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Original Article

Molecular Characterization of Cyclophilin Protein Gene in Skin Normal Microflora: *Malassezia furfur*

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Abstract

Background: *Malassezia* are dimorphic, lipid-dependent yeasts, which are responsible for causing several cutaneous and systemic conditions. Although cyclophilins (CyPs) are highly conserved cytosolic proteins that catalyze the peptidyl-prolyl *cis-trans* isomerazation reaction before protein folding process, it has been suggestive of an allergen in a few numbers of fungi such as *Aspergillus fumigatus* and *Malassezia* species. Allergenic cyclophilins are IgE-binding components, which have been characterized in other species of *Malassezia*; and are considered as Mala s 6 in *Malassezia sympodialis*. In the present study we tried to identify the molecular characterization of cyclophilin gene in *M. furfur*.

Methods: Pairs of oligonucleotide primers were designed from highly conserved regions of the gene counterparts in other fungi. The primers were then applied to amplify the primer-specific DNA fragment. Afterward, PCR product fragments were sequenced to be used in further analysis.

Results: About 573 nucleotides, encoding a polypeptide of 190 amino acids, have been sequenced. Sequence comparison was performed in Gene Bank, both for the nucleotides and their deduced amino acid sequence. It revealed a significant homology with cyclophilin genes and proteins of other eukaryotic cells. The amino acid sequence of the encoded protein was about 86% identical to the sequence of cyclophilin protein from other fungi.

Conclusion: The molecular characterization of cyclophilin gene may open the way to disclosure of the functional characteristics of cyclophilin and is a fundamental step for understanding the molecular basis of its pathogenesis in AEDS disease.

Keywords: Malassezia furfur, *Atopic dermatitis, Fungal DNA, Cyclophilin, IgE binding proteins*

Introduction

Members of the genus *Malassezia* are dimorphic, lipid-dependent yeasts comprising 11 different species, including *Malassezia furfur* (1-4). The yeasts are all members of the skin microflora of human and warm-blooded animals. However, under certain conditions, they can act as opportunistic pathogens (5). It has been documented that several cutaneous diseases such as, pityriasis versicolour, seborrheic dermatitis, foliculitis, atopic eczema/dermatitis syndrome (AEDS) and also systemic infections including fungaemia, meningitis and pulmonary vasculitis are associated with *Malassezia* species (1,6). Apart from causing diseases, *M. furfur* is considered as an allergen-producing organism that can trigger the AEDS symptom (7, 8).

Atopic eczema/dermatitis syndrome (AEDS) is a chronic relapsing inflammatory skin disease, which its aetiology is not completely understood (3, 9). Approximately 30-80% of AEDS patients have an elevated level of *Malassezia*-specific IgE antibodies, positive skin prick test (SPT) and positive atopy patch test reactions (10). On the other hand, treatment of AEDS patient with antifungal agents resulted in clinical improvement (3). Therefore, *Malassezia* species may play a role in pathogenesis of the disease. In addition, a num-

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ber of studies suggest the important role of *M. furfur* in the pathogenesis of AEDS (3, 10, 11).
A number of IgE-binding components have been found in the yeast, of which Mala f 1 (36 kDa), Mala f 2 (21kDa), Mala f 3 (20 kDa) and Mala f 4 (35 kDa), have been characterized and produced as recombinant proteins using a phage display technique (5).
Allergenic cyclophilin (CyP) is another IgE-binding component that has been characterized in other species of *Malassezia*; and is considered as Mala s 6 in *M. sympodialis* (12).
Generally, CyPs are members of highly conserved cytosolic proteins, which play crucial role in protein folding through enzymatic catalysis of the peptidyl-prolyl *cis-trans* isomerazation reaction (13, 14). As the protein belongs to the family of immunophilins, (15) the complex of CsA, an immunosuppressive drug, with CyP, lead to suppress the signal transduction in T-cells (15, 16). However, CyP from a few number of fungi such as *Aspergillus fumigatus* and *Malassezia* species, notably *M. sympodialis* (12, 17), have been found as IgE-binding proteins (17).
The aim of the present study was to molecular characterization and analysis of the gene encoding CyP from lipophilic yeast, *M. furfur*. This study is the fundamental step for understanding the molecular basis of its pathogenesis in AEDS disease.

Material and Methods

Culturing of Malassezia isolates and species identification

Five clinical isolates were provided by Medical Mycology Laboratory of School of Public Health of Tehran University of Medical Science, Tehran, Iran. The isolates were cultured on solid Leeming and Notman Agar (LNA-agar) medium for 5 d at 32° C. The species were identified by using a PCR-RFLP method for identification of *Malassezia* species which has been described before (18). Briefly, to amplify the 26s rDNA region, 2 mm^3 of cultured yeast was placed in a 1.5 ml micro centrifuge tube containing 100µl of HPLC grade water. 1µl of the solution was transferred to a fresh 0.2 ml tube. Afterward, 12µl of PCR amplification mix (Amplicon^{TM}, Denmark) and 11µl of primer suspension was added. PCR amplification was performed in a thermal cycler (Techne, UK) with the following temperature profile: 1 cycle of 5 min at 94° C, followed by 30 cycles of 45s at 94° C, 1min at 55° C and 1.5 min at 72° C and a final extension step at 72°C for 5min. Five µl of PCR products were added to a new 1.5ml microcentrifuge tube containing 1.5 µl restriction enzyme buffer, 0.5µl of restriction enzyme *Cfo*I and 8µl of HPLC grade water. It was incubated at 37º C for 2 h.

Isolation of nucleic acids

Fungal genomic DNA was extracted from harvested *M. furfur* colonies using glass bead disruption (19). Briefly, about 5 mm^3 of cultured yeast was transferred to a 1.5 ml microcentrifuge tube and 300 mg of 0.5 mm diameter glass beads, 300 µg of lysis buffer (100mM Tris-Hcl pH 8, 10mM EDTA, 100mM NaCl, 1% sodium dodecyl sulfate) and 300µl of phenol chloroform-isoamyl alchohol (25:24:1) was added. The samples were shacked vigorously for 5 min, centrifuged for 5 min at 5000 rpm and the supernatant was transferred to a fresh tube. The supernatant extracted again with chloroform and DNA was precipitates by adding the same volume of isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2). The solution was vortexed and incubated for 10 min at -20° C and centrifuged for 15 min at 12000 rpm. The precipitant was washed with cold 70% ethanol, dried in air, dissolved in 50 µl of double distilled water and stored at -20° C till used for PCR.

PCR analysis

CyP gene was analyzed in two steps using different primer pairs. The primers were designed according to the data obtaining from DNA sequence fragments of cyclophilin gene in other fungi. The first part of the gene was defined using primer pair Moz-S1 (5'-ATG TCT AAC GTT TTC TTC G -3') as forward and Moz-As1 (5’ CAC

CGC ACT TGG CGA TC-3') as reverse primer. Both amplification reaction was performed in a final volume of 50µl containing 1µl of genomic DNA, 1.25U of thermo stable *Taq* DNA polymerase (Smar Taq™, Sinagene, Iran), 0.2 mM of deoxynucleoside triphosphate mix (dATP, dTTP, dGTP, dCTP), 1 mM of $MgCl_2$, 0.2µM of each primer and 10µl of 10X PCR buffer. PCR was carried out in a thermal cycler (Techne, UK) with the following tempreture profile: 1 cycle of 5 min at 94° C , followed by 35 cycles of 30s at 94° C, 1 min at 49° C and 1.5 min at 72° C and a final extension step at 72° C for 8min . The second primer pair Moz-S2 (5'-GAT GTC TAA CGT TTT CTT CGA CAT-3') as forward and Moz-As2 (5'-ATT TAGCAC ACA CCG CAC TT-3') as reverse primer were used to characterized the complete sequence of the gene. The same PCR protocol was used to carry out the second amplification reaction but the annealing temperature which has been changed to 52° C. Fifteen µl of each amplification products were loaded onto 1% agarose gel and run in TAE buffer (0.09M Tris, 0.09 M glacial acetic acid and 2mM EDTA, [pH 8.3]). The products were detected by staining with ethidium bromide (10mg/ml) and photographed.

Sequencing of the PCR fragments

Sequencing of the amplified DNA fragments were performed with the Dye Terminator Cycle Sequencing Kit (MWG, Germany), by using the amplified double stranded DNA fragments as template and synthetic 21-meric primers. Sequencing of each part was repeated at least three times for both strands. The nucleotide sequence of the amplified DNA fragment was compared with the sequences of the same gene (cyclophilin) in other organisms using data on GenBank at National Centre for Biotechnology Information.

Results

Species identification

A single band of approximately 0.580 kbp was obtained from all five isolates after PCR amplification of 26s rDNA region (data not shown) and two distinct patterns were observed while the subsequent enzymatic digestion was performed. As Fig. 1 shows, two different bands of 0.05 and 0.25 kbp were generated by four isolates which demonstrated the banding pattern of *M. furfur* species. Whereas according to the obtained banding pattern of the latter isolate giving two different bands of 0.357 and 0.2 kbp, only one isolate is corresponded to *M. sympodialis.*. One strain of the four identified *M. furfur* was selected randomly to continue the investigation.

Isolation and characterization of cyclophilin (CyP) gene

Characterization of cyclophilin has been performed by PCR amplification of two parts of gene using overlapping primers. These two parts have covered the complete sequence of *M. furfur* cyclophilin. The first and second amplified part reveals a band with approximate amplicon size of 0.6 and 0.68 kbp respectively. (Fig. 2A, 2B). The cyclophilin nucleotide sequence and its deduced amino acid sequence are shown in Fig. 3. According to the result, the gene consists of 573 nucleotides encoding 190 amino acids. Comparison of the sequence of *M. furfur* cyclophilin gene with its counterparts in other organisms was carried out afterward. It demonstrated a significant homology with the cyclophilin genes of other *Malassezia* species, especially *M. yamatoensis* (97%) and of other eukaryotes such as *Aspergillus clavatus* (82%) and *Cryptococcus neoformance* (77%). Moreover, analysis of the amino acid sequence of cyclophilin protein reveals (98%) of homology with *M. yamatoensis*, (83%) with *Aspergillus clavatus*, (76%) with *Cryptococcus neoformance*, (72%) with *S. cerevisiae,* (81%) with *A. fumigatus* and (84%) with *Shizosaccharomycec pombe*.

Nucleotide and amino acid sequences of cyclophilin have been submitted to National Centre for Biotechnology Information GenBank and are available for public access under the accession Number; EU395812.

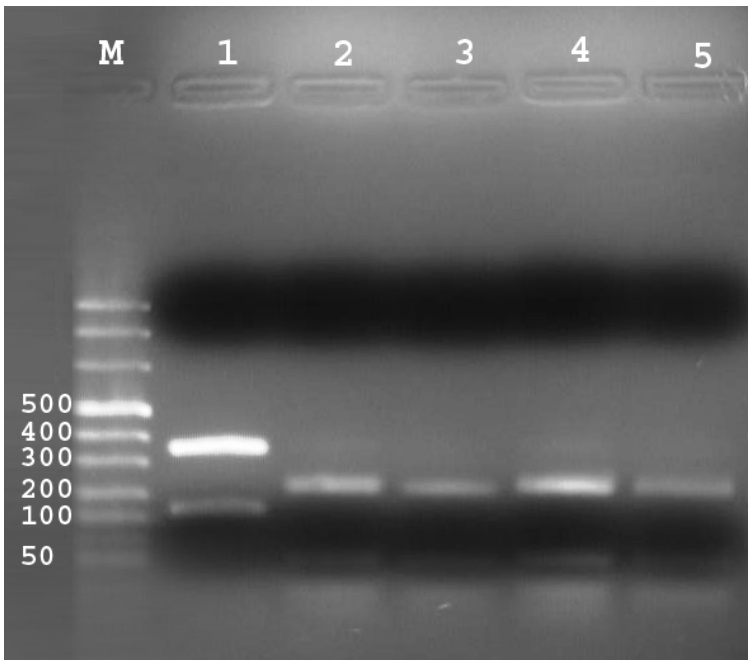

Fig. 1: Banding pattern of PCR product of five *Malassezia* clinical isolates digested by restriction enzyme *Cfu*I. From left to right: Marker (Roche, Germany), lane 1: *M. sympodialis*, lane 2-5: *M. furfur*

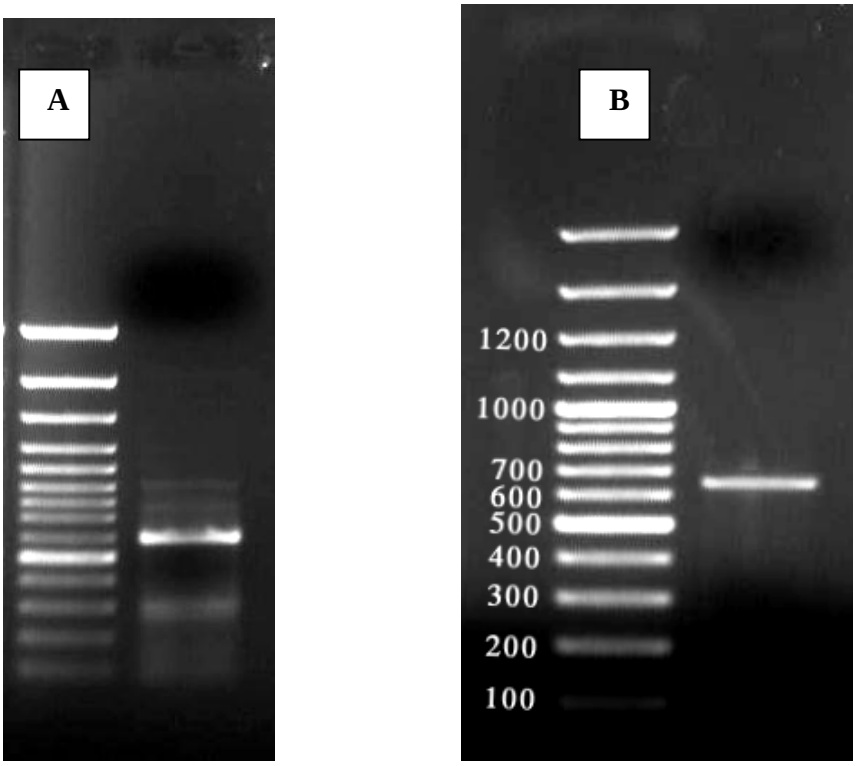

Fig. 2A: PCR product of the first part of the *M. furfur* cyclophilin gene. **Fig 2B.** PCR product of complete cyclophilin gene. Markers in both pictures were provided by Gene Ruler™ (fermentase, Canada) and were the same. Sizes are indicated in base pair

MSNVFFD ITKNGSPLGS IKFKLFDEVVPKTAANFRALATGEKGFGYEGSNFHRVIPDF
MLQGGDFTAGNGTGGKSIYGQKFADENFNLKHNKPGLLSMANAGPNTNGSQF

1 tctaacatca agattgattc ctgccagcgt aacactccga tcacgggcac actcaagatg
61 gccgcagtct ctgcacaaca ctgggtcagg ccaagtaacc tactcgtcaa agagcttgaa
121 cttgatggtg ccgaggggcg agccgttctt ggtg**atg**tct aacgttttct tcgacatcac
181 caagaacggc tcgcccctcg gctccatcaa gttcaagctc tttgacgatg ttgtgcccaa
241 gactgcggcc aacttccgtg cgctcgccac cggcgagaag ggcttcggct acgagggctc
301 caacttccac cgtgtgatcc ccgacttcat gctccagggc ggtgacttca ccgccggcaa
361 cggtaccggt ggcaagtcga tctacggcca gaagttcgct gacgagaact tcaacctcaa
421 gcacaacaag cctggcctgc tctcgatggc caacgccggc cccaacacga acggctcgca
481 gttcgtacgt atgctttgtg gatgtatgga gactaacact gctacagttc atcaccaccg
541 tggtgacccc ctgggtacgc acacgcctat gcacgctagt actaaccgca gctcgacggc
601 aagcacgtcg tcttcggcga ggtcgtcgac ggcatggacg tcgtcaaggc cgttgaggcc
661 gagggcaccg gcagcggcaa gcccaagagc caggtccaga tccacaagtg cggtgtgtgc
721 taaa**taa**

Fig. 3: Complete nucleotide sequence of DNA and deduced amino acid sequence (Accession number; EU395812). The initiation codon (ATG) and the stop codon (TAA) have been typed bold

Discussion

In the present study, we reported the identification and molecular characterization of a vital gene of *M. furfur* expressing a protein called cyclophilin, which belongs to the peptidyl-prolyl *cis-trans* isomerase enzyme family. Analysis of the amino acid sequence of this gene revealed a considerable homology with other eukaryotic cyclophilin proteins such as those of *A. clavatus* (83%), *S. cerevisiae* (72%) and *C.neoformance* (76%). This high homology may lead to confirm the previous data about the low diversity of cyclophilins. Moreover, it was demonstrated that serine (11.6%), alanine (10.5%), and glysine (10%) are the most common amino acids comprising the protein. In contrast, the amounts of tryptophane and tyrosine were poor, so that they comprise 0.52% and 1.05% of the amino acids sequence, respectively. Sequence conservation was highest at the N-terminus and decreased towards the C-terminus. In addition, comparison of the nucleotide sequence of the DNA fragment with mRNAs of the same identified gene in other eukaryotes revealed that the newly characterized gene lacks intron. The presence of initiation and stop codons at the real positions of the cyclophilin DNA sequence, together with the information deduced from the alignment with other cyclophilin genes, indicated that it encompassed the full-length gene coding sequence.

Sequence alignment shows that there are several individual identical amino acids present among allergenic cyclophilins such as Mala s 6 (from *Malassezia sympodialis*), Asp f 11 (from *Aspergillus fumigatus*) and sa-CyP A (from *Saccharomyces servisiae*). Two identical regions [SMANAGPNTNGSQFFI and WLDGKHVVFG] mainly consist of exposed loops and β- sheet strands and may be involved in cross-reactivity reactions. Models of Asp f 11, Mal s 6 and sa-CyP A with the crystallographically, revealed a similarity in overall structure. It has been found that these proteins belong to a common protein fold of allergens where eight anti-parallel β-sheets are intimately associated with one or more α-helices. In addition, it was demonstrated that the whole surface of those three proteins is antigenic (17). Due to the highly observed similarity among cyclophilin proteins, it is suggested that the whole structure of the investigated cyclophilin may contains the same domains and the same numbers of β-sheets and α-helices. But, unfortunately, there is no information about the structure of cyclophilin in *M. furfur*.

Identification of possible roles of this newly characterized gene in the physiological function of *M. furfur* is still under investigation. The molecular characterization of cyclophilin gene, which has been described here, may open the way to disclosure of the functional characteristics of cyclophilin and to assessment of its possible role in the pathogenesis of Atopic Dermatitis.

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The authors declare that there is no conflict of interests.

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