

ORIGINAL ARTICLE

Molecular analysis of *Malassezia* microflora in the lesional skin of psoriasis patients

Misato AMAYA,¹ Mami TAJIMA,¹ Yukari OKUBO,¹ Takashi SUGITA,²
Akemi NISHIKAWA,³ Ryoji TSUBOI¹

¹Department of Dermatology, Tokyo Medical University, Shinjuku-ku, Departments of ²Microbiology and ³Immunology, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan

ABSTRACT

Systemic and focal infections by microorganisms have been known to induce or exacerbate psoriasis. To investigate the role of *Malassezia* species in the development of psoriasis, we analyzed the *Malassezia* microflora in psoriasis patients using a nested polymerase chain reaction (PCR) assay, and compared it with those in atopic dermatitis (AD) patients and healthy subjects. Fungal DNA was directly collected from the lesional and non-lesional skin of the trunk of 22 psoriasis patients by applying a transparent dressing. The extracted DNA was amplified by using specific primers designed for the PCR in the intergenic spacer or internal transcribed spacer area of the ribosomal RNA. All nine of the *Malassezia* species were detected at different rates from the 22 psoriasis patients. The overall detection rates in lesional and non-lesional skin of *M. restricta*, *M. globosa* and *M. sympodialis* were high (96%, 82% and 64%, respectively), whereas the detection rates of the other species were relatively low. However, there was no difference in the rates between lesional and non-lesional skin areas. The average number of *Malassezia* species detected in overall sites of the psoriasis patients was 3.7 ± 1.6 species, although this fact showed no correlation with the severity of the symptoms. The number of *Malassezia* species detected was 4.1 ± 1.9 in the AD patients, and 2.8 ± 0.8 in the healthy subjects, suggesting that the skin microflora of psoriasis patients and AD patients show greater diversity than that of healthy subjects.

Key words: *Malassezia*, nested polymerase chain reaction, psoriasis.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease associated with genetic, immunological and environmental factors.^{1–3} Systemic and focal infections by microorganisms including hemolytic streptococci have been known to trigger guttate psoriasis or exacerbate chronic plaque psoriasis.^{3,4} Peripheral blood mononuclear cells (PBMC) from psoriasis patients showed a significantly increased proliferative response to group A streptococcal superantigens,^{5,6} and produced more inflammatory cytokines.^{6–9} We previously demonstrated that peripheral monocytes from psoriasis patients expressed a higher level of

bacterial 16S ribosomal RNA and fungal 18S rRNA genes than those from healthy subjects, suggesting that psoriatic monocytes are activated by infection or colonization by certain microorganisms.¹⁰

Yeasts of the genus *Malassezia* are resident microflora of human and animal skin, and colonize the sebaceous gland-rich areas of the skin, including the chest, back, face and head. *Malassezia* has been identified as a causative organism in pityriasis versicolor,^{11,12} and has also been cited as an aggravating factor in various skin diseases, including atopic dermatitis (AD) and seborrheic dermatitis.^{11,13–15}

Previous studies have suggested the involvement of *Malassezia* in the development of psoriasis. In an

Correspondence: Ryoji Tsuboi, M.D., Ph.D., Department of Dermatology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. Email: tsuboi@tokyo-med.ac.jp

Received 19 Feb 2007; accepted 15 March 2007.

early study, all 10 of the psoriasis patients developed psoriatic lesions at sites where a heat-killed, sonicated suspension of *Malassezia* was applied.¹⁶ PBMC as well as T-cell lines cultured from the psoriasis lesions had a proliferative response¹⁷ or produced more cytokines⁹ to the *Malassezia* extracts, and chemotactic C5a anaphylatoxin was generated by the incubation.¹⁸ In addition, systemic ketoconazole was effective in limited cases of scalp psoriasis.¹⁹

Due to the difficulties in culturing *Malassezia* species, the correlation between cutaneous microflora and the clinical manifestations of certain cutaneous diseases have not been well investigated. In 1996, Guého *et al.* revised the taxonomy of the genus *Malassezia* based on a molecular phylogenetic analysis.²⁰ Since then, further studies using molecular biological techniques subdivided the genus *Malassezia* into 11 species,^{21–23} and examined the *Malassezia* microflora of various skin diseases.^{12,14,15} *M. globosa* and *M. restricta* are the two major isolates from Japanese subjects,¹⁴ while *M. pachydermatis* and *M. nana* were found to colonize animal skin.²⁴

As far as we could determine, there is only one report on the distribution of *Malassezia* species in

psoriasis vulgaris patients, which analyzed fungal microflora sampled from three psoriasis patients.²⁵ The aim of our study is to analyze the *Malassezia* microflora in psoriasis patients using a nested polymerase chain reaction (PCR) assay, and to compare the results with those from AD patients and healthy subjects. Fungal DNA was directly collected from the lesional skin of psoriasis patients by applying a transparent dressing, and the extracted DNA was amplified by using specific primers for each *Malassezia* species.

MATERIALS AND METHODS

Subjects sample collection

Twenty-two psoriasis patients with moderate symptoms, who visited Tokyo Medical University Hospital as outpatients, were enrolled in this study (Table 1). They included 19 males and three females whose ages ranged 22–71 years (average, 54.5 years). The psoriatic area and severity index (PASI) scores ranged 6.6–33.6 (average, 13.7). Twenty patients had received treatment including topical corticosteroid, vitamin D3 analog ointment, systemic etretinate, systemic cyclosporine A, or psoralen and ultraviolet

Table 1. Summary of the clinical severity, treatments and the number of *Malassezia* species detected in psoriasis patients

Patients (no.)	Age (years), gender	PASI (score)	Treatments [†]	No. of <i>Malassezia</i> spp.
1	43, M	8.4	VitD3	5
2	38, M	33.6	TS	4
3	66, M	7.8	ETR, VitD3	7
4	75, F	18.6	TS, VitD3	3
5	47, M	21.6	TS, VitD3, CYA	5
6	72, M	16.2	VitD3	3
7	57, M	6.6	TS, VitD3	2
8	60, M	10.8	ETR, VitD3	2
9	54, M	12.3	ETR, VitD3, TS	4
10	55, M	7.5	TS	2
11	56, M	13.2	TS, VitD3	5
12	71, M	11.4	TS, VitD3	5
13	44, M	10.2	TS, VitD3	2
14	55, M	11.2	TS, PUVA	7
15	66, F	7.5	–	4
16	31, M	11.2	TS, VitD3	4
17	22, F	14.1	TS, VitD3	2
18	67, M	8.4	VitD3, CYA	4
19	37, M	25.5	TS, VitD3	4
20	52, M	15.7	TS, VUD3	4
21	67, M	9.7	–	2
22	64, M	19.8	TS, PUVA	2

[†]Treatments: TS, topical steroids; VitD3, vitamin D3 analogue ointment; ETR, systemic etretinate; CYA, systemic cyclosporine A; PUVA, psoralen and ultraviolet A light. PASI, psoriasis area and severity index.

A (PUVA) therapy (Table 1). Two patients had not received any treatment. Written informed consent was obtained from each subject prior to the procedure. To assess the distribution of *Malassezia* microflora in the psoriatic skin, our previous data from 36 AD outpatients (24 males and 12 females), aged 20–64 years (average, 33.3), and 30 healthy control subjects (12 males and 18 females), aged 20–53 years (average, 22.8), were used for comparison.¹⁵

Malassezia samples were collected by applying OpSite transparent dressings (3 cm × 7 cm; Smith and Nephew Medical, Hull, UK) three times to both lesional and non-lesional skin of the trunk of the psoriasis patients.

DNA extraction and detection of *Malassezia* DNA

Malassezia DNA was extracted according to the previously reported method.¹⁴ Briefly, the OpSite film was trimmed at the center (9 cm²), placed in a 1.5-mL Eppendorf tube with 1 mL of lysing solution (100 mmol Tris-HCl [pH 8.0], 30 mmol ethylene

diamine tetra acetate [EDTA; pH 8.0], and 0.5% sodium dodecyl sulfate) and incubated for 15 min at 100°C. The suspension was transferred to another new tube, combined with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and centrifuged at 13,200 g. The aqueous phase was transferred to another tube, combined with chloroform-isoamyl alcohol (24:1, vol/ vol) and centrifuged at 13,200 g. The DNA was precipitated with 2.5 volume of ethanol in the presence of 3 mol sodium acetate and Ethatimate (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The DNA pellet was resuspended in 30 µL of TE (10 mmol Tris-HCl [pH8.0], 1 mmol EDTA [pH8.0]) and stored at –20°C until use.

Nested PCR was conducted using two sets of primers, as shown in Table 2, by the method of Morishita *et al.*¹² The species-specific primers were derived from the internal transcribed spacer (ITS) region or intergenic spacer region (IGS) of the rRNA gene. The DNA extracted (10 µL) from each sample

Table 2. Oligonucleotides used in the nested polymerase chain reaction (PCR) at the intergenic spacer region or internal transcribed spacer region of the rRNA gene

Species and primers used		Sequence (5'U 3')	Annealing temperature (°C)
First PCR for six <i>Malassezia</i> species [†]	Forward	ATCCTTTGCAGACGACTTGA	55
	Reverse	TGCTTAAC TTCGAGATCGG	
First PCR for three <i>Malassezia</i> species [‡]	Forward	ACCTGCAGAAGGATCATTAGTGA	56
	Reverse	TCCTCCGCTTATTGATATG	
First PCR for each <i>Malassezia</i> species			
<i>M. dermatis</i>	Forward	CGCACCTTGCCTCCATGGT	58
	Reverse	AGCCTGGTTTCCCAGGCAGCGG	
<i>M. furfur</i>	Forward	TGTGTACCATAGGCACCCAC	58
	Reverse	CACGGTGATAAAGGGATGCA	
<i>M. globosa</i>	Forward	TCGAGTGCATACCACACTCGAG	57
	Reverse	TACGGTGCTTTCACGGTTCT	
<i>M. japonica</i>	Forward	CGTATGTGGATCTTATCCTAT	44
	Reverse	TGACTAGTGTCTAGGCACGGTA	
<i>M. obtusa</i>	Forward	CATGGTCCATCTCCCACACA	60
	Reverse	AGAGAGTGCGTGGCGCATGGT	
<i>M. restricta</i>	Forward	CGACCTAGTCGACTACATCCTACT	55
	Reverse	TTCGGAGATACAAGCCTCCAT	
<i>M. slooffiae</i>	Forward	ACGCACGCTAACACAACGTG	60
	Reverse	TGTGCGATTGAAGCGCACA	
<i>M. sympodialis</i>	Forward	CGCACCTTGCCTCCATGGT	58
	Reverse	GGTACAATCCCCAGGCAGVAA	
<i>M. yamatoensis</i>	Forward	CGATCAAACCTCTCTGTGTCCAG	59
	Reverse	TGTGTGGGAGGTAGAAGAGGCA	

[†]Common primers for *M. furfur*, *M. globosa*, *M. japonica*, *M. obtusa*, *M. restricta* and *M. yamatoensis*. [‡]Common primers for *M. dermatis*, *M. slooffiae*, *M. sympodialis*.

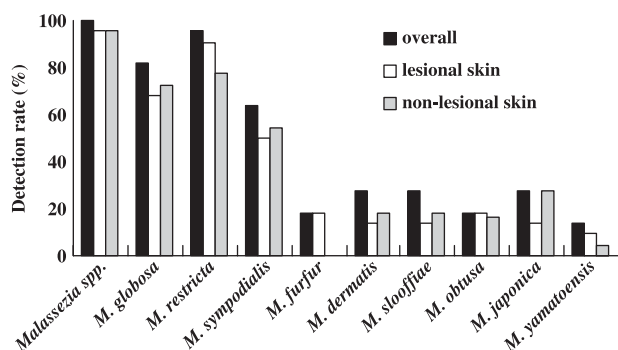


Figure 1. Detection rate of *Malassezia* DNA in samples from the lesional and non-lesional skin of psoriasis patients.

was added to 40 μ L of PCR master mixture, which consisted of 5 μ L of 10 $\times$ PCR buffer (Takara), 4 μ L of 200 μ mol deoxynucleoside triphosphates, 10 pmol of each primer, and 0.5 U of Takara Ex *Taq* DNA polymerase (Takara). PCR was performed in a thermocycler (model 9700, Applied Biosystems, Foster City, CA, USA). In the nested PCR step, 1 μ L of the first amplification product was added to a new reaction mixture with the same composition as the first. Of the currently accepted eleven *Malassezia* species, the animal-associated species, *M. nana* and *M. pachydermatis* were excluded from the present study.

RESULTS

Figure 1 shows the detection rate of each *Malassezia* species in the samples obtained from the overall, lesional and non-lesional trunk skin of psoriasis patients. All of the nine *Malassezia* species were detected at various rates from the 22 psoriasis patients. The overall detection rates in the lesional and non-lesional skin of *M. globosa*, *M. restricta* and *M. sympodialis* were high (82%, 96% and 64%, respectively), whereas the rates of *M. furfur*, *M. dermatis*, *M. slooffiae*, *M. obtusa*, *M. japonica* and *M. yamatoensis* were relatively low (18%, 27%, 27%, 18%, 27% and 14%, respectively). Unfortunately, there was no difference in the detection rates between lesional and non-lesional skin areas. Also, species-specific colonization was not observed to be dependent on age, gender, site, severity of psoriasis, or treatment (data not shown). As shown in Figure 2, the average number of *Malassezia*

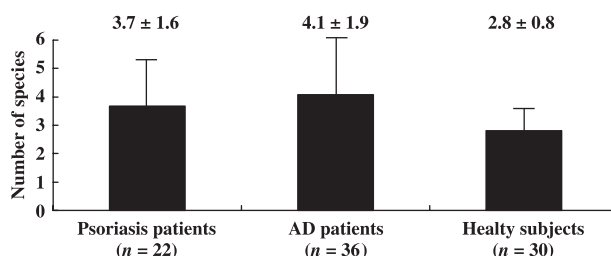


Figure 2. Number of *Malassezia* species detected in psoriasis patients, atopic dermatitis (AD) patients and healthy subjects. Mean $\pm$ SD.

species detected in overall sites of the psoriasis patients was 3.7 ± 1.6 species (varying from 2–7 species). This value did not correlate with the PASI score of the psoriasis patients (Table 1). We then compared the results with variations in AD patients and healthy subjects (Figure 2). The number of *Malassezia* species was 4.1 ± 1.9 in the AD patients, and 2.8 ± 0.8 in the healthy controls.¹⁵ The skin microflora of psoriasis patients and AD patients showed greater diversity than that of healthy subjects. However, the predominant species in psoriasis patients, AD patients and healthy subjects were *M. restricta* and *M. globosa*, and no significant species variation was observed (data not shown).

DISCUSSION

In the present study, we detected *M. restricta*, *M. globosa* and *M. sympodialis* at high rates, 96%, 82% and 64%, respectively, from the skin of psoriasis patients. This result suggests that more than one species routinely colonize a given psoriatic plaque on the trunk. However, we could not see any significant difference in the detection rates between lesional and non-lesional skin. In addition, there was no correlation between the number of *Malassezia* species found in psoriasis patients and the severity of the condition or previous treatments for the disease.

The high detection rate of *Malassezia* species was achieved by collecting fungal DNA directly from the lesional skin and amplifying it by PCR. By using conventional cultures and identifying the physiological properties of the organisms, Prohic reported the following rates for *Malassezia* microflora in scrapings of scalp skin from psoriasis patients: *M. globosa*,

55%; *M. slooffiae*, 18%; *M. restricta*, 10%; *M. furfur*, 8%; and *M. sympodialis*, 3%.²⁶ Similarly, Gupta *et al.* reported the microflora on the scalp and other body sites from 28 psoriasis patients and found higher detection rates from the scalp and forehead rather than the trunk and extremities. The detection rate overall was: *M. globosa*, 57.7%; *M. sympodialis*, 30.8%; and *M. slooffiae*, 11.5%.²⁷ The detection rate in the conventional culture method is significantly lower compared with our culture-independent direct method. Our data are noteworthy in that the samples from which they were derived were taken from the trunk and not from the scalp, an area frequently colonized by *Malassezia* species. High detection rates for *M. restricta* using a non-culture method were also reported in two out of three psoriasis patients.²⁵ The higher detection rate for *Malassezia* species, especially in *M. restricta*, by the culture-independent direct method was observed not only in psoriasis patients but also in pityriasis versicolor, seborrheic dermatitis and AD patients, as well as healthy subjects in Japan.^{12,14,15}

Although our data suggested that *Malassezia* microflora in psoriasis and AD patients is more diverse than in healthy subjects, we could not observe any significant differences in species distribution regarding (depending on) lesional versus non-lesional skin, or psoriasis patients versus healthy subjects. As mentioned previously, PBMC from psoriasis patients has been known to be activated by the *Malassezia* extracts,^{9,10,17,18} and induce a T-helper (Th)1 type immune response.^{1-3,9} The possibility that the *Malassezia* antigen induces a delayed-type hypersensitivity reaction leading to the Köbner phenomenon in the development of psoriasis cannot be ruled out.^{16,28}

At the current stage, it is uncertain why the frequency in the detection of *Malassezia* species remains unchanged in lesional and non-lesional skin. This article is the first to document the use of nested PCR as an alternative to the fungal culture in psoriasis patients. Further studies will likely involve the quantitative analysis of *Malassezia* microflora using a real-time PCR assay. Determining the genotype of the organisms will also provide more useful data in determining whether specific *Malassezia* species are involved as a pathogenetic factor in the development of psoriasis.

ACKNOWLEDGMENTS

This work was supported by the "High-Tech Research Center" Project for private universities: matching fund subsidy from the MEXT (Ministry of Education, Culture, Sports, Science and Technology of Japan, 2003–2007) (R.T.). This study was also supported in part by research grants from the Japan Society for the Promotion of Science (no. 16590127), the MEXT for an Open Research Center Project, and the IFO Foundation (T.S.).

REFERENCES

- 1 Gaspari AA. Innate and adaptive immunity and the pathophysiology of psoriasis. *J Am Acad Dermatol* 2006; **54**: S67–80.
- 2 Nickoloff BJ, Nestle FO. Recent insights into the immunopathogenesis of psoriasis provide new therapeutic opportunities. *J Clin Invest* 2004; **113**: 1664–1675.
- 3 Gudjonsson JE, Johnston A, Sigmundsdottir H, Valdimarsson H. Immunopathogenic mechanisms in psoriasis. *Clin Exp Immunol* 2004; **135**: 1–8.
- 4 Skov L, Baadsgaard O. Bacterial superantigens and inflammatory skin disease. *Clin Exp Dermatol* 2000; **25**: 57–61.
- 5 Baker BS, Powles AV, Malkani AK, Lewis H, Valdimarsson H, Fry L. Altered cell-mediated immunity to group A haemolytic streptococcal antigens in chronic plaque psoriasis. *Br J Dermatol* 1991; **125**: 38–42.
- 6 Baker BS, Bokth S, Powles A *et al.* Group A streptococcal antigen-specific T lymphocytes in guttate psoriatics lesion. *Br J Dermatol* 1993; **128**: 493–499.
- 7 Teranishi Y, Mizutani H, Murata M, Shimizu M, Matsushima K. Increased spontaneous production of IL-8 in peripheral blood monocytes from the psoriatic patients: Relation to focal infection and response to treatments. *J Dermatol Sci* 1995; **10**: 8–15.
- 8 Okubo Y, Koga M. Peripheral blood monocytes in psoriatic patients overproduce cytokines. *J Dermatol Sci* 1998; **17**: 223–232.
- 9 Kanda N, Tani K, Enomoto U, Nakai K, Watanabe S. The skin fungus-induced Th1- and Th2-related cytokine, chemokine and prostaglandin E2 production in peripheral blood mononuclear cells from patients with atopic dermatitis and psoriasis vulgaris. *Clin Exp Allergy* 2002; **32**: 1243–1250.
- 10 Okubo Y, Koga M, Kawashima H *et al.* Increased microorganisms DNA levels in peripheral blood monocytes from psoriatic patients using PCR with universal ribosomal RNA primers. *J Dermatol* 2002; **29**: 547–555.
- 11 Gupta AK, Batra R, Bluhm R, Boekhout T, Dawson TL. Skin diseases associated with *Malassezia* species. *J Am Acad Dermatol* 2004; **51**: 785–798.

- 12 Morishita N, Sei Y, Sugita T. Molecular analysis of *Malassezia* microflora from patients with pityriasis versicolor. *Mycopathologia* 2006; **161**: 61–65.
- 13 Nakabayashi A, Sei Y, Guillot J. Identification of *Malassezia* species isolated from patients with seborrheic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. *Med Mycol* 2000; **38**: 337–341.
- 14 Sugita T, Suto H, Unno T et al. Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects. *J Clin Microbiol* 2001; **39**: 3486–3490.
- 15 Tajima M, Sugita T, Nishikawa A, Tsuboi R. Molecular analysis of *Malassezia* microflora in seborrheic dermatitis patients: comparison with other diseases and healthy subjects. *J Invest Dermatol* (2007 in press).
- 16 Lober CW, PW Belew, EW Rosenberg, Gbale. Patch tests with killed sonicated microflora in patients with psoriasis. *Arch Dermatol* 1982; **118**: 322–325.
- 17 Baker BS, Powles A, Garioch JJ, Hardman C, Fry L. Differential T-cell reactivity to the round and oval forms of *Pityrosporum* in the skin of patients with psoriasis. *Br J Dermatol* 1997; **136**: 319–325.
- 18 Terui T, Rokugo M, Kato T, Tagami H. Analysis of the proinflammatory property of epidermal cyst contents: chemotactic C5a anaphylatoxin generation. *Arch Dermatol Res* 1989; **281**: 31–34.
- 19 Rosenberg EW, Belew PW. Improvement of psoriasis of the scalp with ketokonazole. *Arch Dermatol* 1982; **118**: 370–371.
- 20 Guého E, Midgley G, Guillot J. The genus *Malassezia* with description of four new species. *Antonie Leeuwenhoek* 1996; **69**: 337–355.
- 21 Sugita T, Takashima M, Shinoda T et al. New yeast species, *Malassezia dermatis*, isolated from patients with atopic dermatitis. *J Clin Microbiol* 2002; **40**: 1363–1367.
- 22 Sugita T, Takashima M, Kodama M, Tsuboi R, Nishikawa A. Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. *J Clin Microbiol* 2003; **41**: 4695–4699.
- 23 Sugita T, Tajima M, Takashima M et al. A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. *Microbiol Immunol* 2004; **48**: 579–583.
- 24 Hirai A, Kano R, Makimura K et al. *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. *Int J Syst Evol Microbiol* 2004; **54**: 623–627.
- 25 Paulino LC, Tseng CH, Strober BE, Blaser MJ. Molecular analysis of fungal microbiota in samples from healthy human skin and psoriatic lesions. *J Clin Microbiol*. 2006; **44**: 2933–2941.
- 26 Prohic A. Identification of *Malassezia* species isolated from scalp skin of patients with psoriasis and healthy subjects. *Acta Dermatovenerol Croat* 2003; **11**: 10–16.
- 27 Gupta AK, Kohli Y, Summerbell RC, Faergemann J. Quantitative culture of *Malassezia* species from different body sites of individuals with or without dermatoses. *Med Mycol* 2001; **39**: 243–251.
- 28 Rosenberg EW, Noah PW. The Köbner phenomenon and the microbial basis of psoriasis. *J Am Acad Dermatol* 1988; **18**: 151–158.