PH.D. THESIS

INVESTIGATION OF HOST - PATHOGEN INTERACTION DURING *CANDIDA* INFECTIONS

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Szeged

2013

Introduction

The incidence of *Candida* infections raised dramatically during the last three decades. Although these yeasts are know as the members of the normal human flora, if the host control is compromised, they can cause life-treatining systemic infections (candidaemia). The predominant species isolated from candidaemia is C*andida albicans*, hence early investigations included exclusively this yeast. However the incidence of other *Candida* species has increased since 2000. These so-called non-*albicans* species showed remarkable differences in resistance to antibiotics, in epidemiology and in interaction with the host compared those to the ones of *C. albicans*. Nowadays *C. parapsilosis* is the second most commonly isolated *Candida* species from candidaemia, that in some cases outranks even *C. albicans* depending on the geographic area and the time period of the given study. Since the emergence of *C. parapsilosis* occured a decade ago its physiology, interaction with the host cells, virulence factors are poorly understood. To fill this gap an *in vitro* model system was developed to investigate the interaction

between *C. parapsilosis* and mammalian phagocytes. The process was followed by microscopic techniques, the transcriptional changes in the host and the pathogen were examined by RNA microarray and RNA-seq. The whole genome of three *C. parapsilosis* strains were sequenced and compared to the reference one.

Methods

Cultivation and coincubation: Isolation and cultivation of primary phagocytes, cultivation of mammalian cell lines and yeast strains, *in vitro* coincubation of yeast cells and phagocytes, yeast elimination assay

Microscopic techniques: scanning electron microscopy, Lysotracker Red/FITC fluorescent staining and fluorescent confocal microscopy, acridin orange/crystal violet fluorescent staining and fluorescent microscopy

Molecular techniques: DNA isolation from yeast, RNA isolation from yeast and mammalian phagocytes, RNA microarray, cDNA synthesis, qRT-PCR, flow cytometry, PCR, gelelectrophoresis, Southern hybridisation, Sanger-sequencing, pulsed field gelelectrophoresis

Sequencing and *in silico* analysis (in collaboration): whole genome sequencing and library preparation, detection of SNPs, genomic recombination and structural variants, RNA sequencing and library preparation, gene prediction, transcriptome analysis

Results

Development of an *in vitro* model system to study the interaction between murine macrophages and *C. parapsilosis*

The response of J774.2 murine macrophages given to *C. parapsilosis* clinical isolate was observed by flow cytometer, scanning electron microscope, flourescent microscope and confocal fluorescent microscope. It was established that J774.2 macrophages start to uptake *C. parapsilosis* wild-type cells after 30 minutes of incubation. The uptake reaches a plateau-phase by the third hour. Phagosome-lysosome colocalisation and elimination of the yeasts occurs by the eighth hour.

Since three and eight hours seemed to be important milestones of the interaction microarray analysis of the host transcriptome was performed with RNA samples isolated at these timepoints. Respectively 115 and 511 genes were found to be differently expressed compared to the non-infected control. Genes taking part mostly in wound healing, stress or immune response were upregulated. Six genes were choosen to validate the microarray results by qRT-PCR. The relative expressions

of *CD83*, *IL1β*, *IL15*, *TNFα*, *TNFRSF9* (a gene of transmembrane protein, a costimulatory molecule) and *PTGS-2* (gene of a cytoplasmic enzyme responsible for prostaglandin biosynthesis) genes were examined in samples from three different timepoints, 3, 8 and 24 hour. Overexpression data were normalised to the non-infected controls by using $2^{-\Delta(\Delta Ct)}$ method, actin was used as an internal control. QRT-PCR validated the microarray results.

The overexpression of the *TNFRSF9* gene was examined after stimulation by other *Candida* spp (*C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. metapsilosis*, *C. orthopsilosis*, *C. tropicalis*) and both of them were able to induce the upregulation of *TNFRSF9*.

Flow cytometry analysis established that *C. parapsilosis* wild-type was able to increase the level of the functional protein on the surface of the phagocytes compared to the control (0 h).

The expression of this specific molecule was also examined *in vitro* in a model using mouse peritoneal macrophages and human PMBC derived macrophages after 3, 6, 12 and 24 hours of coincubation with wild-type

C. parapsilosis GA1 strain. Both induced the overexpression of *TNFRSF9* in these cells. In mouse macrophages the longer the incubation time was, the higher the expression occured. In primary human cells however the highest expression was observed at twelve hours post-infection.

Whole transcriptome analysis of the host and the pathogen from *in vitro* interactions of THP-1 human monocytic cells with the members of the *C. parapsilosis sensu lato* group

Complete transcriptional profiles of samples originated from incubation of THP-1 monocytes with the members of *C. parapsilosis sensu lato* group **(**involving clinical, environmental and mutant strains of *C. parapsilosis*) in different timepoints were determined by RNA-seq. This technique seemed to be capable of managing the transcriptome profile of the host and the yeast simultaneously in a single RNA sample. Applying this novel method not only the response of phagocytes but the one of the pathogens could also be examined. The

evaluation of the data generated is still in progress. Our current results are as follows.

Experiments involving *C. parapsilosis* CDC317, *C. orthopsilosis* MCO 456 and *C. metapsilosis* PL 429 (three closely related species having highly different clinical relevance) made possibe to analyse the changes of the yeast transcriptome in the response to the monocytes. Unfortunately the phagocyte transcriptome was hardly detectable therefore the changes caused by the fungi in the monocyte could not be examined yet. *In silico* analysis determined the distribution of ortolog genes in the three species and the differentially expressed genes during infection. RNA-seq identified more than 300 previously unknown genes in *C. parapsilosis*. It was also established that *C. metapsilosis* underwent a whole genome duplication recently.

The interaction of THP-1 with two environmental (CBS 1954 and CBS 6318) and two clinical (CDC317 and GA1) strains was also examined. Based on the heatmap of the Euclidean-distances of the samples from this arrangement the clinical and environmental *C. parapsilos*is isolates were classified in completely

different clusters suggesting notable differences in the transciptomes. In the four strains a total of 5.167 transcripts were identified. Out of these 729 transcripts were found to be differentially expressed in the presence of monocytes. The thirty genes with the highest expression difference were choosen at any given timepoints to calculate the Euclidean-distances of the samples. This analysis included the following four layout: 1.) basic expression (without monocytes) of the two clinical versus the two environmental isolates, 2.) the two environmental strains uninduced versus induced at 1h, and 3.), 4.) the two clinical isolates uninduced versus induced at 3 h and 12 h. Each two groups of the four comparison aligned into separate clusters, that means differences in the expression between the examined groups.

In monocytes 118.641 transcripts were identified, but surprisingly only 29 of them showed significantly different expression compared to the non-infected control. Out of these 21 encode protein. The 29 transcripts were used to calculate the Euclidean-distances between the samples. This analysis revealed differences

in the monocyte response given to clinical and environmental strains.

Comparative genomic analysis of clinical and environmental isolates of *C. parapsilosis*

Our earlier results revealed notable differences between clinical and environmental isolates of *C. parapsilosis* regarding to their resistance to phagocytosis and monocyte elimination mechanisms, the distribution of the members of the *ALS* (Agglutinin-like sequences) genefamily, and chromosome arrangements. To map the genomic variation of *C. parapsilosis* isolates originated from different sources whole genom sequencing was carried out involving two environmental (CBS 1954 and CBS 6318) and GA1 clinical isolates, then comparative genomic analysis was performed including CDC317 strain as a reference. The project was managed in tight collaboration with Toni Gabaldón and Leszek Pryszcz from the Comparative Genomics group of CRG (Barcelona, Spain).

In silico analysis identified a total of 5147 SNPs and determined the distribution of these in CBS 1954, CBS

6318 and GA1 compared to the CDC317. Although evidence of recombination has never been described in this species, the frequency and distribution of the SNPs refers to this molecular event.

The comparison predicted forty chromosomal mutations: five duplications (DUP) and 35 deletions (DEL). The DUP#5 causes the copy number variation of a physiologically important gene named *ARR3*, that is an ortholog of the *S. cerevisiae ARR3* (arsenite transporter). This was found to be responsible for arsenite resistance in baker's yeast. The copy number variation and the flanking sequences of the copies of this gene in clinical isolates supports the idea that the "environment to host" transition occured more than once during the phylogeny of this species. This recognition contradict the present point of view that considers all clinical *C. parapsilosis* isolates being clonal.

The 35 deletions range from 17 to 23.475 bps. Out of these 31 affected protein coding regions and 18 seem to led gene fusions. Most of them are specific to one single strain. Five deletions were found to be heterozygous. Twenty out of the 35 deletions were choosen for

experimental validation. PCR and Southern hybridisation strategies were designed and applied together with Sanger sequencing. The nucleotide order determined by Sangerseq made possible to identify the breakpoints of the deletions precisely. In ten out of twenty cases Sangerseq failed possibly due to secondary structures of the fragments. The analysis of the flanking sites of deletions affecting the *ALS* genes revealed nucleotide patterns referring to recombination events. Results of molecular techniques verified the *in silico* findings in 19 cases out of 20 the deletions choosen for experimental validation. Out of the five heterozygous region only three were confirmed. (Pryszcz *et al.*, 2013)

Summary

- Development of an *in vitro* model to study the interaction between mammalian phagocytes and *Candida* cells

- Characterisation of the interaction by using microscopic and molecular techniques

- RNA-seq analysis of the whole transcriptomes isolated from the host and yeast cells upon infection

- Detection of whole genom duplication in *C. metapsilosis*

- Identification of more than 300 previously unknown genes in *C. parapsilosis*

- Revealing of pattern referring to recombination in *C. parapsilosis*

- Performing whole genome sequencing involving three *C. parapsilosis* isolates and comparative genomics including the reference *C. parapsilosis* strain

- Molecular validation of the *in silico* results

Publication

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Total impact factor: 18,119