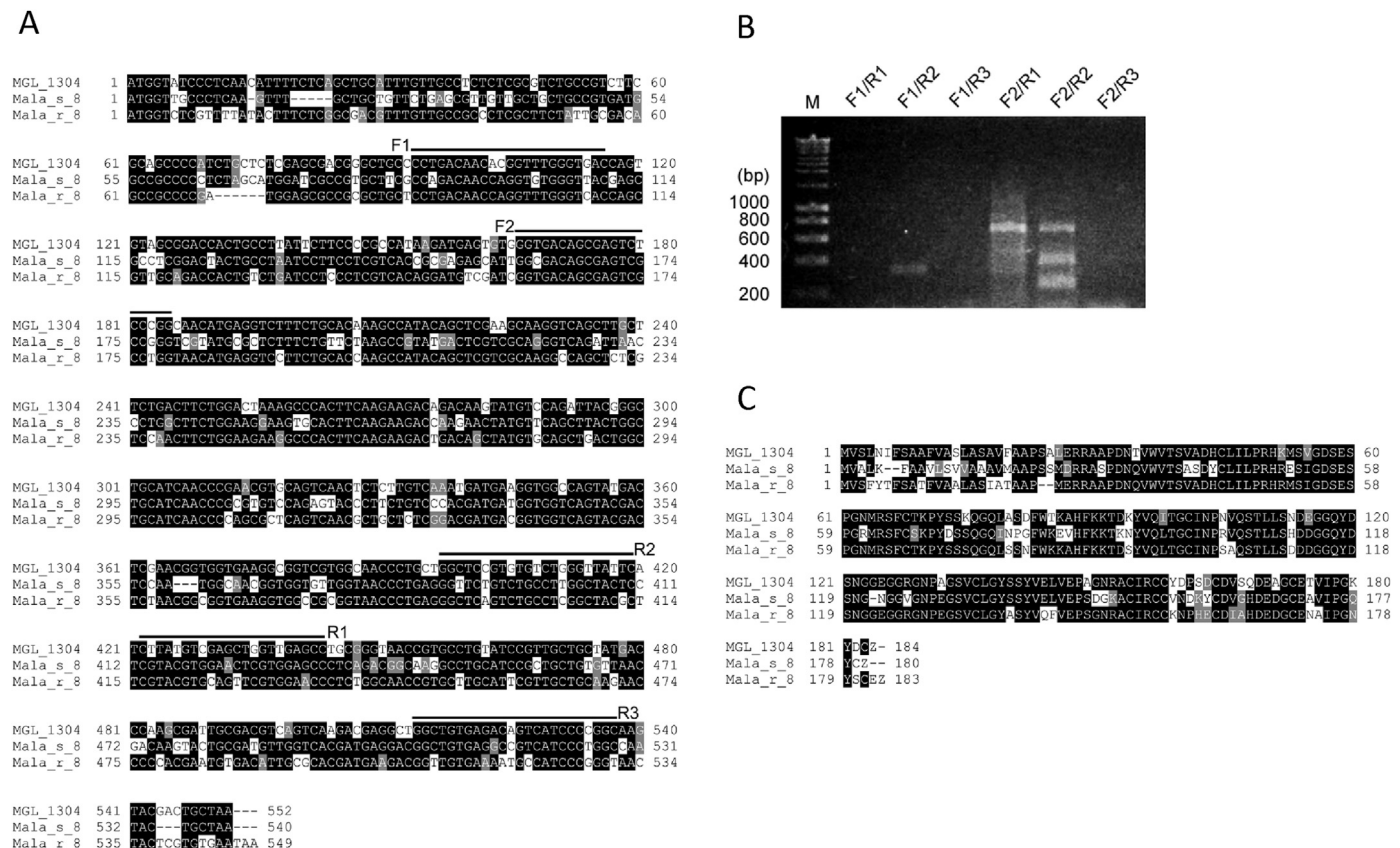


Fig. 1. (A) Multiple DNA sequence alignment of MGL_1304 and its homologs. Positions of degenerate primers (F1, F2, R1, R2, and R3) were highlighted by the lines. A black-, gray-, and normal-background character indicated a residue identical, not identical but similar, not identical nor similar, to the column-consensus. (B) Degenerate PCR with genomic DNA of *M. restricta* and degenerate primer pairs. Amplified PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide. (C) Multiple protein sequence alignment of MGL_1304 and its homologs. Multiple DNA (A) and protein (C) sequence alignments were performed by ClustalW and shaded with BoxShade.

Table 1

Primers.

Degenerate primers
F1: 5'-CCNGACAACNNGGTNTGGGTNAC-3'
F2: 5'-GGNGACAGCGAGTCNCCNGG-3'
R1: 5'-GGCTCNACNAGYTCNACRTANG-3'
R2: 5'-GARTANCCNAGRCANACRGARCC-3'
R3: 5'-GCCNGGGATGACNGNCTCACAGCC-3'
3'-RACE primer
5'-GGGGTACCACTGACAGCTATGTGCAGCTGAC-3'
5'-RACE primers
A1: 5'-GATCGACATCCTGTGACGAG-3'
A2: 5'-GACAGTGGTCTGCAACGCTG-3'
S1: 5'-GCGAGTCGCTGCTGAACATG-3'
S2: 5'-TTCTGACCAAGCCATACAG-3'
Cloning primers
Mala s 8 forward: 5'-GGGGTACCGTTGCCCTCAAGTTTGTGCTGCTG-3'
Mala s 8 reverse: 5'-CCCAGCTTGACGTTACACTTTAGCAGTATTGG-3'
Mala r 8 forward: 5'-GGGGTACCGTCTCTGTTTATCTTCTCTCGGC-3'
Mala r 8 reverse: 5'-GCTCTAGAACACAAGCCATTATTCACACGAG-3'

Underlines indicated restriction enzyme sites.

Generation of recombinant proteins by Escherichia coli

The recombinant MGL_1304 was generated as described previously.³ The cDNA of Mala s 8 (GenBank: AJ011958) and Mala r 8 (Fig. 1A) were amplified by PrimeSTAR GXL DNA Polymerase (Takara Bio) with specific primer pairs shown in Table 1. These DNA fragments were digested by *Kpn* I/*Hind* III or *Kpn* I/*Xho* I, respectively, and then ligated into the same sites of pCold TF vector (Takara Bio). The recombinant proteins of Mala s 8 and Mala r 8 were generated by *E. coli* (JM109, Takara Bio) as previously described.³ Briefly, the cultures from single clones of JM109 which contained pCold TF vectors were lysed with xTractor buffer kit (Takara Bio) and purified by HisTARON™ gravity columns purification kit (Takara Bio), according to the manufacturer's instructions. Protein concentrations were measured by Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot analysis

The recombinant proteins were denatured with SDS sample buffer in non-reducing conditions, loaded into SDS-PAGE gel (e-Pagel, ATTO, Tokyo, Japan), and then transferred to a polyvinylidene fluoride membrane (Immobilon P, EMD Millipore, Billerica, MA, USA) as reported elsewhere.¹¹ The membrane was incubated with anti-Penta-His antibody (Qiagen, Hilden, Germany) at 4 °C overnight. The membrane bound primary antibodies were visualized with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA) and chemiluminescence (ECL Prime, GE Healthcare, Buckinghamshire, UK).

Dot blot analysis

The recombinant proteins (5 µg) were spotted onto nitrocellulose membrane (BioRad, Hercules, CA, USA) and air dried. The membranes were blocked with Blocking One (Nakalai Tesque, Kyoto, Japan) and then incubated with 1:1000 diluted sera of patients with AD at 4 °C overnight. The membrane bound IgE was visualized with HRP-conjugated anti-human IgE antibody (KPL, Gaithersburg, MD, USA) and chemiluminescence. The blotting data were acquired by LAS-1000 Image analyzer (Fujifilm, Tokyo, Japan) as TIFF files and the integrated densities of individual spots were quantified by Image J.¹²

Histamine release tests

HRT with basophils obtained from peripheral blood were performed as described previously.¹³ Cells were stimulated with 1 µg/

ml of goat anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA), 1 µg/ml of TF-rMGL_1304, TF-rMala s 8, and TF-rMala r 8. The basophil donors did not respond to the positive control (anti-IgE) were omitted for the analysis.

Results*Cloning of the counterpart of MGL_1304 in M. restricta*

Degenerate PCR of genomic DNA from *M. restricta* using a primer pair, F1/R2 (Fig. 1A) successfully amplified single DNA fragment which migrated at approximately 320 bp (Fig. 1B). Based on the DNA sequence of the fragment, 3'- and 5'-RACE were performed. The full length sequence of Mala r 8, the counterpart of MGL_1304 in *M. restricta*, was identified (Fig. 1A). The identities of DNA sequences between MGL_1304 and Mala s 8, MGL_1304 and Mala r 8, and Mala s 8 and Mala r 8 were 69%, 75%, and 72%, respectively (Fig. 1A). In protein sequences, the identities between MGL_1304 and Mala s 8, MGL_1304 and Mala r 8, and Mala s 8 and Mala r 8 were 68%, 78%, and 76%, respectively (Fig. 1C).

The levels of IgE binding against MGL_1304 and its homologs

The recombinant proteins of MGL_1304, Mala s 8, and Mala r 8 were generated as fusion proteins with Trigger Factor (TF).³ Western blot analysis with anti-His tag antibody revealed that the amounts and migrate distances of these antigens were similar (Fig. 2A). To estimate the levels of IgE binding against these antigens in sera of patients with AD, dot blot analysis was performed. As shown in Figure 2B, the levels of IgE binding against these homologs were almost similar. However, IgE binding against Mala s 8 was slightly, but significantly higher than that against MGL_1304. The disease severity of AD and IgE binding to MGL_1304 (Fig. 2C), Mala s 8 (Fig. 2D), and Mala r 8 (Fig. 2E) showed weak but significant correlation. Among them, the highest correlation coefficient was seen in MGL_1304 (Fig. 2C) and was similar to the result with ELISA.⁴

Histamine release against MGL_1304, its homologs and crude lysates of Malassezia fungi

To estimate histamine release ability of these homologous antigens, we performed histamine release tests using basophils of patients with AD. In contrast to the result of dot blot analysis, the levels of histamine release against MGL_1304 and Mala r 8 were significantly higher than that of Mala s 8 (Fig. 3A). The level of histamine release against the crude lysate of *M. globosa* was also significantly higher than that of *M. sympodialis* (Fig. 3B). No apparent difference in histamine release was observed between lysates of *M. restricta* and those of *M. sympodialis* (Fig. 3B). The levels histamine release by all MGL_1304 homologs correlated with each other (Fig. 4A–C). Among them, the highest correlation coefficient ($r = 0.8679$) was observed between MGL_1304 and Mala r 8 (Fig. 4B). In contrast to these components, all levels of histamine release induced by lysates of three *Malassezia* fungi correlated well with each other ($r > 0.7$) (Fig. 4D–F). The levels of basophil histamine release induced by MGL_1304 homologs and crude lysates of their source *Malassezia* fungi correlated but were not identical (Fig. 5).

Discussion

In this study, we identified and cloned a counterpart of MGL_1304 in *M. restricta* (i.e. Mala r 8), then generated recombinant proteins of MGL_1304, Mala s 8, and Mala r 8 by *E. coli*. Dot blot

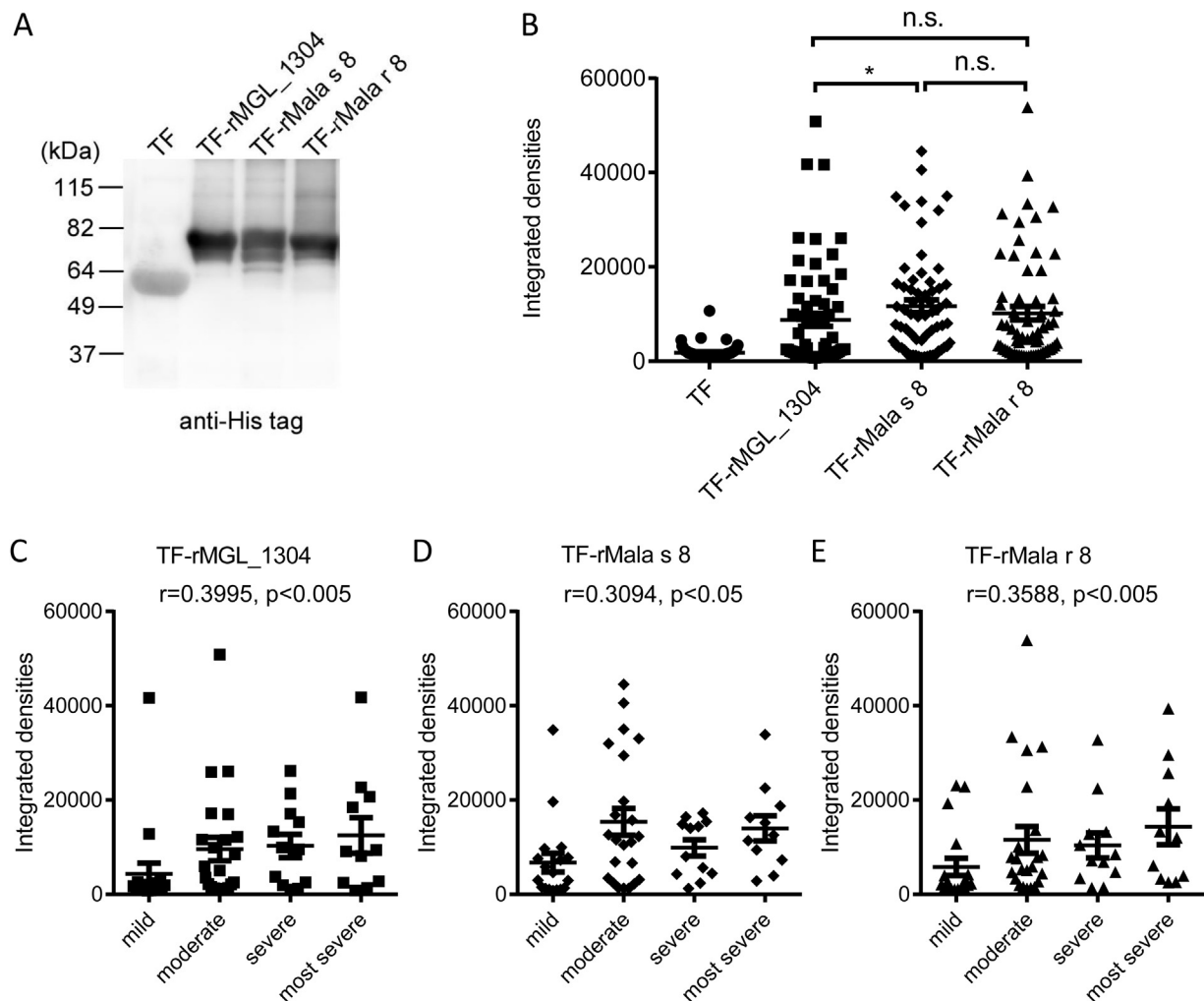


Fig. 2. (A) Trigger factor (TF)-fused recombinant MGL_1304 (TF-rMGL_1304) and its homologs. Samples were electrophoresed and blotted with anti-His tag antibody. (B) Dot blot analysis of recombinant MGL_1304 and its homologs by sera of whole patients with AD. Data were expressed as integrated densities of individual spots corresponding to TF alone, MGL_1304, Mala s 8, and Mala r 8, and analyzed by the Kruskal–Wallis test. * $p < 0.05$; n.s., not significant. (C–E) Data of dot blots (B) were replotted with 4 patient groups with AD showing different severities (mild, moderate, severe, and most severe) and Spearman's correlation coefficients (r) were calculated.

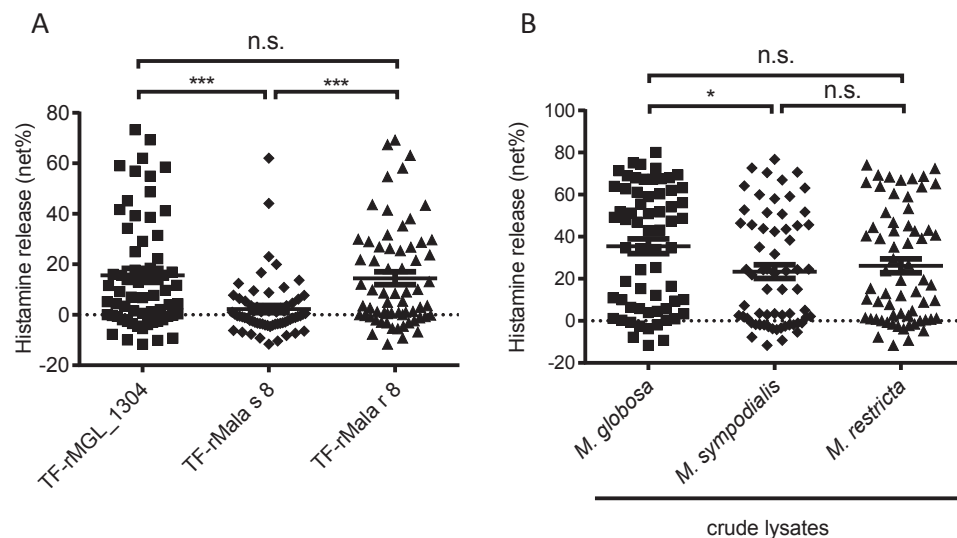


Fig. 3. Histamine release tests of recombinant MGL_1304 and its homologs (A), and crude lysates of major *Malassezia* fungi (B) by basophils of patients with AD. Data were expressed as histamine release (net%) and analyzed by the Kruskal–Wallis test. *** $p < 0.0001$; * $p < 0.05$; n.s., not significant.

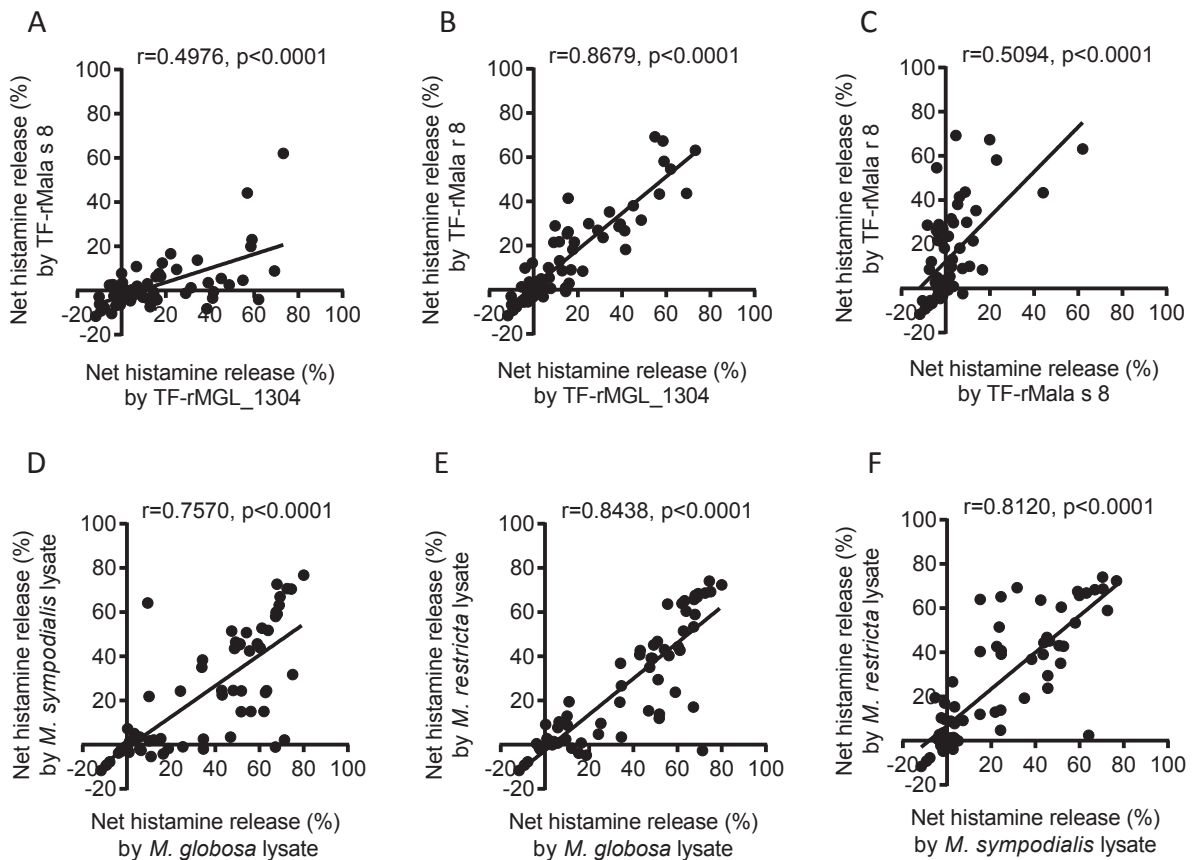


Fig. 4. Correlation of histamine release between MGL_1304 homologs (A–C) and between by crude lysates of their source *Malassezia* fungi (D–F). Data were expressed as histamine release (net%) and Spearman's correlation coefficients (r) were calculated.

analysis with sera of patients with AD showed that serum IgE binding to Mala s 8 was higher than that of MGL_1304. In contrast to IgE binding to solid-phased antigens, histamine release tests revealed that MGL_1304 and Mala r 8 showed higher histamine release than that of Mala s 8. Histamine release against crude lysates of *Malassezia* fungi showed that the lysate of *M. globosa*, but not *M. restricta*, showed higher histamine release ability than that

of *M. sympodialis*. The correlation coefficients between histamine release by the components and those by the crude lysates were quite different. The reason might be that Mala s 8 was not a representative antigen generated by *M. sympodialis*.

The 13 *Malassezia* antigens (Mala f 2–4, Mala s 1, and Mala s 5–13) have been submitted and recognized by the WHO/IUIS Allergen Nomenclature Sub-committee and two antigens were

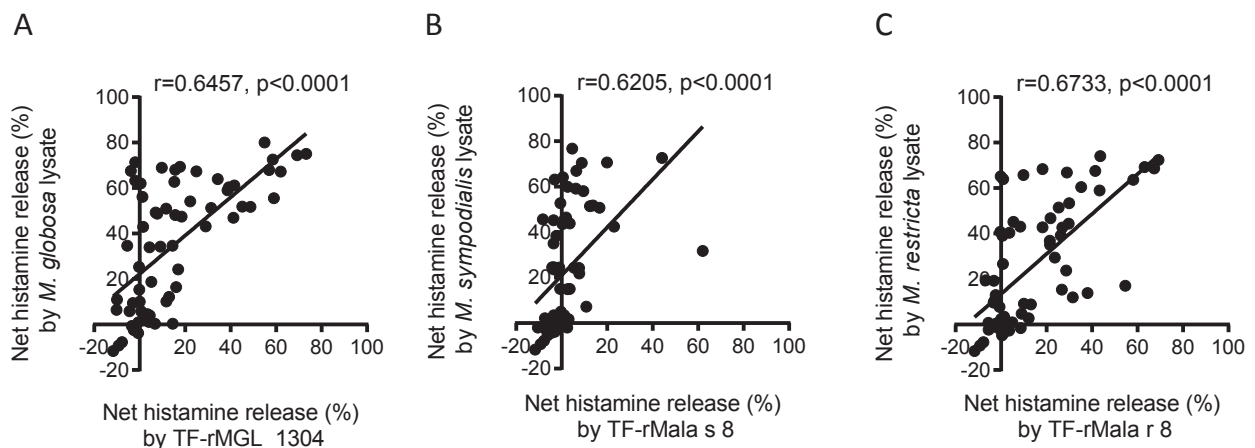


Fig. 5. Correlation of histamine release between MGL_1304 homologs and crude lysates of their source *Malassezia* fungi. Data were expressed as histamine release (net%) and Spearman's correlation coefficients (r) were calculated.

recently reported.^{3,14} The 13 official antigens were from *M. sympodialis* and *Malassezia furfur* which grow rapidly in agar. *M. sympodialis* is considered as most predominant *Malassezia* fungi in European countries,¹⁵ mainly by culture-based methods. In contrast to European countries, in Asian countries including Japan, *M. restricta* and *M. globosa* were reported as predominant *Malassezia* fungi in healthy¹⁶ and diseased skin¹⁷ by non-culturing PCR-based method. Since *M. restricta* is slow-growing, it had been covered by rapid-growing *Malassezia*, such as *M. sympodialis*, in the culture-based method. In this study, the MGL_1304 homolog in *M. restricta* showed comparable histamine release ability in patients with AD, suggesting its abundance in skin of AD and the pathogenic role in AD.

The similarity between MGL_1304 and Mala s 8 was less than 70% in protein sequences. On the other hand, the similarity between MGL_1304 and Mala r 8, and Mala r 8 and Mala s 8 were higher than 70%. Considering the patterns of similarity of these homologs, it is feasible that patients with AD may be primarily sensitized with MGL_1304 and then cross react with Mala r 8 and Mala s 8. Since the epitope of MGL_1304 for IgE is likely a tertiary structure of protein,³ amino acid residues crucial for the difference in allergenicity between MGL_1304 and Mala s 8 remained unclear. Moreover, the solubility to human sweat on the skin may also important in terms of the antigenicity of *Malassezia*-derived proteins. We recently established an assay for MGL_1304-like immunoreactivity and revealed that human sweat contain 3.76 ± 2.64 ng/ml MGL_1304-like immunoreactivity. The antibody to detect MGL_1304 used in this assay weakly cross-reacted with Mala r 8, but not Mala s 8 (data not shown). The measurement of the other MGL_1304 homologs in human sweat, using assays specific for each homolog is warranted to reveal the causative mechanism of sweat allergy and adequate target for treatments to be developed.

In conclusion, patients with AD showed heterogeneous hypersensitivity against MGL_1304 and its homologs. MGL_1304 and Mala r 8 showed higher histamine release activities for basophils of patients with AD than Mala s 8. The crude lysates of source *Malassezia* fungi could not be substitutes of the components, MGL_1304 homologs. Estimating the function and concentrations of MGL_1304 homologs, but not crude *Malassezia* fungi, on the skin surface is needed to further our understanding of sweat allergy.

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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, TH, MaH, AK, and KI contributed to data collection. All authors read and approved the final manuscript.

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