Analysis of gene expression changes in *Trichophyton rubrum* after skin interaction

Tao Liu, Xingye Xu, Wenchuan Leng, Ying Xue, Jie Dong and Qi Jin

MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, PR China

*Trichophyton rubrum*, an anthropophilic and cosmopolitan fungus, is the most common agent of superficial mycoses. In this study, *T. rubrum* infection was modelled by adding human skin sections to a limited medium containing glucose and cDNA microarrays were used to monitor *T. rubrum* gene expression patterns on a global level. We observed that exposure to human skin resulted in upregulation of the expression levels of *T. rubrum* genes related to many cellular and biological processes, including transcription and translation, metabolism and secondary transport, the stress response, and signalling pathways. These results provide a reference set of *T. rubrum* genes whose expression patterns change upon infection and reveal previously unknown genes that most likely correspond to proteins that should be considered as virulence factor candidates and potential new drug targets for *T. rubrum* infection.

INTRODUCTION

*Trichophyton rubrum* is a filamentous fungus found throughout the world that can infect human keratinized tissue (skin, nails and, rarely, hair), and is the causal agent of 80–90% of all chronic and recurrent dermatophytoses (Costa et al., 2002; Jennings et al., 2002; Monod et al., 2002). This pathogen, which normally causes well-characterized superficial infections, also produces skin infections in unusual parts of the body in immunosuppressed patients (Sentamliselvi et al., 1998; Smith et al., 2001; Squeo et al., 1998). Although not normally life-threatening, dermatophyte infections are often difficult to eliminate completely and they have a 25–40% recurrence rate (Sentamliselvi et al., 1998). Additionally, resistance to antifungal drugs poses an increasing problem in clinical treatment; over the past decade, an increasing number of cases of azole- and terbinafine-resistant dermatophyte infections have been reported (Fachin et al., 1996; Smith et al., 2001).

The prevalence of infections caused by *T. rubrum* and its human-specific nature make it a good model for the study of human pathogenic filamentous fungi. The epidemiology, clinical case reports, strain-relatedness and drug susceptibilities of the organism are well documented (Kane et al., 1997). Several studies on *T. rubrum* pathogenicity have provided evidence that genes involved in secretory activity and membrane transporters play roles in the infection process of the fungus (Fachin et al., 2006; Maranhão et al., 2009). Despite its limited total number of genes, a cDNA microarray analysis devised to monitor the gene expression profile of *T. rubrum* during culture in keratin–soy-protein-containing medium revealed some genes and dermatophyte-specific mechanisms involved in the putative processes related to *T. rubrum* infection (Staib et al., 2010; Zaugg et al., 2009). All these findings provided important information about the biological characteristics of *T. rubrum* and enhanced our understanding of its pathogenicity.

Previously, our group reported a sequencing program of over 40,000 expressed sequence tags (ESTs) derived from 10 different stages of the *T. rubrum* life cycle, which represented the first significant step towards a comprehensive description of the cellular functions involved in *T. rubrum* biology (Wang et al., 2004; Yang et al., 2007). In this study, we added human skin sections to a limited *T. rubrum* growth medium, and used cDNA microarray analysis to compare the transcription patterns of dermatophyte fungi in skin suspension medium and limited medium (LM). The results reveal a number of genes for which expression may be specifically regulated during infection, as well as genes related to virulence and adaptation, thereby suggesting new potential drug targets for the treatment of infections mediated by this pathogen.

METHODS

Ethics statement. This study was approved by the Review Board of the Institute of Pathogen Biology. The skin samples used were
collected from an abdominoplasty and were removed during the normal course of surgery. This was only done on the one occasion. Written informed consent was obtained from the patient who donated the skin sample.

Skin sections. The skin sections used in this study were collected from a woman who underwent an extended abdominoplasty. The normal thigh skin was incised into pieces measuring approximately 0.1–0.2 cm² with full epidermal and dermal thickness, and the pieces were placed in small Petri dishes. The skin pieces were immersed in cold sterilized PBS. Whenever possible, the skin samples were used within 1 h of removal.

Strain and culture conditions. The *T. rubrum* clinical isolate BMU01672 used throughout this study was obtained from a patient suffering from tinea unguium. The strain was confirmed as *T. rubrum* by morphological identification, as well as by PCR amplification and sequencing of the 18S rDNA and internal transcribed spacer regions. Strain reference samples were stored at −20 °C in the Research Centre for Medical Mycology, Beijing, PR China (Wang et al., 2004).

*T. rubrum* microconidia were isolated as reported previously and adjusted to a concentration of 5–8 × 10⁷ conidia ml⁻¹ and used as inoculum (5 × 10⁶ conidia ml⁻¹) in the following experiments (Liu et al., 2007). Incubation was at 28 °C in liquid Sabouraud medium (containing 49 g glucose, 20 g Difco Bacto Peptone in 1 l distilled water) with constant shaking (orbital, 200 r.p.m.) for 72 h to the exponential growth phase. Mycelia were harvested by filtration and washed twice with a saline solution (0.9 % NaCl), transferred into LM [glucose, 20 g 1⁻¹; (NH₄)₂HPO₄, 6.6 g 1⁻¹; KH₂PO₄, 0.46 g 1⁻¹; KH₂PO₃·3H₂O, 1.3 g 1⁻¹; MgSO₄·7H₂O, 0.49 g 1⁻¹] and incubated for 24 h. The aim of this incubation step was to eliminate the interference of the Sabouraud medium, which contained protein components that may have influenced gene expression. To analyse the gene expression of the clinical isolate under infection conditions, 2 g human skin sections were added to each of 12 flasks (250 ml), each with 80 ml LM. Another group of 12 flasks with an equal volume of LM but lacking the human skin sections was used as a control. Fungal mycelia (200 mg) were introduced into every flask and the flasks were then incubated at 28 °C with constant shaking. Three samples taken directly from the fungal mycelia were used as the 0 h samples. Subsequent samples were independently cultured, and harvested at 1, 3, 6 and 12 h.

RNA extraction and cDNA microarray construction. At every RNA extraction and cDNA microarray construction. The skin sections used in this study were collected from a woman who underwent an extended abdominoplasty. The normal thigh skin was incised into pieces measuring approximately 0.1–0.2 cm² with full epidermal and dermal thickness, and the pieces were placed in small Petri dishes. The skin pieces were immersed in cold sterilized PBS. Whenever possible, the skin samples were used within 1 h of removal.

**RESULTS**

Highly and differentially expressed genes in the *T. rubrum* infectious model

To assess gene expression patterns during the infection process, the mycelia of a *T. rubrum* isolate were grown in a human skin suspension medium and equal amounts of mycelia were introduced into LM as a control. During the
12 h culture process, the growth of the mycelia in these two media showed no obvious differences. The skin sections in the medium were also not consumed visibly. Changes in gene expression were monitored from 0 to 12 h using a cDNA microarray. A total of 2452 genes were selected based on the significant changes in expression by ANOVA (P<0.01) during this period. Using ANOVA (P<0.01) by TIGR MeV software (Saeed et al., 2003), the expression patterns of the 2452 selected genes were compared with the corresponding data from the control LM culture. Of these 2452 genes, the expression changes of 1258 genes were estimated by quantitative real-time RT-PCR. The results showed a strong positive correlation between the two techniques (Table 1). These 768 genes were then subjected to KMC analysis using TIGR MeV software to evaluate the relative expression pattern of T. rubrum in skin induction (for detailed results, see Table S2). To verify the microarray results, the relative expression levels of 15 genes in the KMC clusters were determined by quantitative real-time RT-PCR and microarray hybridization results for these two technologies was calculated using SPSS 13.0 software.

Clusters I and IV contained 434 and 151 of the 768 genes, respectively. Transcripts of genes in these two clusters were induced to increase from the zero time point, with a peak expression at 6 h. Genes in cluster IV maintained high expression levels at each time point examined, while the expression level of the cluster I genes declined rapidly after 6 h. There were 122 genes assigned to cluster II, whose mRNA accumulated throughout the skin induction process. The transcript levels of the 61 genes in cluster III had low expression levels throughout the process and they exhibited their lowest expression at 6 h.

**Metabolic changes induced by human skin sections**

The genes that were induced by the addition of human skin sections were listed in Table S1 and the distribution of the functional categories is shown in Table 2. Compared with the control LM culture, the addition of human skin sections induced prominent changes in metabolism in T. rubrum. In our analysis, ~231 of the 768 genes functioned in the 'metabolism' category.

In the metabolism category, 59 genes were involved in primary carbohydrate metabolism, including glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation (Table S3). Most of these genes were in clusters I and IV, which exhibited induced expression from the zero time point and reached peak expression at 6 h.

Three genes involved in the chitin metabolic process were induced during incubation (Table S4), nine of which were potential secreted proteases or peptidases, such as aspartyl esterase, which encodes the cell wall enzyme 1,3-β-glucanosyltransferase, were induced upon addition of human skin sections.

Thirty-seven genes relative to protein metabolism were induced during incubation (Table S4), nine of which were potential secreted proteases or peptidases, such as aspartyl esterase, which encodes the cell wall enzyme 1,3-β-glucanosyltransferase, were induced upon addition of human skin sections.

**Table 1.** Relative fold change for 15 genes listed determined by quantitative real-time RT-PCR and microarray hybridization results

Column R shows the fold change after adding human skin sections relative to LM, determined by quantitative real-time RT-PCR. Column M shows the fold change after adding human skin sections relative to LM, determined by the microarray hybridization results. The correlation coefficient (r) for these two technologies was calculated using SPSS 13.0 software.

| ESTs     | Cluster | 0 h | 1 h | 3 h | 6 h | 12 h | R   | M   | R   | M   | R   | M   | r     |
|----------|---------|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|-------|
| DW685106 | I       | 1   | 1   | 1.0324 | 0.5678 | 0.52159 | 1.07093 | 4.53154 | 2.91846 | 1.2781 | 0.26321 | 0.89 |
| EL788295 | IV      | 1   | 1   | 1.20497 | 1.40353 | 0.92916 | 1.27826 | 7.12554 | 3.01009 | 1.18509 | 1.52157 | 0.976 |
| DW680891 | I       | 1   | 1   | 1.31859 | 1.19658 | 0.82817 | 1.60904 | 2.16145 | 3.0985 | 0.46041 | 0.77202 | 0.884 |
| DW680904 | I       | 1   | 1   | 0.993781 | 1.103348 | 0.56331 | 1.183078 | 2.540302 | 2.775284 | 1.585568 | 1.464977 | 0.929 |
| DW685928 | I       | 1   | 1   | 0.959264 | 0.706954 | 1.624505 | 1.261124 | 1.58447 | 2.169198 | 1.024557 | 0.673213 | 0.801 |
| DW680574 | I       | 1   | 1   | 0.984866 | 1.127878 | 0.628507 | 1.221062 | 2.760635 | 2.466178 | 1.071031 | 0.770112 | 0.906 |
| DW706077 | IV      | 1   | 1   | 1.449947 | 1.466285 | 0.842063 | 1.236475 | 3.874473 | 3.165263 | 0.951978 | 1.872483 | 0.919 |
| DW678823 | I       | 1   | 1   | 0.681601 | 1.245822 | 1.701727 | 1.2606 | 3.805273 | 2.114076 | 1.063633 | 0.89313 | 0.946 |
| DW679448 | I       | 1   | 1   | 1.303529 | 1.423327 | 0.541488 | 1.290017 | 2.270484 | 3.213281 | 1.138394 | 1.52867 | 0.875 |
| DW680706 | I       | 1   | 1   | 0.986233 | 1.246938 | 0.315126 | 1.022999 | 1.91587 | 1.752762 | 2.040609 | 2.356341 | 0.891 |
| EL786224 | I       | 1   | 1   | 3.228804 | 0.708054 | 0.571173 | 1.022053 | 5.490738 | 1.709587 | 1.313121 | 1.424985 | 0.646 |
| EL792939 | IV      | 1   | 1   | 2.363623 | 0.774499 | 0.566049 | 0.986895 | 5.43018 | 2.6246 | 2.16595 | 0.617256 | 0.831 |
| DW678242 | I       | 1   | 1   | 1.139183 | 0.831055 | 0.384752 | 1.272711 | 5.333195 | 2.962537 | 2.475981 | 0.462158 | 0.765 |
| EL785855 | I       | 1   | 1   | 1.504204 | 0.706083 | 0.428094 | 0.998018 | 3.292081 | 2.441484 | 1.135242 | 1.038479 | 0.871 |
| DW703795 | I       | 1   | 1   | 1.201636 | 0.568963 | 0.741234 | 1.054972 | 2.171964 | 1.680151 | 0.455019 | 0.236967 | 0.815 |
protease, serine-type peptidase, metallopeptidase and prolidase pepP, which are likely involved in proteolysis and related to pathogenicity (Krappmann & Braus, 2005; Monod et al., 2002; Naglik et al., 2003).

Twenty-four other genes encoding components of the proteasome and ubiquitin-conjugating enzymes E2 and E3 were also identified (Table S4).

Forty-eight genes were identified that functioned in amino acid metabolic pathways, including those for arginine, glutamine, proline, lysine, methionine, threonine, histidine and glutamate, as well as pathways related to aromatic amino acid biosynthesis, glutamate metabolism, methionine metabolism and arginine metabolism (Table S5). Apart from one gene that belonged to cluster III, the other 47 genes belonged to clusters I, II and IV, which were overexpressed during the culturing process. In our analysis, two putative genes, DW678242 and DW680904, encoding homocitrate synthase and α-aminoadipate reductase involved in lysine biosynthesis, were induced throughout the infection process. As α-aminoadipate reductase is a key enzyme in the branched pathway for lysine and β-lactam biosynthesis of filamentous fungi, and impaired lysine biosynthesis can severely attenuate virulence in Aspergillus nidulans and Aspergillus fumigatus (Liebmann et al., 2004; Tang et al., 1994), our results suggest that lysine biosynthesis is related to the process of T. rubrum infection as well.

Methionine production is also essential for protein biosynthesis in micro-organisms. Our data indicate that the gene EL785855, which encodes aspartate semialdehyde dehydrogenase, was upregulated; this enzyme functions at the first branch point in the biosynthetic pathway through which bacteria, fungi and higher plants use aspartate to synthesize certain amino acids, including lysine and methionine.

Secondary metabolites from fungi are usually active as mycotoxins and virulence factors that may be involved in the pathogenic development of fungal infections (Bennett & Klich, 2003; Rementeria et al., 2005). Two genes, DW680904 and DW680891, involved in ergot alkaloid biosynthesis, encoding l-αminoadipate-semialdehyde dehydrogenase and dimethylallyl tryptophan synthase, were observed in cluster I. The ergot alkaloids are a family of indole-derived mycotoxins that have a variety of important biological activities (Coyle & Panaccione, 2005; Tudyński et al., 2001). Knocking out the gene encoding dimethylallyl tryptophan synthase
resulted in the loss of all known ergot alkaloids in *Aspergillus fumigatus* and clavicipitaceous fungi, which indicated that dimethylallyl tryptophan synthase controls the determinant step in the ergot alkaloid pathway (Coyle & Panaccione, 2005).

**Table 2. Functional annotation and cluster distribution of genes induced by human skin sections**

Individual genes can take part in multiple biological processes. The detailed results are provided in Table S2. Detailed annotation and function characterization of the *T. rubrum* ESTs is also depicted in Table S1 and our *T. rubrum* database (http://www.mgc.ac.cn/TrED/).

| Biological process                               | Cluster I | Cluster II | Cluster III | Cluster IV |
|--------------------------------------------------|-----------|------------|-------------|------------|
| Metabolism                                        | 48        | 11         | 3           | 15         |
| Carbohydrate metabolic process and energy pathways | 22        | 2          | 0           | 13         |
| Protein metabolic process, proteolysis             | 35        | 6          | 1           | 6          |
| Amino acid metabolic process                       | 8         | 3          | 1           | 4          |
| Lipid metabolic process                            | 10        | 0          | 0           | 5          |
| Nucleic acid metabolic process                     | 6         | 3          | 2           | 4          |
| Cell organization and biogenesis                   | 9         | 3          | 1           | 0          |
| Cell division, reproduction                        | 12        | 4          | 10          |            |
| Transcription                                     | 17        | 10         | 2           | 5          |
| Translation and protein biosynthesis               | 73        | 2          | 0           | 9          |
| Response                                          | 5         | 4          | 0           | 5          |
| Regulation of biological process                   | 7         | 0          | 0           | 1          |
| Transport                                         | 29        | 17         | 0           | 13         |
| Other                                             | 25        | 13         | 1           | 11         |
| Biological process unknown                        | 132       | 57         | 50          | 52         |

**Changes in transport gene expression upon the addition of human skin sections**

The expression of 60 genes related to transport was modified in response to the addition of human skin sections (Table S8). Thirty of these genes were assigned to cluster I, 17 of them were assigned to cluster II and 13 of them were assigned to cluster IV. Of these 60 genes, 12 upregulated genes were related to vesicle-mediated autophagy and exocytosis. Additionally, 12 genes encoding major facilitator superfamilly transporter proteins, two genes encoding ATP-binding cassette transporters, and three genes encoding proteins involved in iron and copper transport were also induced. These transporter proteins have remarkably broad substrate specificity, and are able to transport a wide variety of natural and synthetic toxic products of either endogenous or exogenous origin (Pao *et al.*, 1998; Stergiopoulos *et al.*, 2002). When functioning as virulence factors, these membrane transporters are required to overcome host defence mechanisms and are important for *T. rubrum* pathogenicity (Maranhão *et al.*, 2009).

**Changes in stress response genes upon the addition of human skin sections**

We observed changes in the expression of 14 genes encoding proteins with a known or putative role in the response to temperature shift or oxidative stress (Table S9). These proteins included two molecular co-chaperones associated with cellular responses to temperature shifts, HSP70 (DW681773) and HSP90 (DW693707); one superoxide dismutase (EL788295), and three novel cytochrome c oxidases associated with antioxidant function (DW686878, DW700639 and DW696907). Three homologues of genes encoding secondary antioxidant enzymes belonging to the
glutathione S-transferase family (DW679047, DW679402 and DW406127) were also identified.

The signal transduction pathways are conserved and mediate adaptive changes in many fungi, including cellular development and morphogenesis (d’Enfert, 1997; Fillinger et al., 2002; Liebmann et al., 2004). In pathogenic micro-organisms, these signalling pathways may also be used to regulate the determinants of virulence and host infection (Hamilton & Holdom, 1999; Rementeria et al., 2005). We identified seven genes involved in mitogen-activated protein kinase signalling and Rab/RAS small GTPase-mediated signalling pathways, and we assigned them to clusters I and IV (Table S10). Some of these genes encoded proteins in these signalling pathways that have been associated with virulence in Aspergillus fumigatus and Candida albicans (Hogan et al., 1996; Rementeria et al., 2005). The role of these signalling pathways in T. rubrum infection requires further study.

DISCUSSION

In this study, we modelled a T. rubrum infection by adding human skin sections to LM containing glucose to monitor global T. rubrum gene expression patterns using cDNA microarrays. Our results reflect the adaptation that results from the interaction of T. rubrum with its human host. In host–pathogen interactions, the gene expression of the pathogen is modulated by signals from the host and understanding the resulting expression patterns may provide insight into the disease mechanism. The transcription profile monitoring revealed that the expression levels of genes related to many cellular and biological processes of T. rubrum were induced by the addition of human skin sections, including transcription and translation, metabolism and secondary transport, stress response, and signalling pathways. The cell cycle is the basic cellular process involved in cell division, polar construction, morphogenesis and adaptation to different environments. We observed that a number of genes associated with cell cycle events, including the assembly of chromatin, chromosome segregation and cell cycle control, were upregulated. The transcription of genes encoding certain ribosomal subunits and translation factors was also upregulated highly after the human skin sections were added. These results suggest that the expression of many genes changes dramatically as T. rubrum adapts to the presence of human skin sections.

The secretion of proteolytic enzymes by dermatophytes is a key factor in the invasion and utilization of the stratum corneum of the host (Grumbt et al., 2011; Maranhão et al., 2009; Monod, 2008). Recently, seven dermatophyte genomes have been completely sequenced and all of them were found to encode high numbers of proteases compared with other non-dermatophytic fungi (Achterman & White, 2012; Burmester et al., 2011; Martinez et al., 2012). Secretion of several T. rubrum proteolytic enzymes has been observed and is thought to be involved in infection (Apodaca & McKerrow, 1989; Leng et al., 2009; Maranhão et al., 2007; Monod, 2008). Using a T. rubrum cDNA microarray, pathogenic dermatophyte Arthroderma benhamiae and the molecular basis of keratin degradation in T. rubrum indicated that numerous genes encoding secreted proteases were induced in these two fungi (Staib et al., 2010; Zaugg et al., 2009). In the present study, certain genes involved in protein metabolism were induced, including several types of potential secreted proteases. Most genes involved in multiple protein transport pathways, autophagy and exocytosis were also upregulated. These results may improve our understanding of the pathogen and its ability to use extracellular enzymes to degrade the structural barriers of the host. We also observed the induction of T. rubrum genes involved in the biosynthesis of various amino acids and in amino acid metabolism. In these pathways, lysine and methionine biosynthesis have been identified as contributors to virulence in many fungal pathogens (Liebmann et al., 2004; Tang et al., 1994), and they may also have a role in the T. rubrum infection process. These pathways are non-existent or they function differently in animals and humans. We hypothesize that certain genes that encode enzymes involved in the key steps of these pathways are promising antifungal drug targets. Some genes and pathways involved in secondary metabolism, the response to stress and the transport of toxic components were also induced after the addition of human skin sections. These genes and related pathways may be involved in virulence and determining the sensitivity to fungicides and other antymycotic agents.

T. rubrum is a human-specific pathogenic fungus. Due to the lack of an animal model to study biological processes involved in T. rubrum infection, the functional analysis of T. rubrum described here will provide important information regarding its pathogenicity and virulence.

ACKNOWLEDGEMENTS

This work was supported by the National Nature Science Foundation of China (grant no. 30870104), the National High Technology Research and Development Program of China (grant no. 2012AA020303), the National Science and Technology Major Project of China (grant no. 2013ZX10004-601), and an intramural grant from the Institute of Pathogen Biology, Chinese Academy of Medical Sciences (grant no. 2006IPB008).

REFERENCES

Achterman, R. R. & White, T. C. (2012). Dermatophyte virulence factors: identifying and analyzing genes that may contribute to chronic or acute skin infections. Int J Microbiol 2012, 358305.

Apodaca, G. & McKerrow, J. H. (1989). Regulation of Trichophyton rubrum proteolytic activity. Infect Immun 57, 3081–3090.

Bennett, J. W. & Klich, M. (2003). Mycotoxins. Clin Microbiol Rev 16, 497–516.

Burmester, A., Shelest, E., Glöckner, G., Heddergott, C., Schindler, S., Staib, P., Heidel, A., Felder, M., Petzold, A. & other authors (2011). Comparative and functional genomics provide insights into the pathogenicity of dermatophytic fungi. Genome Biol 12, R7.
A fungal genetic study involving the development of resistant mutants to antimycotic drugs, focusing on tioconazole.

Deletion of the TruMDR2 gene and clustered hypothetical genes from Aspergillus fumigatus and its role in pathogenicity.

Expression analysis of Trichophyton rubrum using ESTs.

Secretory proteinases from dermatophytes.

Deep dermal invasion from the epidermis during growth on keratin.

Secreted proteinases in virulence and pathogenesis of Trichophyton rubrum.

Secreted proteinases from pathogenic fungi.