Original article

Identification of 30-kDa heat shock protein gene in *Trichophyton rubrum*

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Summary

Small heat shock proteins (sHSPs) are chaperones that are crucial in the heat shock response but also have important non-stress roles within the cell. HSP70 in *Trichophyton rubrum* is already detected and carefully characterised; however, no study was carried out for HSP30 in this pathogenic fungus. In the present study, *T. rubrum* was obtained from patients with dermatophytosis and cultured in appropriate conditions. High-molecular-weight DNA was extracted using standard extraction methods. Pairs of 21 nt primers were designed from highly conserved regions of the similar genes in other eukaryotic cells. Mentioned primers were utilised in PCR using isolated genomic DNA and extracted RNA templates of *T. rubrum*. The PCR fragments were then sequenced and 415 nucleotides of HSP30 in this pathogenic fungus were detected; the open reading frame had 156 nucleotides and was coding 51 amino acids. This gene (called *TrHSP30*) is registered in GenBank at National Center for Biotechnology Information (NIH, USA) database. Detection of *TrHSP30* gene may open the way to determination of its possible role in the pathogenesis of dermatophyte infections due to *T. rubrum*.

Key words: *Trhsp30*, *Trichophyton rubrum*, PCR, dermatophytosis, heat shock proteins.

Introduction

The heat shock response was first discovered by Italian scientist F. Ritossa, who used heat shock to the *Drosophila buschii* and then observed puffing bodies in the polytene chromosome of this fly.[1] Today, we know that heat shock proteins (HSPs) are a ubiquitous, highly conserved family of proteins that are synthesised by the cells of all organisms studied thus far, i.e. prokaryotes and eukaryotes.[2–4] These proteins have been characterised by their high degree of amino acid homology, dominance in protein profiling and role as molecular chaperones.[5,6] In addition, HSPs have been demonstrated to have numerous cytoprotective effects *in vivo* and play roles in many disease processes.[7,8]

Heat shock protein 30 which has been studied here is placed in the class of small HSPs that has certain short-sequence motifs, so-called α-crystallin domains[9] and some sequences in the N-terminal parts, are conserved.[10] Additional features attributed to these proteins range from RNA storage in heat shock granules to inhibition of apoptosis, actin polymerisation and contribution to the optical properties of eye lens in the case of α-crystallin.[11,12] The significant characteristic of these molecules is the presence of a specific sequence at the end of the hydrophilic carboxyl terminal region.[2] Reports show that small HSPs which are found in one organism have more similarity than small HSPs in different organisms. These proteins form a polymeric structure called heat shock granules, whose sedimentation coefficient is 15–20 s.[13] Although HSPs play essential roles in cells under both stress and non-stress conditions, there is a lack of information on small HSPs of the

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Accepted for publication 21 May 2008

doi:10.1111/j.1439-0507.2008.01561.x

dermatophyte fungi, especially of those which are involved in human infections.

Dermatophytes are keratinophilic fungi, all of them produce keratinases that cause infections of the skin, hair and nails called dermatophytoses, 'tineas' or 'ringworms'. *Trichophyton rubrum* is the most common dermatophyte in the world and causes the majority of non-scalp skin infections.[14–16] Despite its wide distribution, not too much study has been carried out on molecular characteristics of this pathogen. In the present study, we report the identification of a 30-kDa HSP of this dermatophyte.

Materials and methods

Sampling

Trichophyton rubrum was isolated from infected skin and nails in the Department of Medical Mycology & Parasitology at the School of Public Health of Tehran University of Medical Sciences. Isolates were collected over a 6-month period in the Mycology Laboratory. All isolates were identified by standard methods, which included identification based on the macroscopic and microscopic characteristics of culture strains. Additional tests included ability to produce a red pigment on potato dextrose agar (PDA) and biochemical tests such as urease activity, as well as hair perforation test.

DNA and RNA isolation

Total RNA from *T. rubrum* was isolated by a method which had been developed previously.[17] The poly(A)$^+$ RNA was obtained from total RNA and used for cDNA synthesis. High-molecular-weight DNA from *T. rubrum* was isolated by a modification of the method of Rezaie *et al.* [18]. Briefly, the harvested mycelial mass was washed with PBS 1x and then stored at −20 °C for 24 h. Then, the washed mycelia were flash-frozen in liquid nitrogen and ground to a fine powder in a porcelain mortar. The mycelial powder was suspended to DNA extraction buffer containing 50 mmol l^{-1} Tris–HCl (pH 8.0), 50 mmol l^{-1} EDTA, 3% SDS, 1% β-mercaptoethanol and 50 µl of proteinase-K (20 mg ml^{-1}). The suspension was then incubated at 65 °C for 1 h and the cellular debris was removed by centrifugation at 2500 *g* for 15 min. After addition of 25 µl RNase-H (10 mg ml^{-1}), the suspension was incubated at 37 °C for 30 min, extracted once with phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and once with chloroform–isoamyl alcohol (24 : 1). The DNA was precipitated by the addition of an equal volume of isopropanol, followed by centrifugation at 15 000 *g* for 30 min. The DNA pellet was rinsed with 70% ethanol and resuspended in distilled water.

Primer designing

The genomic DNA and final double-stranded cDNA fragments encoding *T. rubrum* HSP30 were each amplified by one round of PCR. Oligonucleotide primers for the HSP30 were designed from multiple alignment of homologous sequences of fungi (*Arthroderma benhamiae* AB047592, *Emericella nidulans* D32071, *Exophiala dermatitidis* AF475907, *Neurospora crassa* M55672 and *Magnaporthe grisea* MG05719). PCR amplification performed using synthetic oligonucleotide primers including: S, 5′-ATG ACC GAA CGC TCC GTT GG-3′ as sense and AS, 5′-TAC AGT GTC ATA GAA AGC ACC-3′ as reverse primers. In short, 20 pmol l^{-1} of each primer was added in a volume of 50 µl containing 20 mmol l^{-1} $(NH_4)_2SO_4$, 75 mmol l^{-1} Tris–HCl (pH 8.8), 1 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP mix, 1.2 U of thermostable DNA polymerase (Advance Biotechnologies, Surrey, UK), and 1 µl of template (genomic or plasmid DNA). The PCR cycle employed was 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, with a total of 36 cycles. PCR products were analysed by electrophoresis through a 1% agarose gel.

Sequencing of the RT-PCR fragments

Sequencing of the amplified DNA and cDNA fragments was performed with the Dye Terminator Cycle Sequencing Kit (MWG Biotech, Ebersberg, Germany), using amplified double-stranded cDNA and DNA fragments as template and synthetic 21-meric primers designed according to the obtained DNA sequence fragments from other fungi. Sequencing of each part was repeated at least three times for both strands. The nucleotide sequence of DNA was compared with the sequences in gene data banks at National Center for Biotechnology Information (NCBI) (NIH, Bethesda, MD, USA).

Results

Trichophyton rubrum identification

Microscopic examination showed clavate to pyriform microconidia and moderate numbers of smooth, thin-walled multiseptate, slender cylindrical macroconidia. On macroscopic examination, colonies were flat to slightly raised, white to cream, suede-like with a red reverse. On PDA, culture produced red pigment within

15 days; in addition, perforation of hair as well as the urease production were negative.[19]

Molecular characterisation

After amplification of the HSP gene by PCR and RT-PCR, a DNA with the approximate molecular weight of 394 bp was identified (Fig. 1). However, the molecular weight of amplified cDNA was approximately the same as the DNA (Fig. 2). The amount of 419 bp of the cDNA was sequenced which is presented in Fig. 3. The sequenced DNA contains an open reading frame (ORF) of approximately 156 bp which encodes a protein containing 51 amino acids. The start and stop codons are, respectively, at 7th and 160th positions of obtained sequence. The comparison of nucleotide sequences in gene data banks (NCBI; NIH) for the DNA and its deduced amino acid sequence revealed significant homology with members of the eukaryotic 30-kDa HSP family. The amino acid sequence of the encoded protein has homology with *A. benhamiae* (94%), *Aspergillus nidulans* (72%), *Aspergillus oryzae* (70%) and *N. crassa* (67.5%) (Fig. 4).

Nucleotide and amino acid sequences of TrHSP have been submitted to the NCBI GenBank and are available for public access under the accession numbers: AY763791 for cDNA and AAV33735 for encoded protein sequence.

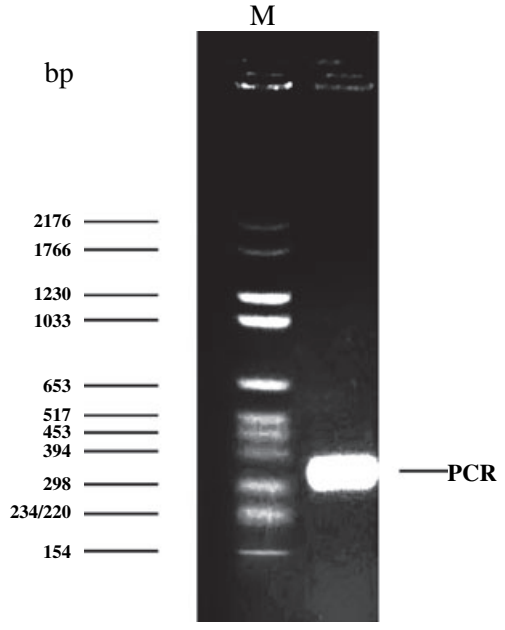

Figure 1 PCR products of the *Trichophyton rubrum* heat shock protein. M: molecular weight marker VI (Roche Diagnostics, Mannheim, Germany).

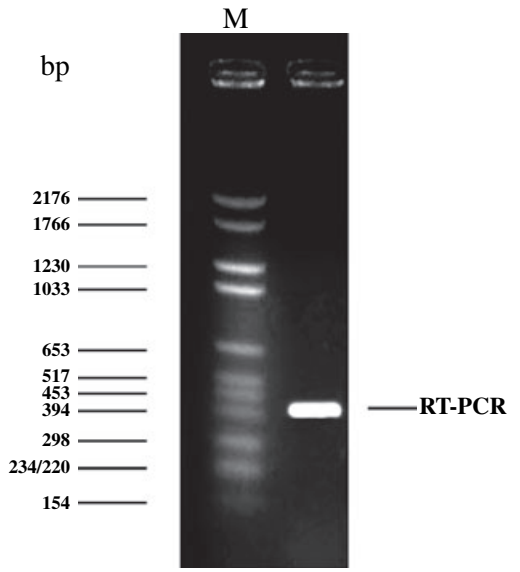

Figure 2 RT-PCR products of the *Trichophyton rubrum* heat shock protein. M: molecular weight marker VI (Roche).

Discussion

In the present study, we report the identification of a *T. rubrum* gene encoding a protein of low-molecular-weight heat shock protein (sHSP), which will here be referred to as TrHSP30. Dermatophyte pathogen *T. rubrum* is an anthropophilic fungus and may be involved in scalp and cutaneous ringworms. It also causes more intense, chronic infection of foot.[14–16,20] Therefore, this fungus is very important for genetical and molecular biology studies. In the previous survey, TrHSP70 has been studied completely;[18] however, small HSPs in this fungus and some other dermatophytes are not well examined.

Analysis of the amino acid sequence of the encoded protein showed a considerable identity with other eukaryotic sHSPs, such as those of *A. benhamiae* (BAB12047), *A. nidulans* (EAA64635), *A. oryzae* (BAD02411) and *N. crassa* (XP_332056). Comparison of the TrHSP cDNA to genomic DNA showed no intron within the identified gene which appears consistent with previous reports by Heikkila [21]. ORF in this gene contains 25.2% adenine, 20.1% thymine, 30.8% cytosine and 23.9% guanine. The study of amino acid composition of TrHSP30 reveals valine (13.7%) as the most common amino acid and indicates the amount of serine, lysine and alanine as 9.8% in all three, consequently in *A. benhamiae* 9.8%, 11.8% and 9.8% *A. nidulans* 8%, 10% and 6% and *A. oryzae* 10%, 10% and 8%. This composition shows that 30-kDa HSP is

1	**GGC**	**ACG**	***ATG***	**ACC**	**GAA**	**CGC**	**TCC**	**GTT**	**GGC**	**GAG**	**TTC**
1			**M**	**T**	**E**	**R**	**S**	**V**	**G**	**E**	**F**
34	**AAT**	**CGC**	**GTC**	**TTC**	**AAG**	**TTC**	**CCA**	**TCC**	**CGA**	**GTC**	**GAC**
10	**N**	**R**	**V**	**F**	**K**	**F**	**P**	**S**	**R**	**V**	**D**
67	**CAG**	**GAT**	**GCA**	**GTC**	**AGT**	**GCT**	**AGC**	**TTG**	**AAG**	**GAT**	**GGC**
21	**Q**	**D**	**A**	**V**	**S**	**A**	**S**	**L**	**K**	**D**	**G**
100	**ATC**	**CTC**	**TCC**	**GTC**	**AAC**	**GTG**	**CCC**	**AAG**	**GCA**	**GCT**	**CCA**
32	**I**	**L**	**S**	**V**	**N**	**V**	**P**	**K**	**A**	**A**	**P**
133	**CCA**	**ACT**	**GTC**	**AAG**	**AAG**	**ATC**	**AAC**	**ATC**	**GCG**	***TAA***	**ATG**
43	**P**	**T**	**V**	**K**	**K**	**I**	**N**	**I**	**A**		
166	**AGA**	**AAT**	**GAG**	**GAA**	**CAA**	**TAT**	**GCC**	**TTG**	**CCC**	**TTT**	**TAA**
199	**TGA**	**TGC**	**ATG**	**CGA**	**CTT**	**TAC**	**GAT**	**CCT**	**GCG**	**CAT**	**TTA**
232	**TGA**	**TGG**	**TTA**	**TGG**	**GAA**	**ATT**	**GAT**	**GAG**	**TCT**	**GGC**	**GCA**
265	**TTC**	**CAA**	**CTT**	**TTT**	**CTT**	**TCG**	**CTG**	**TTC**	**TGT**	**ATT**	**CCA**
298	**ATT**	**TGA**	**TGT**	**TGT**	**GAC**	**TTT**	**GTT**	**TCC**	**TTG**	**GTG**	**CTT**
331	**TCT**	**ATG**	**ACA**	**CTG**	**TAT**	**TTA**	**TTA**	**TAG**	**CCA**	**GCC**	**TCT**
364	**TTT**	**AAC**	**AAA**	**AAA**	**AAA**	**AAA**	**AAA**	**AA**	**397**		

Figure 3 Complete nucleotide sequence of DNA and deduced amino acid sequence of TrHSP (GenBank accession numbers: AY763791 and AAV33735). The start codon (ATG) and stop codon (TAA) are in italic.

T.rubrum:	7	*MT* **ERS** *VG* **EF** *N* **R** *V* **F** *K* **FP** *S* **RV** *D* **Q** *DA* **V** *S* **ASL** *KD* **GIL** *S V N* **VPKA** *APP* **T** *V* **KKI** *N* **I** *A* 159
T.mentagrophytes:	1	*MT* **ERS** *VG* **EF** *N* **R** *V* **F** *K* **FP** *S* **RV** *D* **Q** *DA* **V** *S* **ASL** *KD* **GIL** *S I K* **VPKA** *APP* **T** *L* **KKI** *N* **I** A 51
A.nidulans:	131	*VS* **ERS** *VG* **EF** *Q* **R** *T* **F** *T* **FP** *T* **RV** *N* **Q** *DD* **V** *K* **ASL** *KD* **GIL** *S LV* **VPKA** *VPP* **T** *A* **KKI** T **I -** 180
A.oryzea:	116	*VS* **ERS** *VG* **EF** *H* **R** *T* **F** *T* **FP** *S* **RV** *D* **Q** *EN* **V** *K* **ASL** *KN* **GIL** *S LV* **VPKA** *AAY* **T** *G* **KKI** T **I -** 165
N.crassa:	178	*VS* **ERS** *I G* **EF** *S* **R** *T* **F** *N* **FP** *G* **RV** *D* **Q** *NA* **V** *S* **ASL** *NN* **GIL** *T I T* **VPKA** ---------- 217

Figure 4 Alignment of amino acid sequence from TrHSP30 with selected eukaryotic HSP30 family members.

also rich in proline (7.8%). In contrast, the amounts of methionine (2%) and glutamine (2%) are poor.

With regard to the importance of the HSP in different organisms and its chaperonic role in individual cells[1–3,7–13,21–23] and on their immune system,[24,25] the molecular structure of small HSPs was studied carefully in *T. rubrum*. Today, the most important use of sHSPs is in recombinant vaccines and their protecting role against stressing agents such as heavy metals and different irradiations must be considered. In this case, possible role of TrHSP30 against anti-fungi drugs is worth noticing.

Finally to our knowledge, TrHSP30 is the first sHSP gene and protein of this dermatophyte characterised so far. The detection of this gene in *T. rubrum* which is described here may open the way to reveal the functional characteristics of *TrHSP* in *T. rubrum* and to evaluate its possible role in the pathogenesis of dermatophyte infections because of *T. rubrum.*

Acknowledgments

We are much obliged to Dr Sassan Rezaie and Dr Fathollah Fallahiyan for the completion of this work. We are grateful for the support we received from the School of Public Health of Tehran University of Medical Sciences and Islamic Azad University, Science and Research Campus, Tehran, Iran.

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