Molecular Epidemiology of Clinical Cryptococcus neoformans Isolates in Seoul, Korea

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Abstract Cryptococcal infection is primarily caused by two species, Cryptococcus neoformans and C. gattii. Between the two species, C. neoformans var. grubii is the major causative agent of cryptococcosis in Asia. We investigated the molecular characteristics of 46 isolates of C. neoformans from patients with cryptococcosis between 2008 and 2012 in Seoul, Korea. All the isolates were determined to be C. neoformans var. grubii (serotype A), mating type MATα and molecular type VNI by PCR-restriction fragment length polymorphism of the URA5 gene. Multilocus sequencing type (MLST) analysis using the International Society of Human and Animal Mycoses (ISHAM) consensus MLST scheme identified two sequence types (ST). Out of the 46 strains, 44 (95.7%) were identified as ST5, and remaining 2 were identified as ST31. Our study revealed that the clinical strains of C. neoformans in Korea are genetically homogeneous with the VNI/ST5 genotypes, and new appearance of VNI/ST31 genotype may serve as an important indicator of global genetic analysis.

Keywords Cryptococcus neoformans, Molecular type, Multilocus sequencing type

Cryptococcosis is a life-threatening fungal infection affecting both immunocompromised and immunocompetent hosts. It is caused by two species of the genus Cryptococcus: C. neoformans and C. gattii. Although both species can cause pulmonary and central nervous system infections, they contain a number of genetically diverse subgroups and differ in their ecology and epidemiology [1, 2]. C. neoformans has been isolated worldwide from pigeon excreta, and comprises of two varieties and three serotypes: C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (serotype D), and a hybrid (serotype AD), whereas C. gattii comprises two serotypes, B and C based on differences in the capsular components [3, 4]. Among the Cryptococcus species, C. neoformans var. grubii (serotype A) is found worldwide and is responsible for >90% of cryptococcal infections and with >99% of infections in patients with acquired immunodeficiency syndrome [5]. C. neoformans var. neoformans (serotype D) has a comparable distribution worldwide, and in some areas such as sub-Saharan Africa up to 20% of the isolates are serotype D strains [6]. C. gattii, previously classified as C. neoformans var. gattii (serotypes B and C), was until recently considered to be restricted to tropical and subtropical climates zones and thought to be found mainly on Eucalyptus trees. In addition, this species has a predilection for infecting immunocompetent hosts [7, 8]. A recent outbreak of C. gattii in the Pacific Northwest of North America, a temperate area, indicated an environmental shift for this species [9]. C. neoformans has two mating types, MATα and MATα, controlled by a single locus two allele mating system with MATα being the prevalent mating type among both clinical and environmental isolates [10, 11].

Several molecular typing methods have been used to study the molecular epidemiology of the C. neoformans and C. gattii. These methods include M13-PCR fingerprinting, amplified fragment length polymorphism, PCR-restriction fragment length polymorphism (RFLP) analysis of the orotidine monophosphate pyrophosphorylase (URA5) or phospholipase B (PLB1) genes, random amplification of polymorphic DNA analysis, multilocus microsatellite typing, and multilocus sequence typing (MLST) [12-16]. Based on their genetic differences, eight distinct molecular types have been recognized in two species: VNI and VNII (C.
*Cryptococcus neoformans* var. *grubii*, serotype A), VNIII (serotype AD, hybrid), VNIL (C. *neoformans* var. *neoformans*, serotype D), and VGI, VGII, VGIII, and VGVIV (C. *gattii*, serotypes B and C). Within *C. neoformans* var. *grubii*, VNI is the most prevalent genotype worldwide, especially in Asian countries, whereas VGI has been the most prevalent genotype of infections caused by *C. gattii* [17-19]. In addition, a new genotype in *C. neoformans* var. *grubii*, VNB, was recently discovered as a unique cryptococcal population in Botswana [5].

MLST is a typing method based on the sequence analysis of a set of polymorphic loci and has been used to trace the putative origin of pathogen populations. Recently, the International Society of Human and Animal Mycoses

| Strain | Gender | Age | Source                | Serotype | Mating type | Region | Year |
|--------|--------|-----|-----------------------|----------|-------------|--------|------|
| Sh79   | F      | 74  | CSF                   | A        | α           | Seoul  | 2008 |
| Sh98   | M      | 58  | Blood                 | A        | α           | Seoul  | 2008 |
| Sh99   | F      | 75  | Bronchial fluid       | A        | α           | Seoul  | 2008 |
| Sh100  | M      | 73  | CSF                   | A        | α           | Seoul  | 2008 |
| Sh102  | F      | 68  | Blood                 | A        | α           | Seoul  | 2008 |
| Sn103  | M      | 70  | CSF                   | A        | α           | Seoul  | 2008 |
| Sh107  | M      | 48  | Tissue                | A        | α           | Seoul  | 2008 |
| Sh108  | M      | 62  | Abdominal fluid       | A        | α           | Seoul  | 2008 |
| Cn129  | M      | 86  | Blood                 | A        | α           | Seoul  | 2008 |
| Cn130  | M      | 39  | Blood                 | A        | α           | Seoul  | 2008 |
| Cn131  | M      | 82  | Blood                 | A        | α           | Seoul  | 2008 |
| Cn132  | F      | 68  | PCNA                  | A        | α           | Seoul  | 2009 |
| Cn133  | M      | 48  | CSF                   | A        | α           | Seoul  | 2009 |
| Cn134  | M      | 86  | CSF                   | A        | α           | Seoul  | 2009 |
| Sh109  | M      | 35  | CSF                   | A        | α           | Seoul  | 2009 |
| Sh110  | M      | 77  | Pleural fluid         | A        | α           | Seoul  | 2009 |
| Sh111  | F      | 70  | Bronchial fluid       | A        | α           | Seoul  | 2009 |
| Sh112  | M      | 80  | CSF                   | A        | α           | Seoul  | 2009 |
| Sh113  | F      | 27  | Blood                 | A        | α           | Seoul  | 2009 |
| Sh114  | F      | 70  | Blood                 | A        | α           | Seoul  | 2009 |
| Sh115  | F      | 68  | Abdominal fluid       | A        | α           | Seoul  | 2009 |
| Sh116  | F      | 51  | Tissue                | A        | α           | Seoul  | 2009 |
| Sh117  | M      | 57  | Blood                 | A        | α           | Seoul  | 2009 |
| Sh119  | F      | 60  | Urine                 | A        | α           | Seoul  | 2009 |
| Sh120  | F      | 65  | Blood                 | A        | α           | Seoul  | 2009 |
| Sh121  | F      | 67  | Bronchial fluid       | A        | α           | Seoul  | 2010 |
| Sh122  | F      | 65  | Blood                 | A        | α           | Seoul  | 2010 |
| Sh123  | F      | 63  | Tissue                | A        | α           | Seoul  | 2010 |
| Sh124  | F      | 61  | Sputum                | A        | α           | Seoul  | 2010 |
| Sh126  | M      | 49  | Blood                 | A        | α           | Seoul  | 2010 |
| Sh127  | M      | 66  | Blood                 | A        | α           | Seoul  | 2010 |
| Sh128  | F      | 54  | Ascitic fluid         | A        | α           | Seoul  | 2010 |
| Cn135  | F      | 78  | CSF                   | A        | α           | Seoul  | 2010 |
| Cn136  | M      | 70  | Blood                 | A        | α           | Seoul  | 2010 |
| Cn137  | M      | 39  | Blood                 | A        | α           | Seoul  | 2010 |
| Cn138  | F      | 75  | Blood                 | A        | α           | Seoul  | 2011 |
| Cn139  | M      | 52  | Urine                 | A        | α           | Seoul  | 2011 |
| Cn140  | F      | 74  | CSF                   | A        | α           | Seoul  | 2011 |
| Cn141  | M      | 44  | Blood                 | A        | α           | Seoul  | 2011 |
| Cn142  | M      | 71  | Blood                 | A        | α           | Seoul  | 2012 |
| Cn144  | F      | 72  | CSF                   | A        | α           | Seoul  | 2011 |
| Cn145  | F      | 55  | CSF                   | A        | α           | Seoul  | 2011 |
| Cn146  | F      | 51  | Ascitic fluid         | A        | α           | Seoul  | 2012 |
| Cn147  | F      | 42  | Blood                 | A        | α           | Seoul  | 2012 |
| Cn149  | F      | 53  | Blood                 | A        | α           | Seoul  | 2012 |
| Cn150  | M      | 60  | Abdominal fluid       | A        | α           | Seoul  | 2012 |
| K52    | F      | 64  | CSF                   | A        | α           | Choi et al. [17] |
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MATERIALS AND METHODS

Strains and serotyping. Forty six clinical Cryptococcus isolates obtained from Asan Medical Center and Seoul National University Hospital in Seoul, Korea, between 2008 and 2012 were used. Information about the clinical isolates is summarized in Table 1. All the isolates were grown on yeast peptone dextrose medium (Difco, Detroit, MI, USA) prior to subsequent analysis. The species identification of each strain was determined using the canavanine-glycine-bromothymol blue agar test [21], and serotyping was performed by slide agglutination (Crypto Check Kit; Iatron Laboratory, Tokyo, Japan), following the manufacturer's instructions. The reference strains used in this study were WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype AD, VNIII), and WM629 (serotype D, VNIv). In addition, K52 (VNIc/M5 strain) [17] was also included as a reference. All the strains were stored in 25% glycerol at −80°C.

DNA extraction and mating type analysis. Genomic DNA was extracted from each isolate using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The mating types were determined by PCR with specific primers to the MATα or MATα allele of the STE20 locus for C. neoformans strains, as described previously [11].

Molecular typing by URA5-RFLP. PCR of the URA5 gene was performed followed by restriction digestion with the enzymes, Hhal and SaeI, as previously described [17]. The digested PCR fragments were run on 2.5% agarose gel against 4 reference strains to determine their molecular types.

MLST analysis. MLST analysis was performed according to ISHAM consensus scheme of seven unlinked genetic loci (CAP59, GPD1, LAC1, PLB1, SOD1, URA5, and IGS1). DNA from each isolate was amplified by PCR in a 20 μL reaction volume for the each of the seven MLST loci by using the primers and protocols as described previously [15]. Each locus was subsequently sequenced using the Applied Biosystems 3730 sequencer with the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA). Alleles at each locus were assigned numbers (allele types [ATs]) following comparison with those previously identified in the global collection [20], resulting in a 7-digit profile for each isolate. Each unique allelic profile was concatenated and assigned a sequence type (ST) according to the MLST scheme (http://cneformans.mlst.net). The phylogenetic tree from the combined DNA sequences of the 7 genes was inferred by neighbor-joining using the MEGA 4 software (http://www.megasoftware.net) [22].

RESULTS

We collected 46 clinical strains of the C. neoformans from 2 hospitals located in Seoul, Korea. All of the 46 strains were C. neoformans var. grubii, serotype A, and mating type MATα, whereas C. gattii strain was not identified within any of the isolates during the study period. In addition, all strains proved to be VNI molecular types by URA5-RFLP analysis compared to the appropriate standard patterns for each molecular type (Fig. 1).

MLST sequence data were obtained for all 46 VNI strains based on seven unlinked genetic loci including conserved and variable regions of CAP59, GPD1, LAC1, PLB1, SOD1, URA5, and IGS1. The seven loci yielded 10 allele types (CAP59, 1; GPD1, 2; LAC1, 2; PLB1, 1; SOD1, 1; URA5, 1 and IGS1, 2 [AT]), and 2 STs, ST5 and ST31 within the isolates (Table 2). Of the 46 strains, 44 strains (95.7%) were ST5, and 2 strains (4.3%) were ST31. Most isolates in Korea were ST5, which was found to be the major MLST type from the Chinese, Hong Kong, and Japanese populations. A phylogenetic tree was constructed

Fig. 1. URA5-restriction fragment length polymorphism profiles obtained after double digestion with the restriction enzymes, Hhal and SaeI. Lane 1–4, Reference strains of Cryptococcus neoformans molecular type VNI, VNI, VNIII, and VNIV; lanes 5–16, selected clinical isolates (sh79, sh98, sh100, Cn129, Cn130, Cn131, Cn136, Cn137, Cn138, Cn140, Cn146, Cn147, and Cn150); M, molecular marker (100 bp DNA ladder).
by the neighbor joining method based on a concatenated data set from 7 loci (Fig. 2). In addition, these ST5 strains correlated with the VNIc/M5 genotype (K52 strain) which represents the major molecular type in Korea [17]. However, ST31 strains represented a new genotype of *C. neoformans* var. *grubii*, which has not been reported in Korea until now.

**DISCUSSION**

In this study, all 46 isolates from Seoul belonged to *C. neoformans* var. *grubii* (serotype A) and molecular type

![Table 2. The allelic profiles and sequence types of Cryptococcus neoformans isolates](image)

| Strain | Molecular type | AT  | ST  |
|--------|----------------|-----|-----|
|           | CAP59 | GPD1 | IGS1 | LAC1 | PLB1 | SOD1 | URA5 |
| Sh79     | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh98     | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh99     | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh100    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh102    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sn103    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh107    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh108    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn129    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn130    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn131    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn132    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn133    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn134    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh109    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh110    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh111    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh112    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh113    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh114    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh115    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh116    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh117    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh119    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh120    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh121    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh122    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh123    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh124    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh126    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh127    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Sh128    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn135    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn136    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn137    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn138    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn139    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn140    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn141    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn142    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn144    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn145    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn146    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn147    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn149    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| Cn150    | VNI   | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |
| K52      | VNIc/M5 | 1    | 3    | 1    | 5    | 2    | 1    | 1    | 5 |

AT, allele type; ST, sequence type.
Recent genotype analyses of global, clinical, and environmental isolates of *C. neoformans* var. *grubii* identified three genetic subpopulations, VNI, VNII, and VB. The molecular type VNI strain is the most prevalent causative agent of cryptococcosis worldwide, while VNII is globally distributed but rare. VB strain is highly diverse and apparently restricted geographically to Southern Africa, especially Botswana [5]. Khayhan *et al.* [18] reported that in Asian countries, 99.8% (n = 475) of *C. neoformans* var. *grubii* isolates belonged to VNI, 0.2% (n = 1) were VNII, and no VNB types were found. Our data demonstrate that the Korean clinical strains of *C. neoformans* are a genetically homogeneous population with the same VNI (100%) molecular type similar to the major type found in clinical strains of *C. neoformans* in China [23] and Japan [24].

According to the global analysis of population genetics using MLST, *C. neoformans* var. *grubii* database contains 110 STs, which have highly polymorphic alleles on the MLST gene loci [25]. Previously, Choi *et al.* [17] have reported that cryptococcosis in Korea was found to be mainly caused by *C. neoformans* var. *grubii* (serotype A) VN1c/M5 type by M13 fingerprinting method and MLST analysis. Our MLST data showed that 44 of the 46 VNI isolates were genotype ST5 and the remaining 2 were ST31. Additionally, we confirmed that the K52 (VN1c/M5) strain belonged to ST5 genotype in this study. Most *C. neoformans* var. *grubii* isolates from East Asia (Korea, China, and Japan), belong to ST5 with low genetic diversity within the population [23, 24]. However, in Thailand, ST4 and ST6 have been found to be the major MLST types, while ST93 is dominant in India and Indonesia. In addition, Thai cryptococcal isolates among VNI strains showed 13 different STs, revealing a greater genetic diversity than those of the East Asian region [20]. In this study, our data shows that the predominant MLST genotype of *C. neoformans* var. *grubii* isolates in Korea is ST5 indicating less diversity compared to other Asian populations. In other recently reported studies, 6 cases of ST31 strains isolated from clinical and environmental sources have been reported: China (n = 1, clinical), Japan (n = 1, environmental), Thailand (n = 5, environmental), India (n = 7, clinical), and Uganda (n = 1, clinical) [18]. In Korea, there are no previous data on the ST31 MLST genotype, isolated from clinical and environmental sources. The occurrence of ST31 genotype of *C. neoformans* var. *grubii* represents a significant association between genetic relatedness and geographical populations among the East Asian regions.

In conclusion, our data revealed that the clinical strains of *C. neoformans* in Korea are genetically homogeneous with the VNI/ST5 genotypes, and the new appearance of VNI/ST31 genotype may serve as an important indicator of global genetic analysis.
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