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Research Article

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 *MAT*α, 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, *MAT*α and *MAT*a, controlled by a single locus two allele mating system with *MAT*α 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.*

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neoformans var. *grubii*, serotype A), VNIII (serotype AD, hybrid), VNIV (*C. neoformans* var. *neoformans*, serotype D), and VGI, VGII, VGIII, and VGIV (*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. gatti* [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

Table 1. List of clinical isolates of *Cryptococcus neoformans* used in this study

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	68	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]	

F, female; CSF, cerebrospinal fluid; M, male; PCNA, percutaneous needle aspiration.

(ISHAM) established a standard MLST scheme for *C. neoformans* and *C. gattii* to accurately distinguish between the two and to enable the rapid acquisition of global genotypic data. The ISHAM group selected 7 unlinked genetic loci, *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and *IGS1*, that represent housekeeping genes, virulence factor coding genes and intergenic spacers of ribosomal DNA [15].

Cryptococcosis in Korea is commonly caused by *C. neoformans* var. *grubii*, serotype A, VNIc molecular type [17]. This serotype is similar to several major types found within clinical isolates of *C. neoformans* in China and Japan. Therefore *C. neoformans* var. *grubii* population from the East Asian region has less genotypic variation than those from Southeast Asian and the Middle East regions [18, 20]. Despite the availability of considerable amounts of data on the global epidemiology and geographically structured populations of *C. neoformans*, information on the molecular epidemiology of the Korean strains remains limited.

In the present study, we investigated the molecular epidemiology of 46 clinical *C. neoformans* isolates from 2008 to 2012 using *URA5*-RFLP and MLST analyses, and compared the molecular types and distribution of diversity among the Korean strains of *C. neoformans*.

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 *MAT***a** 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, *Hha*I and *Sau*96I. 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://cneoformans.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

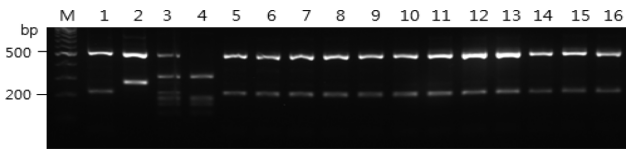

Fig. 1. *URA5*-restriction fragment length polymorphism profiles obtained after double digestion with the restriction enzymes, *Hha*I and *Sau*96I. Lane 1~4, Reference strains of *Cryptococcus neoformans* molecular type VNI, VNII, VNIII, and VNIV; lanes 5~16, selected clinical isolates (sh79, sh98, sh100, Cn129, Cn130, Cn131, Cn136, Cn137, Cn138, Cn140, Cn146, Cn147, and Cn150); M, molecular marker (100 bp DNA ladder).

Table 2. The allelic profiles and sequence types of *Cryptococcus neoformans* isolates

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	1	10	3	2	1	1	**31**
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	1	10	3	2	1	1	**31**
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.

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

Fig. 2. Neighbor joining phylogenetic analysis of the 46 clinical isolates, K52 reference strain, and known sequence types (STs) of *Cryptococcus neoformans* using a concatenated seven multilocus sequencing type loci. Numbers at each branch indicate the bootstrap values of >50%, based on 500 replicates.

VN1. 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) VNIc/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 (VNIc/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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