Cryptococcus at Work: Gene Expression during Human Infection

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ABSTRACT Meningitis is a frequent manifestation of infection due to *Cryptococcus neoformans* and a major cause of increased morbidity in patients with AIDS. Numerous *in vitro* gene expression and genetic studies of the fungus have predicted a myriad of genes, pathways, and biological processes that may be critical for pathogenesis, and many studies using animal models have supported the role of these processes during infection. However, the relevance of these hypotheses based on *in vitro* and animal models has often been questioned. A recent study by Chen et al. [Y. Chen, D. L. Toffaletti, J. L. Tenor, A. P. Litvintseva, C. Fang, T. G. Mitchell, T. R. McDonald, K. Nielsen, D. R. Boulware, T. Bicanic, and J. R. Perfect, mBio 5(1):e01087-13, 2014] represents an important step in understanding the cryptococcal response during human infection.

Cryptococcus neoformans is distributed worldwide, grows in soil, fends off a variety of phagocytic predators, and can infect a wide variety of animals and plants. The presence and survival of the fungus in these diverse environmental and host conditions imply that the fungus has evolved a well-orchestrated gene expression program that enables it to adapt and proliferate under various conditions. The data from testing a multitude of genetic variants under defined *in vitro* growth conditions and in multiple animal infection models for *C. neoformans* have been used to formulate and test hypotheses about pathogenesis in humans.

Examination of gene expression profiles of *C. neoformans* under specific *in vitro* stress conditions that are thought to mimic conditions encountered by the fungus within the human host has been widely used to explore the molecular response of *C. neoformans* to predicted host stress conditions. A major advantage of the *in vitro* studies is that many variables can be carefully controlled and sufficient biological and technical replicates can be generated to obtain highly reproducible and significant data. These *in vitro*-derived data sets have suggested roles for different biological processes during pathogenesis. However, data from one narrowly defined set of experiments may not be generalizable to animal or human infection. Even within one animal host, the fungus has to survive under diverse tissue and immunologic stress conditions. Our understanding of how the fungus adapts to these changing conditions within the host is limited, and *in vitro* experiments are unlikely to fully explain these adaptative responses. However, obtaining sufficient statistical power in studies that are limited by having few biological samples makes it more difficult to generate firm conclusions.

Therefore, one way to ascertain the involvement of mechanisms of pathogenesis gleaned from experiments carried out under laboratory conditions is to directly examine the genomic response of the fungus growing inside an infected human patient. A major technical hurdle to examining gene expression during human infection has been obtaining sufficient high-quality material to generate the RNA for analysis, due to the limited number of fungal cells and the presence of large amounts of polysaccharide capsule, which interferes with the purity of RNA. More importantly, these experiments are particularly challenging to interpret due to the difficulty of controlling for multiple variables, including stage of infection, host responses, complications of other infections, and the strain of *Cryptococcus* that is causing the infection. The variability introduced by different infecting genotypes was well demonstrated in a study by Wiesner et al. (1) in which they analyzed 140 *Cryptococcus* strains isolated from 111 human AIDS patients presenting with *Cryptococcus* meningitis. The found a significant correlation between the genotype and the phenotypic response elicited by the fungal strain in the human host.

Chen and colleagues in John Perfect's laboratory at Duke University (2) have pioneered the examination of *C. neoformans* gene expression during human infections. They have succeeded in extracting sufficient amounts of good-quality RNA from *C. neoformans* growing in the cerebrospinal fluid (CSF) of two AIDS patients. They subjected the RNA samples to whole-genome transcriptome analysis employing transcriptome sequencing (RNA-Seq). In addition, they purified RNA from the same yeast strains after culturing them in yeast extract-peptone-dextrose (YPD) and in human CSF under laboratory conditions. They were able to produce high-quality sequence data from the RNA isolated from the yeast cells collected from human patients, with 95 to 98% of the reads mapping to the predicted genes of the H99 genome. One of the surprising revelations of this study is the high similarity of the gene expression profiles between the samples from cells growing in the CSF of the human patient and cells growing in YPD under laboratory conditions. This is intriguing because there are substantial differences in the growth conditions, including CO_2 levels, stress, exposure to light, and nutrient composition. The ontology analysis of the differentially expressed genes in the patient's CSF and in YPD in comparison to cells grown in CSF *in vitro* indicated that the expression of genes in the cellular metabolism category increased, suggesting that yeast cells are actively growing in CSF inside the human patient. Active metabolism was also observed in *C. neoformans* in the CSF of infected rabbits by the use of serial analysis of gene expression (SAGE) (3), along with induction of various other biological processes.

The environment in the lung may be very different from the environment in the CSF. Comparative SAGE analysis of yeast cells isolated from infected mouse lungs and those from infected rabbit

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CSF showed that the gene expression profiles of *C. neoformans* growing in mouse lung was significantly different from that of the yeast isolated from rabbit CSF. The cells from rabbit CSF most closely resembled cells grown *in vitro* in low-iron medium (LIM) (4). Moreover, when a library of ~1,400 gene deletion strains were subjected to a virulence study employing a mouse inhalation model, there were 164 mutants which showed decreased ability to infect and grow inside the mouse lung (5). On the other hand, when the same deletion strain library was used for survival studies of the mutant strains in human CSF, there were only 13 mutants that showed impaired growth in human CSF (6). These data further support the requirement of more robust mechanisms of gene regulatory networks when the fungus is grown in the lung than when the fungus is grown in human CSF. One possibility is that in human CSF, *C. neoformans* may experience mild stress, which in turn may positively affect fungal growth. When *C. neoformans* cells are grown under *in vitro* conditions, we have observed that mild stress, such as low oxidative stress conditions, increases the rate of growth significantly (reference 7 and unpublished data).

Chen et al. (2) did find 20 genes that were upregulated only in the cells growing in the human CSF, and these included genes predicted to be critical for fungal virulence. One of the genes upregulated inside the human body was a homolog of *SRX1*, a gene involved in oxidative stress resistance, and *SRX1* was recently shown to be critical for *C. neoformans* pathogenesis (8). Two other genes, *ENA1* and *RIM101*, were upregulated under CSF conditions and have been shown to be important for cryptococcal virulence in animal models (9, 10).

To associate the fungal gene expression profile with a specific genotype, Chen et al. identified the genotype of the two strains isolated from human CSF. They found that one isolate belonged to the G0 subtype and the other to the HC1 subtype of *C. neoformans* var. *grubii*, an observation consistent with fact that the two strains were obtained from individuals residing in different geographical locations. Comparison of the gene expression patterns of these two strains indicated that around 100 genes are differentially expressed between the G0 and HC1 subtypes. Further single-nucleotide polymorphism (SNP) analysis revealed the presence of a single-nucleotide variant (SNV) in these isolates, and the consequence of this SNV was demonstrated by the increased sensitivity of the HC1 strain, compared to the G0 strain, to exogenous peroxide, providing molecular evidence for the potential influence of SNPs on fungal virulence and subsequent clinical outcome.

Understanding the role and expression of various cryptococcal biological processes during the different stages of human infection is important to help develop effective therapeutic interventions for fungal infections. Depending on their environment of growth, *C. neoformans* isolates may exhibit varied sensitivity to growth inhibitory molecules. The results presented by Chen and colleagues are important first forays into the exploration of genomic regulation in the human host.

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