Molecular epidemiology of *Cryptococcus neoformans* species complex isolates from HIV-positive and HIV-negative patients in southeast China

Min CHEN1*, MM, Xiao-Ran LI2*, BS, Shao-Xi WU3, MD, Xiao-Ping TANG4, MD, Bi-Wei FENG2, BS, Zhi-Rong YAO5, MD, Wei-Hua PAN1, MD, Wan-Qing LIAO (✉)1, MM, Zhe-Xue QUAN (✉)2, PhD

1 National Laboratory of Cryptococcus Neoformans, Department of Dermatology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China
2 Department of Microbiology & Microbial Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China
3 Chinese Cultural Collection Commission for Microbiology, Institute of Dermatology, Chinese Academy of Medical Science, Nanjing 210042, China
4 Guangzhou No.8 People’s Hospital, Guangzhou 510060, China
5 Department of Dermatology, Xinhua Hospital, Shanghai Jiao Tong University, Shanghai 200092, China

© Higher Education Press and Springer-Verlag Berlin Heidelberg 2010

Abstract  This study investigated the molecular types of the *Cryptococcus neoformans* species complex isolates and their clinical manifestations among human immunodeficiency virus (HIV)-positive and HIV-negative patients in southeast China in the past 15 years. The molecular types of 109 isolates from 108 patients were analyzed by the PCR fingerprinting method, sequences of internal transcribed spacers of rDNA (ITS region), and sequences of the capsule-associated gene (*CAP59*). In HIV-positive patients, clinical isolates were grouped into molecular types VNI (75%, 15/20), VNII (15%, 3/20), and VNIII (10%, 2/20). In HIV-negative patients, the majority of the clinical isolates were grouped into molecular types VNI (72%, 64/89), VNII (13%, 12/89), VGI (12%, 11/89), VNIII (1%, 1/89), and VGII (1%, 1/89). In reference to the mating type of the isolates, 97% (106/109) were of the *MATα*, 2% (2/109) were of the *MATα/-* and 1% (1/109) were of the *MATα/a*. As for the clinical manifestations of the molecular types among the patients, the average cerebrospinal fluid (CSF) pressure of the patients infected by the *C. gattii* was higher than that of the patients infected by the *C. neoformans*. These results suggest that both HIV-positive and HIV-negative cryptococcal patients in the southeast of China are mostly infected by the *C. neoformans* strains. No *C. gattii* strains were found in HIV-positive patients.

Keywords  *Cryptococcus neoformans*; *Cryptococcus gattii*; cryptococcosis; molecular epidemiology; molecular type; mating type

1 Introduction

*Cryptococcus neoformans*, a fatal fungal pathogen, was previously divided into two varieties comprising *C. neoformans* var. *neoformans*, which is the opportunistic agent of cryptococcosis in immunodepressed hosts, and *C. neoformans* var. *gattii*, which is a probable cause of cryptococcosis in immunocompetent hosts [1,2]. Recently, *C. neoformans* var. *gattii* has been defined as a separate species, *C. gattii*, due to the divergence of ecological, biochemical, and molecular characteristics [3]. Today, these *C. neoformans* species complex contains *C. neoformans* var. *neoformans* (serotype D), the hybrid isolates (serotype AD), *C. neoformans* var. *grubii* (serotype A) [4] and *C. gattii* (serotypes B and C) [3]. In addition, *C. neoformans* species complex is a heterothallic yeast with two alternative mating types, α or a, which can multiply by budding or sexual reproduction, respectively [5].

Several molecular typing methods have been widely used in epidemiological molecular analyses of the *C. neoformans* species complex including electrophoretic karyotyping [6], random amplified polymorphic DNA (RAPD) [7], restriction fragment length polymorphism (RFLP) [8], amplified fragment length polymorphism...
(AFLP) [9], internal transcribed spacer rDNA (ITS region) [10] and multilocus sequence typing (MLST) analysis [11]. For example, polymerase chain reaction (PCR) fingerprint patterns based on M13 microsatellite DNA identified eight major molecular types among the C. neoformans species complex isolates [7].

To summarize the molecular types and serotypes within C. neoformans, the molecular types VNI and VNII isolates belong to serotype A (C. neoformans var. grubii) and VNIII or VNIV isolates belong to serotype AD or serotype D (C. neoformans var. neoformans). Although the majority of molecular types VGI and VGII isolates belong to serotype B (C. gattii), molecular types VGI, VGII, VGIII and VGIV have no homologous relationship with serotype B and C in C. gattii isolates [12].

Despite recent reports for the molecular types of clinical isolates from China, the cryptococcal infections have been mostly reported from human immunodeficiency virus (HIV)-negative patients [13]. The isolates and clinical manifestation of cryptococcosis from Chinese HIV-positive or other immunocompromised patients have rarely been included. Interestingly, China has also been confronted with a significant increase in acquired immune deficiency syndrome (AIDS) cases with cryptococcosis, which continues to be associated with HIV infections in China in recent years [14, 15]. According to the most recent data of The Ministry of Health of the People’s Republic of China, southeast China was also an important region where the incidence of HIV infections was increased (http://www.gov.cn/gzdt/2009-02/17/content_1233236.htm).

To fill in the gap of available data, this study was performed to analyze the different molecular types of C. neoformans species complex isolates and the clinical manifestations of cryptococcosis between HIV-positive and HIV-negative patients in southeast China.

2 Materials and methods

2.1 Cryptococcal isolates

A total of 109 clinical strains from 108 patients were obtained from ten Chinese university hospitals, and the patients came from 26 cities of ten provinces in southeast China where the subtropical climate dominates. Each strain submitted for analysis was subcultured and a single colony was selected. After identification was confirmed by caffeic acid agar, positive urease test, and its ability to grow at 37°C, species were differentiated by culturing the isolates on L-canavanine-glycine-bromothymol blue medium [16]. The reference strains of each PCR fingerprint pattern were: WM148 (CBS10085, VNI, serotype A), WM626 (CBS10084, VNII, serotype A), WM628 (CBS10080, VNIII, serotype AD), WM629 (CBS10079, VNIV, serotype D), WM179 (CBS10078, VGI, serotype B), WM178 (CBS10082, VGI, serotype B), WM161 (CBS10081, VGIII, serotype B), and WM779 (CBS10101, VIV, serotype C). Ethical approval was granted by the Second Military Medical University Ethics Committee, Shanghai, China.

2.2 DNA extraction

Genomic DNA was extracted with benzyl chloride following the procedures described previously [17] and purified using the Wizard DNA Clean-Up system according to the manufacturer’s protocol. Concentration and quality of the purified DNA was evaluated by 1.4% agarose gel electrophoresis and fluorescence spectrophotometry (Nanodrop 3300, Rockland, DE, USA).

2.3 Internal transcribed spacer (ITS) region and CAP59 gene sequencing

The ITS region including 5.8S rDNA cluster was amplified using the ITS5 and ITS4 primer set [10]. The CAP59 gene was amplified using the primer set CH-CAP59F (5’-CCT YGC CGA AGT YCG AAA CG-3’) and CH-CAP59R (5’-AAT CGG TGG TTG GAT TCA GTG T-3’) designed by Enache-Angoulvant et al. [18] with slight modifications. All amplification reactions were performed with Taq DNA MasterMix (TIANGEN, China) with a total volume of 50 μL and 20 ng of DNA as template. PCR products were directly sequenced on an ABI 3730 automated DNA sequencer twice in opposite directions.

2.4 Phylogenetic analysis

The sequences were aligned with ClustalX version 1.83 [19]. A phylogenetic tree was constructed by MEGA software version 3.0 [20] with the neighbour joining method based on the nucleic acid sequences. Bootstrap analysis with 1000 replications was done to test the robustness of the internal branches.

2.5 PCR fingerprinting

A primer of the minisatellite-specific core sequence of the wild-type phage M13 was used as a single primer, and the PCR condition was in accordance with that originally described by Meyer et al. [7]. Amplification reactions were performed with Taq DNA MasterMix (TIANGEN, China) with a total volume of 50 μL and 20 ng of DNA as template. Amplification products were separated by gel electrophoresis on 1.4% agarose gels in 1× TAE buffer at 110 V for 1 h. DNA bands of each fingerprinting pattern were defined manually by comparison to the reference strains of the eight major molecular types.

2.6 Mating type

Determination of mating type was established by PCR