Microevolution in the Human Fungal Pathogen Cryptococcus neoformans

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Continued growth of immune-deficient patient populations has seen the incidence of secondary infection by fungal pathogens rise sharply to become a significant cause of morbidity and mortality. The haploid basidiomycete yeast *Cryptococcus neoformans* is a leading cause of opportunistic fungal infections amongst the immunocompromised, representing one of the most common life-threatening opportunistic infections in individuals with AIDS (1). The most recent report from the US Centers for Disease Control and Prevention (CDC) estimates there are approximately 958,000 cases of cryptococcosis among individuals with HIV every year and more than 624,000 deaths, with approximately 504,000 of these in sub-Saharan Africa (2).

Cryptococcal infection follows a distinct pathology. Dessicated yeast cells and sexually produced basidiospores are small enough to be inhaled and escape the ciliary action of the lungs. Most individuals are exposed in this way to *C. neoformans* at an early age, when infections are either cleared by the immune system or form granulomas in the lungs or hilar lymph nodes (3). If the infection is not overcome, it may progress to cryptococcal pneumonia, although in immunocompromised individuals, *Cryptococcus* more typically disseminates to the central nervous system (1).

Pharmacologic management of cryptococcosis typically involves induction therapy with the antifungals amphotericin B and flucytosine, followed by consolidation therapy with fluconazole for as long as 12 months, depending on the clinical status of the patient. However, in patients with AIDS, cryptococcal meningoencephalitis is usually incurable. Individuals who survive the initial infection are given lifelong antifungal therapy and can undergo relapse. The incidence of relapse decreases for patients undergoing immune reconstitution through highly active antiretroviral therapy. In the absence of maintenance therapy or patient compliance, high rates of fungal persistence and frequent disease relapse occur, leading to a cohort with repeating rounds of infection (**Fig. 1**). Dealing with relapse events is therefore a critical aspect of treating the disease, as they are often more threatening than the initial infection (4).

Relapse and Microevolution

Analysis of initial and relapse isolates has shown that the majority of clinical recurrences are a result of persistence of the original infection rather than reinfection with a new strain (4,5). Although sequential isolates from a single patient may appear genotypically identical by other tests, they often contain gross chromosomal rearrangements that can only be visualised using pulsed-field gel electrophoresis of whole chromosomes (5) (**Fig. 2**). These karyotypic variants arise readily, and can occur at any stage from early to late infection and in the presence or absence of antimycotic treatment (6). Importantly, these new variants predominate in later stages of infection, suggesting that they may confer a selective advantage. In support of this theory, emergence of a second karyotype during therapy has been observed on more than one occasion coincident with increases in virulence and drug resistance (7,8).

Why could such changes potentially be important during infection? Karyotypic change is an alteration in chromosome size that can generally be attributed to large scale translocations, deletions, insertions, changes in copy number of repetitive DNA sequences and, perhaps most

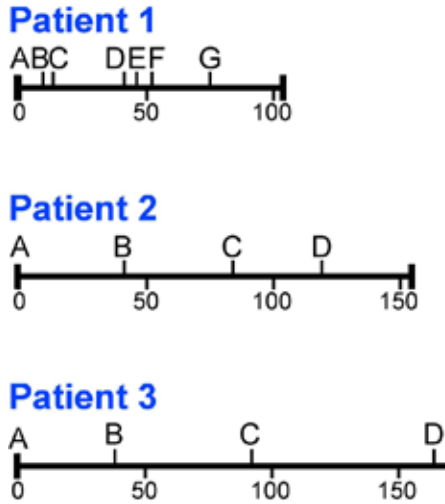

Fig. 1. Relapse of cryptococcal meningoencephalitis.
In the absence of appropriate therapeutic intervention or patient compliance, individuals who have suffered a *C. neoformans* infection can readily undergo relapse. Example timelines are shown for the collection of serial isolates from individual patients (A represents the first isolate collected, B the second, etc.) collected between 1998 and 2004 from three AIDS patients from Duke University Medical Center. While some positive samples are clustered and correspond to a single infection, all three of these patients overcame their initial infection only to undergo relapse. Patient 3 represents a highly unusual case, with numerous rounds of relapse occurring due to patient noncompliance. Scale is in days.

significantly in this case, duplications. First proposed by Susumu Ohno in his seminal work *Evolution by Gene Duplication*, the creation of segmental duplications provides an organism with additional functional genetic material that is now free of selective pressure (9). In the simplest scenario, the extra gene copy provides additional product that may modify a phenotype, such as increasing levels of a virulence factor to enable a more successful infection. In a more complex long-term evolutionary model, these additional copies are now free from selective constraint. They may be mutated to gain new functions without loss of the function encoded by the original copy of the gene, or both copies of the gene may evolve to play only a specialised part of the role that the single copy originally performed.

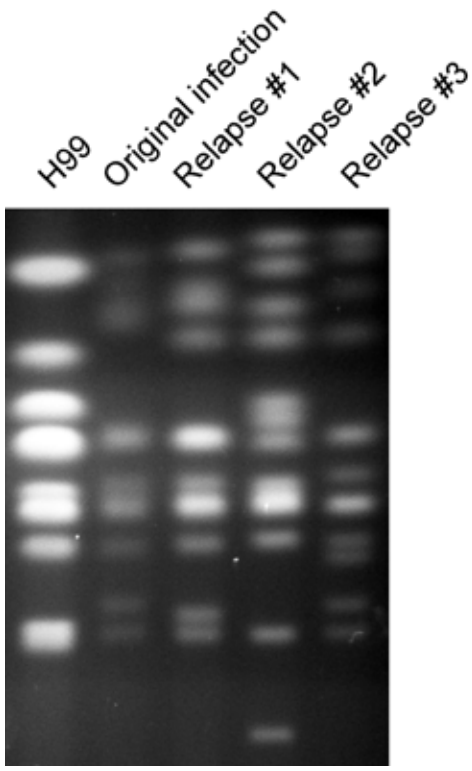

Fig. 1. Evolution of altered karyotype during relapse infections revealed via pulsed field gel electrophoresis. The rapid evolution of karyotypic variants during infection of humans is in stark contrast to the behaviour of *C. neoformans* during normal growth. Karyotypic variation is common among diverse clinical and environmental isolates. However, unlike other medically important fungi such as *Candida albicans*, these karyotypes are stable in the laboratory setting during passage under standard growth conditions, which are far less stressful than those encountered in the human host. Although the common laboratory strain H99 (lane 1) has a different karyotype to other isolates, such as the serial clinical isolate series shown (lanes 2–5), this is surprisingly stable during laboratory passage. In contrast, electrophoretic karyotyping of this series of isolates from four sequential infections of a single patient reveals that these strains, identical by other tests such as MultiLocus Sequence Typing, have undergone extensive gross chromosomal rearrangements.

Examples of Microevolution in *Cryptococcus*

Extensive rearrangements in the *Cryptococcus* genome were first observed at the molecular level during studies of the *MAT* locus, a unique region of the genome that governs the establishment of cell type identity and controls the sexual cycle. DNA sequence at this locus must differ between strains for the traditional sexual cycle to occur. Known as a bipolar system, **a** cells bear the *MAT**a*** mating-type locus allele and α cells bear the *MAT*α allele. Beyond the observation that this locus is unusually large (>100 kb) compared to the equivalent structure in most fungal species (~1 kb), sequencing of this region revealed that the locus has been highly rearranged by a complex series of inversions, leading to structural divergence not only between mating types, but also at the subspecies level (10-12). Analysis of the locus revealed a high repeated element content, including a number of predicted transposons that are theorised to have played a key role in this disruption of synteny between genomes that are otherwise largely colinear. Functionally, these rearrangements are highly significant, as they are likely responsible for the suppression of recombination within the *MAT* locus that enables maintenance of the distinct **a** and α alleles. However, the rearrangement of the *MAT* locus is not a truly microevolutionary event. These structures arose over millions of years during the process of speciation, in contrast to the karyotypic changes observed during infection, which typically arise within days or weeks. However, the *MAT* story nevertheless provides insight into these genomic rearrangement events, as highlighted by two key discoveries made possible by the completion of several genome sequence projects.

In the first example, assembly of the *C. neoformans* var. *neoformans* genome was confounded by a misassembly that consistently indicated only 13 chromosomes were present rather than the known 14 (13). With the aid of karyotypic analysis combined with a study of transposon distribution in the genome, it was revealed that the misassembly was caused by a large segmental duplication (14). Formed during the creation of a *C. neoformans* var. *neoformans* congenic laboratory pair, two chromosomes were thought to have undergone ectopic recombination between subtelomeric copies of a non-LTR retrotransposon, resulting in the formation of a chromosomal translocation and a large segmental duplication (**Fig. 3**). The duplication spanned 62,872 identical nucleotides and generated a second copy of 22 genes. Importantly, the duplication included a cluster of genes thought to play a role in defense against oxidative stress like that encountered in the host, therefore potentially conferring a selective advantage during infection.

In the second example, a comparative genomic approach between the *C. neoformans* var. *neoformans* and the closely related *C. neoformans* var. *grubii* genomes identified a nearly identical ~40 kb region containing 14 genes that resulted from a nonreciprocal transfer event from var. *grubii* to var. *neoformans* (15). This introgression event, present in the vast majority of clinical and environmental var. *neoformans* strains around the world, is predicted to have occurred via an incomplete intervarietal sexual cycle that created a hybrid intermediate where mobile elements

common to both lineages mediated the exchange (15). As with the previous example, transposable elements are predicted to have played a key role in this process. But what is so important about this introgressed fragment that has conferred such a selective advantage? The prediction that this region of DNA contains beneficial genetic information that confers increased fitness over the var. *neoformans* original sequence it has replaced is supported by the fact that a portion of this same region has been subsequently duplicated in laboratory strains (15). Furthermore, this also supports evolutionary theories that instabilities in subtelomeric regions promote adaptive evolution through gene amplification and subsequent adaptation.

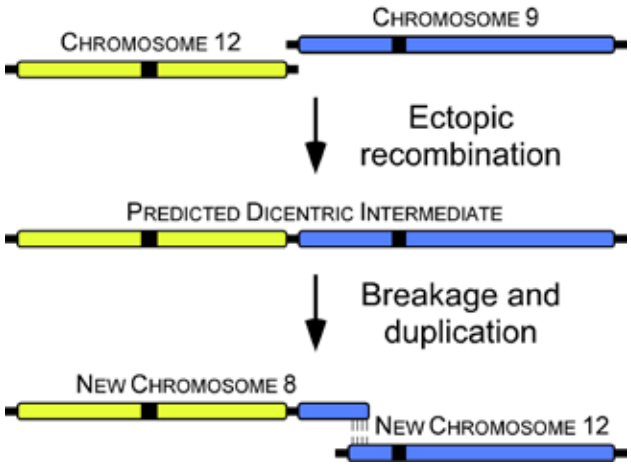

Fig. 3. Characterisation of karyotypic changes in *C. neoformans*.
Comparison of two *C. neoformans* var. *neoformans* genomes revealed that the most common *C. neoformans* laboratory strain contains a large translocation and duplication. Pulsed field gel electrophoresis, Southern blotting and sequencing suggests that this occurred through ectopic recombination between subtelomeric copies of a non-LTR retrotransposon, with the predicted dicentric intermediate resolving to create a translocation and duplication of 22 predicted genes (13).

The Driving Force Behind Karyotypic Change

A common feature arising from analysis of genomic rearrangements in *C. neoformans* is the association of mobile elements, with similar observations reported in experimental evolution studies of the model ascomycete *Saccharomyces cerevisiae*. In the laboratory, prolonged culture of *S. cerevisiae* under nutrient limitation selects for altered karyotype, and there is evidence that these changes confer a selective advantage. For example, strains evolving under glucose limitation acquire chromosomal changes that amplify key genes involved in glucose metabolism or alter their *cis*-acting sequences, with multiple independent strains bearing similar translocations (16). Examination of chromosomal breakpoints in this instance revealed the presence of transposable elements, suggesting a role for these elements as facilitators of karyotypic change. Karyotype alteration therefore provides an avenue for genotypic diversity that can confer a selective advantage, facilitating adaptation to new growth conditions. In addition, comparison of closely related *sensu stricto* species of *Saccharomyces* revealed that the majority of translocations that have occurred during divergence of these lineages involved ectopic recombination between transposable elements, potentially contributing to reproductive isolation and speciation (17).

Transposable elements are dynamic features of the genome. While some elements amplify and proliferate, others become extinct and are gradually eroded away by mutations. By allowing relatively high frequency and adaptively useful chromosomal rearrangements, appropriately positioned transposable element-related sequences may facilitate preferential survival of those lineages in which transposable elements are present. A survey of the *C. neoformans* var. *neoformans* genome revealed that it contains at least 15 LTR retrotransposon species from the Ty1-*copia* or Ty3-*gypsy* families (18). A telomere-localising non-LTR retrotransposon species is also present, in addition to a member of the tyrosine-recombinase-encoding retrotransposons. Together, these elements represent at least 5% of the 20 Mb *C. neoformans* var. *neoformans* genome; however, as these estimates were based on a partial survey of retrotransposons in the genome, it is likely an underestimate. The distribution of transposable elements in *C. neoformans* var. *neoformans* is remarkable; in addition to being scattered throughout the genome, each chromosome bears a single large cluster 40–100 kb in size constructed almost entirely of transposable element fragments that is predicted to be the centromere (13).

As in many other organisms, transposons have been shown to provide a source of genetic variation in *C. neoformans* by inserting into new positions in the genome. In a laboratory study, multiple spontaneous mutants resistant to the antimycotics FK506 and rapamycin were identified (19). Two of these spontaneous mutants resulted from gene-disrupting transposon movement, defining the only two *Cryptococcus* transposons identified by virtue of their mobility. Given that two of the transposons present in the genome have the capacity to facilitate the development of resistance to antimycotic agents, it is important to understand whether these transposition events can also occur in the clinical setting. As observed in *S. cerevisiae*, a recent comparison of the *C. neoformans* var. *neoformans* and var. *grubii* genomes has also identified a range of chromosomal rearrangements that have occurred during divergence of these varieties, many of which were associated with predicted mobile elements (20). Transposable elements may therefore play a key role in generating karyotypic diversity in *C. neoformans* during growth in the host, a condition of prolonged culture that is stressful, nutrient limiting, and known to produce chromosomal length polymorphism.

Conclusion

The completed *Cryptococcus* genomes have provided a stepping stone towards the elucidation of what has now been recognised as a common feature of cryptococcal infection – the rearrangement of the genome in response to infection of the human host. The capacity of this organism to rapidly undergo gross chromosomal rearrangement is remarkable, and it does so at a surprising rate. And while our discoveries so far have begun to illuminate the

types of changes occurring, we have yet to characterise these in serial relapse isolates or correlate them with an alteration in virulence. Hopefully, the availability of next-generation sequencing platforms will herald a new era of understanding this pathogen based on weaknesses identified through analyses of the microevolutionary events occurring in the stressful, nutrient-poor environment that is the human body.

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