Microbiological, Epidemiological, and Clinical Characteristics and Outcomes of Patients with Cryptococcosis in Taiwan, 1997–2010

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Abstract

Background: Among members of Cryptococcus neoformans-Cryptococcus gattii species complex, C. neoformans is distributed worldwide whereas C. gattii is considered to be more prevalent in the sub tropics and tropics including Taiwan. This nationwide study was undertaken to determine the distribution of genotypes, clinical characteristics and outcomes of 219 patients with proven cryptococcosis at 20 hospitals representative of all geographic areas in Taiwan during 1997–2010.

Methods and Findings: Of 219 isolates analyzed, C. neoformans accounted for 210 isolates (95.9%); nine isolates were C. gattii (4.1%). The predominant genotype was VNI (206 isolates). The other genotypes included VNI (4 isolates), VG I (3 isolates) and VG II (6 isolates). Antifungal minimal inhibition concentrations higher than epidemiologic cutoff values (ECVs) were found in nine VNI isolates (7 for amphotericin B). HIV infection was the most common underlying condition (54/219, 24.6%). Among HIV-negative patients, liver diseases (HBV carrier or cirrhosis) were common (30.2%) and 15.4% did not have any underlying condition. Meningoencephalitis was the most common presentation (58.9%), followed by pulmonary infection (19.6%) and “others” (predominantly cryptococcemia) (18.7%). The independent risk factors for 10-week mortality, by multivariate analysis, were cirrhosis of liver (P = 0.014) and CSF cryptococcal antigen titer ≥1:512 (P = 0.020). All except one of 54 HIV-infected patients were infected by VNI genotype (98.1%). Of the 13 isolates of genotypes other than VNI (92.3%) were isolated from HIV-negative patients. HIV-infected patients compared to HIV-negative patients were more likely to have meningoencephalitis and serum cryptococcal antigen ≥1:512. Patients infected with C. gattii compared to C. neoformans were younger, more likely to have meningoencephalitis (100% vs. 57%), reside in Central Taiwan (56% vs. 31%), and higher 10-week crude mortality (44.4% vs. 22.2%).

Conclusions: Cryptococcus neoformans in Taiwan, more prevalent than C. gattii, has a predominant VNI genotype. Isolates with antifungal MIC higher than ECVs were rare.

Introduction

Among members of the Cryptococcus neoformans-Cryptococcus gattii species complex that cause cryptococcosis in humans, C. neoformans (comprising var. grubii [serotype A] and var. neoformans [serotype D]) occur worldwide. In contrast, C. gattii (serotype B and C) is usually limited to the selected regions, particularly the Asia-Pacific region before the occurrence of a C. gattii outbreak in Vancouver Island, Canada [1]. Based on a large global molecular epidemiologic survey Cryptococcus could be divided into eight major genotypes: VNI [serotype A], VNII [serotype A], VNIII [serotype AD], and VNIV [serotype D] of C. neoformans; and VGI, VGII, VGIII, and VGIV of C. gattii using orotidine monophosphate pyrophosphorylase (URA5) gene restriction fragment length
Cryptococcosis in Taiwan

VNI
Subgroup A
(N=99)

VNI
Subgroup B
(N=107)

VNII (N=4)
VGI (N=3)
VGII (N=6)
polymorphism (RFLP) analysis and M13 polymerase chain reaction (PCR) fingerprinting [2].

Cryptococcosis is associated with significant morbidity and mortality. It can present as meningoencephalitis, pneumonia and cryptococcemia in both immunocompetent and immunocompromised hosts. Outcome and treatment failure are usually associated with underlying conditions, a delay in diagnosis, and absence of a fungicidal drug [3–5]. In addition, the emergence of isolates with resistance or elevated minimum inhibition concentration (MIC) above epidemiologic cutoff values (ECVs) is of concern as well [6,7].

We conducted this nationwide multicenter retrospective study for patients with proven cryptococcosis to address two questions. First, what are the genotypes and antifungal susceptibility of Cryptococcus clinical isolates collected from representative regions in Taiwan? Second, are demographic qualities, underlying conditions, and microbiological characteristics associated with cryptococcosis patient mortality?

Population and Methods

This research was approved by the Research Ethics Committees of the National Taiwan University Hospital (No. 201209035RIC), Mackay Memorial Hospital (No.12MMHIS120), Kaohsiung Medical University Hospital (No.KMUH-IRB-20120239), China Medical University Hospital (No. DMR101-IRB1-240), and National Health Research Institute (No.EC 09602024) and was conducted according to the Declaration of Helsinki. The project involved the use of existing data, records, and clinical isolates without intervention. Informed consent was waived and the data were analyzed anonymously.

Hospital settings and Cryptococcus clinical isolates

Cryptococcus clinical isolates were obtained from 219 patients with proven cryptococcosis managed at 20 hospitals located in the four geographic regions of Taiwan during 1997–2010. The initial patient isolate, regardless of anatomical site, was selected and sent to National Taiwan University Hospital (NTUH) for microbiological characterization.

Genotypes

High-molecular-weight DNA was isolated and genotypes were determined by URA5 gene RFLP analysis [2]. Molecular types were evaluated and compared using M13 PCR-fingerprinting [2]. The computer program BioNumerics version 6.0 (Applied Maths, Kortrijk, Belgium) was used to determine the cluster analysis by the UPGMA method [8]. DNA bands were defined manually with a band position tolerance of 0.8% and an optimization setting of 0.2%. Reference strains included WM 148 (VNI), WM 626

Table 1. Susceptibility of 216 cryptococcal clinical isolates to four antifungal agents in Taiwan, 1997–2010.

| Antifungal agent | Genotype | No. of isolates | Minimum inhibitory concentration (μg/mL) | % (No.) above ECV |
|------------------|----------|-----------------|------------------------------------------|-------------------|
|                  |          | Range | Geometric Mean | MIC<sub>50</sub> | MIC<sub>90</sub> | ECV | This study | Global studies* |
| Amphotericin B    | VNI      | 203   | 0.03–1 | 0.48 | 0.5 | 0.5 | 0.5 | 3.4% (7) | 2.8% |
|                  | VNII     | 4     | 0.13–1 | 0.42 | 0.5 | 1 | NA* | |
|                  | VGI      | 3     | 0.25–0.25 | 0.25 | 0.25 | 0.25 | 0.5 | 0% | 0.8% |
|                  | VGII     | 6     | 0.06–1 | 0.31 | 0.5 | 1 | 1 | 0% | 0.8% |
| Flucytosine       | VNI      | 203   | 0.13–32 | 1.14 | 1 | 2 | 8 | 0.5% (1) | 3.4% |
|                  | VNII     | 4     | 0.13–2 | 0.30 | 0.19 | 2 | NA* | |
|                  | VGI      | 3     | 0.5–1 | 0.63 | 0.5 | 1 | 4 | 0% | 4.3% |
|                  | VGII     | 6     | 1–2 | 1.59 | 2 | 2 | 16 | 0% | 2.9% |
| Fluconazole       | VNI      | 203   | 0.03–16 | 2.35 | 4 | 8 | 8 | 0.5% (1) | 2.9% |
|                  | VNII     | 4     | 0.13–8 | 0.84 | 0.75 | 8 | NA* | |
|                  | VGI      | 3     | 1–4 | 2 | 2 | 4 | 8 | 0% | 1.2% |
|                  | VGII     | 6     | 0.13–16 | 5.04 | 8 | 16 | 32 | 0% | 6.9% |
| Voriconazole      | VNI      | 203   | 0.03–0.25 | 0.06 | 0.06 | 0.13 | 0.25 | 0% | 2.4% |
|                  | VNII     | 4     | 0.03–0.13 | 0.05 | 0.05 | 0.13 | NA* | |
|                  | VGI      | 3     | 0.03–0.06 | 0.04 | 0.03 | 0.06 | 0.5 | 0% | 0% |
|                  | VGII     | 6     | 0.13–0.25 | 0.20 | 0.25 | 0.25 | 0.25 | 0% | 4.1% |

*The epidemiologic cutoff values of VNII to antifungal drugs being tested were not available in global studies [6,7].

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Figure 1. Dendrogram of M13 PCR fingerprint analysis of 219 clinical isolates of Cryptococcus neoformans- Cryptococcus gattii species complex collected in Taiwan during 1997 to 2010 and 12 reference strains.

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Table 2. Epidemiological and clinical characteristics of 219 patients with proven cryptococcosis hospitalized at 20 hospitals in Taiwan, 1997–2010.

| Characteristics                                      | Cryptococcus neoformans (N = 210) | Cryptococcus gattii (N = 9) |
|------------------------------------------------------|----------------------------------|-----------------------------|
|                                                      | No. (%)                          | No. (%)                     |
| Geographic distribution                              |                                  |                             |
| Northern                                             | 119 (56.7)                       | 1 (11.1)                    |
| Central                                              | 65 (30.9)                        | 5 (55.6)                    |
| Southern                                             | 21 (10.0)                        | 1 (11.1)                    |
| Eastern                                              | 5 (2.4)                          | 2 (22.2)                    |
| Demographic data                                     |                                  |                             |
| Age, range, years                                    | 12 to 94                         | 22 to 68                    |
| Age, mean ± SD, years                                | 53.1 ± 18.4                      | 38.6 ± 13.0                 |
| Age ≥60 years                                        | 75 (35.7)                        | 1 (11.1)                    |
| Male                                                 | 143 (72.2)                       | 5 (55.6)                    |
| Underlying conditions                                |                                  |                             |
| HIV infection                                        | 53 (27.3)                        | 1 (11.1)                    |
| Liver diseases                                       |                                  |                             |
| Hepatitis B virus carrier                            | 46 (21.9)                        | 0 (0.0)                     |
| Cirrhosis of liver                                   | 31 (14.8)                        | 0 (0.0)                     |
| Malignancy                                           |                                  |                             |
| Hematological malignancy                            | 13 (6.2)                         | 0 (0.0)                     |
| Other malignancy                                     | 31 (14.8)                        | 0 (0.0)                     |
| Diabetes mellitus                                    | 39 (18.6)                        | 1 (11.1)                    |
| Kidney diseases                                      | 21 (9.6)                         | 0 (0.0)                     |
| Systemic lupus erythematosus and other rheumatologic diseases | 11 (5.2) | 0 (0.0) |
| Cerebrovascular accident                             | 8 (3.8)                          | 1 (11.1)                    |
| Tuberculosis                                         | 6 (2.9)                          | 0 (0.0)                     |
| Solid organ transplantationª                         | 3 (1.4)                          | 1 (11.1)                    |
| Idiopathic CD4 lymphocytopenia                       | 3 (1.4)                          | 0 (0.0)                     |
| Other diseases                                       | 3 (1.4)                          | 0 (0.0)                     |
| No underlying conditions                             | 19 (9.0)                         | 4 (44.4)                    |
| Classification of cryptococcosis                     |                                  |                             |
| Meningoencephalitis                                  | 120 (57.1)                       | 9 (100.0)                   |
| Pulmonary cryptococcosis                             | 43 (20.5)                        | 0 (0.0)                     |
| Othersª                                              | 47 (22.4)                        | 0 (0.0)                     |
| Serum cryptococcal capsular antigen                  |                                  |                             |
| Antigen titer ≥512                                   | 73 (34.8)                        | 4 (44.4)                    |
| Antigen titer <512                                   | 57 (27.1)                        | 3 (33.3)                    |
| Not done                                             | 80 (38.1)                        | 2 (22.2)                    |
| CSF cryptococcal capsular antigen                    |                                  |                             |
| Antigen titer ≥1:512                                 | 76 (36.2)                        | 7 (77.8)                    |
| Antigen titer <1:512                                 | 40 (19.0)                        | 2 (22.2)                    |
| Not done                                             | 94 (44.8)                        | 0 (0.0)                     |
| Intracranial pressure                               |                                  |                             |
| Opening pressure ≥250 mmH₂O                          | 48 (22.9)                        | 6 (66.7)                    |
| Opening pressure <250 mmH₂O                          | 42 (20.0)                        | 2 (22.2)                    |
| Not done or not available                            | 120 (57.1)                       | 1 (11.1)                    |
| Neurosurgical intervention                          | 19 (9.0)                         | 3 (33.3)                    |
| All-cause mortality                                  |                                  |                             |
| 2-week mortality                                     | 22 (10.5)                        | 2 (22.2)                    |
| 10-week mortality                                    | 60 (28.6)                        | 4 (44.4)                    |
(VNI), WM 628 (VNIII), WM 629 (VNIV), WM 179 (VGI), WM 178 (VGIi), WM 161 (VGIi), WM 779 (VGIi) [2], two Australia clinical strains T184 (VNI) and T185 (VGI), and Vancouver Island outbreak strains R265 (VGIIa) and R272 (VGIIb).

Antifungal susceptibility
Susceptibility, as displayed by MIC (μg/ml) levels, to amphoterin B, flucytosine, fluconazole, and voriconazole was determined following the Clinical Laboratory Standards Institute (CLSI) M27-A3 broth microdilution method and incubated at 35°C [9]. All results were read visually at 72 h. The reference strains C. neoformans ATCC 90112, Candida albicans ATCC 90028, and Candida parapsilosis ATCC 22019 were used as internal controls. The ECVs are the MIC values that captured 95% of the observed population in RPMI medium provided in recent studies [6,7].

Clinical characteristics and outcomes of patients with cryptococcosis
Data were collected retrospectively after isolates were sent for microbiological characterization and included gender, age, underlying conditions such as human immunodeficiency virus (HIV) status and lowest CD4 count during hospitalization, hepatitis B virus (HBV) carrier defined by positive surface antigen (HBsAg) status, and cirrhosis of liver determined by sonography; clinical characteristics included presentation, initial cryptococcal capsular polysaccharide antigen titer in cerebrospinal fluid (CSF) or serum, baseline intracranial opening pressures, neurosurgical intervention, all-cause mortality at 2- and 10-weeks. One patient could possess more than one underlying condition. We did not collect and record treatment details.

Case definition
Proven cryptococcosis was defined and classified into cryptococcal meningoencephalitis, pulmonary cryptococcosis, and others as described previously [10].

Data analysis
The categorical variables were analyzed by number (No.) (%) and the continuous variables were presented as mean ± standard deviation (SD). The association between categorical variables was analyzed with the Chi-square test or Fisher’s exact test if the expected number was less than five. The independent and joint effects of several variables to identify significant predictors of mortality were investigated by univariate and multivariate logistic regression analyses. Two-sided P value <0.05 was considered statistically significant. All statistical analyses were performed using the SAS software, version 9.2 (SAS Institute Inc., Cary, NC, US).

Results
Cryptococcus genotypes
Of 219 Cryptococcus clinical isolates, 210 were C. neoformans (95.9%) and 9 were C. gattii (4.1%). VNI genotype accounted for 206/210 (98.1%) of C. neoformans. Four isolates were VNII. Among the nine isolates of C. gattii, three were VGI and six were VGII. The details of patients with VNII and C. gattii are shown in Table S1 and Table S2, respectively.

Figure 1 shows the M13 PCR-fingerprinting dendrogram of the 219 cryptococcal isolates (details are presented in Figure S1). Genotype VNI can be divided into two subgroups. Subgroup A accounted for 48.1% (99/206) of VNI with 57.4% similarity and subgroup B accounted for 51.9% (107/206) of VNI with 63.2% similarity.

Antifungal susceptibility
Among the 219 isolates, the susceptibility data of three VNI isolates (T203, T205, and T262) were indeterminate due to very poor growth in RPMI broth at 35°C. The MIC levels of 216 isolates to amphoterin B, flucytosine, fluconazole, and voriconazole are shown in Table 1. Seven of 203 VNI isolates (3.4%) had amphoterin B MIC levels higher than ECV. One VNI isolate had a flucytosine MIC level higher than ECV. Two of six VGII isolates and one of 203 VNI isolates had fluconazole MIC levels >8 μg/ml, but there were none above this level for 4 VNI isolates and 3 VGI isolates. Fluconazole ECV was 8 μg/ml for VNI and VGI, and was 32 μg/ml for VGII. Therefore, only one VNI isolate of 219 isolates had fluconazole MIC higher than ECV. Detailed information regarding cryptococcus due to Cryptococcus VNI isolates with antifungal MICs higher than ECVs is shown in Table S3.

Epidemiological and clinical characteristics
Table 2 shows the epidemiological and clinical characteristics of the 219 patients with proven cryptococcosis. More than half of the patients were in Northern Taiwan. However, 5 of 9 isolates of C. gattii (55.6%) were from Central Taiwan. The most common five underlying conditions were HIV infection (54 patients, 24.6%), HBV carrier (46 patients, 21.0%), malignancies (44 patients, 20.1%), diabetes mellitus (40 patients, 18.2%), and cirrhosis of liver (31 patients, 14.1%). No underlying condition was identified in 23 patients (10.5%). Meningoencephalitis was the most common presentation (58.9%), followed by pulmonary infection (19.6%) and “others” (predominantly cryptococcemia) (18.7%). The nine patients with C. gattii infection, compared to 210 patients with C. neoformans, were younger (mean 38.6 years vs. 53.1 years) and more likely to have no underlying conditions (44.4% vs. 9.0%), to have meningoencephalitis (100.0% vs. 57.1%) and to undergo neurosurgical intervention (33.3% vs. 9.0%). They also had a higher 10-week mortality (44.4% vs. 22.2%), as seen in Table 2.

Of 54 HIV-infected patients, 53 were infected by the VNI genotype (98.1%) and one was infected by the VGI genotype, as seen in Table 3. Excluding five patients without recorded CD4 data, the mean CD4 of 49 HIV-infected patients was 30.0±68.3/ mL (ranging from 2 to 318/mL). Of 13 isolates of genotypes other than VNI, twelve (92.3%) were isolated from HIV-negative patients (Table 3, Table S1, and Table S2). The 54 HIV-infected patients, as compared to the 149 HIV-negative patients, were younger, predominantly male, and more likely to have meningoencephalitis and serum cryptococcal antigen ≥512. Compared to HIV infected patients, HIV-negative patients were more likely to have pulmonary infection and liver diseases (either
### Table 3. Comparisons of genotype distribution and clinical characteristics of cryptococcosis by HIV status, Taiwan, 1997–2010.

| Characteristics                              | HIV-negative patients (N = 149)a | HIV-infected patients (N = 54)a | P value |
|----------------------------------------------|---------------------------------|---------------------------------|---------|
| **Genotype distribution**                    |                                 |                                 |         |
| VNI                                          | 137 (53)                        | 53                              |         |
| VNII                                         | 4                               | 0                               |         |
| VGI                                          | 2                               | 1                               |         |
| VGII                                         | 6                               | 0                               |         |
| **Geographic distribution**                  |                                 |                                 |         |
| Northern                                     | 84 (56.4)                       | 34 (63.0)                       |         |
| Central                                      | 43 (28.9)                       | 14 (25.9)                       |         |
| Southern                                     | 16 (10.7)                       | 5 (9.3)                         |         |
| Eastern                                      | 6 (4.0)                         | 0 (0.0)                         |         |
| **Demographic data**                         |                                 |                                 |         |
| Age ≥ 60 years                               | 75 (50.3)                       | 1 (1.9)                         | <0.001  |
| Male                                         | 94 (63.1)                       | 51 (94.4)                       | <0.001  |
| **Underlying conditions**                    |                                 |                                 |         |
| Liver diseases                               |                                 |                                 |         |
| Hepatitis B virus carrier                    | 33 (22.1)                       | 13 (24.1)                       | 0.845   |
| Cirrhosis of liverb                          | 30 (20.1)                       | 1 (1.9)                         | 0.001   |
| Diabetes mellitus                            | 40 (26.8)                       | 0 (0.0)                         | <0.001  |
| **Malignancy**                               |                                 |                                 |         |
| Hematological malignancy                     | 5 (3.4)                         | 3 (5.6)                         | 0.686   |
| Other malignancy                             | 33 (22.1)                       | 3 (5.6)                         | 0.005   |
| Kidney diseases                              | 20 (13.4)                       | 1 (1.9)                         | 0.014   |
| Solid organ transplantation                   | 4 (2.7)                         | 0 (0.0)                         | 0.576   |
| No underlying conditions                     | 23 (15.4)                       | 0 (0.0)                         | 0.002   |
| **Classification of cryptococcosis**         |                                 |                                 | 0.002   |
| Meningoencephalitis                          | 80 (53.7)                       | 44 (81.5)                       |         |
| Pulmonary cryptococcosis                     | 35 (23.5)                       | 3 (5.6)                         |         |
| Othersc                                       | 34 (22.8)                       | 7 (13.0)                        |         |
| Serum cryptococcal capsular antigen          |                                 |                                 | 0.001   |
| Antigen titer ≥ 512                          | 43 (28.9)                       | 34 (63.0)                       |         |
| Antigen titer < 512                          | 49 (32.9)                       | 11 (20.4)                       |         |
| Not doned                                     | 57 (38.3)                       | 9 (16.7)                        |         |
| CSF c cryptococcal capsular antigen          |                                 |                                 | 0.661   |
| Antigen titer ≥ 1:512                        | 50 (33.6)                       | 33 (61.1)                       |         |
| Antigen titer < 1:512                        | 27 (18.1)                       | 15 (27.8)                       |         |
| Not done[d]                                  | 72 (48.3)                       | 6 (11.1)                        |         |
| **Intracranial pressure**                    |                                 |                                 | 0.101   |
| Opening pressure ≤ 250 mmH2O                 | 32 (21.5)                       | 22 (40.7)                       |         |
| Opening pressure < 250 mmH2O                 | 33 (22.1)                       | 11 (20.4)                       |         |
| Not done or not available[d]                 | 84 (56.4)                       | 21 (38.9)                       |         |
| Neurosurgical intervention                   | 15 (10.1)                       | 7 (13.0)                        | 0.592   |
| **All-cause mortality**                      |                                 |                                 |         |
| 2-week mortality                             | 19 (12.8)                       | 5 (9.3)                         | 0.468   |
| 10-week mortality                            | 52 (34.9)                       | 12 (22.2)                       | 0.100   |

Abbreviation: HIV: human immunodeficiency virus.

aOf 219 patients with cryptococcosis, the HIV status of 16 patients was not available. Therefore, 203 cases were included for analysis.

bOne patient could possess more than one underlying condition; 18 HIV-negative patients had both cirrhosis of liver and HBV infection.

c"Others" included 25 patients with cryptococcemia in HIV-negative group and seven cryptococcemia in HIV-infected group.

dData which were not done or not available were excluded from statistical analysis.
HBV carrier or cirrhosis of liver) as the most common underlying conditions (45 patients, 30.2%). Of nine patients infected by the VNI genotype and with antifungal MICs above ECVs, five patients had HIV infections, six had meningoencephalitis, and three had cryptococcemia. The all-cause mortality at 10 weeks was 33.3% (3/9), as shown in Table S3. We did not collect data, such as prior use of antifungal agent or drug interaction, to explain the reason for elevated MICs.

Risk factors associated with 10-week mortality for 195 patients with cryptococcosis are shown in Table 4. The significant factors under univariate analysis were age ≥60 years (P = 0.016), cirrhosis of liver (P = 0.001), kidney diseases (P = 0.035), meningoencephalitis (P = 0.038), other cryptococcosis (P<0.001) and CSF cryptococcal antigen titer ≥1:512 (P = 0.019). Multivariate analysis showed cirrhosis of liver (P = 0.014; OR, 3.8; 95% CI, 1.3–11.16) and CSF antigen titer ≥1:512 (P = 0.020; OR, 3.3; 95% CI, 1.2–9.0) as independent predictors for mortality.

Discussion

The current study provides the first nationwide description of the microbiological and clinical epidemiology of cryptococcosis in Taiwan. The majority of isolates in Taiwan were C. neoformans genotype VNI (96%). This is in agreement with the worldwide distribution of Cryptococcus which is VNI in Ibero-America (68%) [2], Vietnam (71%) [11], India (89%) [12], Malaysia (89%) [13], China (93%) [14] and Korea (96%) [15]. Cryptococcosis in HIV-negative patients was common (73%) in Taiwan (this study) as well as in China (84% to 96%) [14,16,17]. However, HIV-negative patients accounted for 60% in an Indian study [12], 57% in Australia and New Zealand [18], 23% of a

| Characteristics                  | Died (N = 64) | Lived (N = 131) | Odds ratio | 95% confidence interval | P value |
|----------------------------------|--------------|----------------|------------|--------------------------|---------|
| Demographic data                |              |                |            |                          |         |
| Age ≥60 years                    | 32 (50.0)    | 42 (32.1)      | 2.2        | 1.1–3.9                  | 0.016   |
| Male                             | 41 (64.1)    | 98 (74.8)      | 0.6        | 0.3–1.1                  | 0.12    |
| Underlying conditions            |              |                |            |                          |         |
| HIV infection                    | 12 (18.8)    | 39 (29.8)      | 0.5        | 0.3–1.1                  | 0.10    |
| Hepatitis B virus carrier        | 15 (23.4)    | 28 (21.4)      | 1.1        | 0.5–2.3                  | 0.76    |
| Cirrhosis of liver               | 18 (28.1)    | 12 (9.2)       | 3.9        | 1.7–8.7                  | 0.001   |
| Kidney diseases                  | 11 (17.2)    | 9 (6.9)        | 2.7        | 1.1–7.0                  | 0.03    |
| Classification of cryptococcosis |              |                |            |                          |         |
| Pulmonary                        | 5 (7.8)      | 33 (25.2)      | 1.0        |                          |         |
| Meningoencephalitis              | 37 (57.8)    | 83 (63.4)      | 2.9        | 1.1–8.1                  | 0.04    |
| Others[a]                        | 22 (34.4)    | 15 (11.4)      | 10.4       | 3.3–32.9                 | <0.001  |
| Serum cryptococcal capsular antigen |            |                |            |                          |         |
| Antigen titer ≥1:512             | 26 (40.6)    | 47 (35.9)      | 1.4        | 0.7–2.9                  | 0.41    |
| Antigen titer <1:512             | 17 (26.6)    | 42 (32.1)      | 1.0        |                          |         |
| Not done[b]                      | 21 (32.8)    | 42 (32.1)      |            |                          |         |
| CSF cryptococcal capsular antigen |            |                |            |                          |         |
| Antigen titer ≥1:512             | 29 (45.3)    | 51 (38.9)      | 3.2        | 1.2–8.6                  | 0.02    |
| Antigen titer <1:512             | 6 (9.4)      | 34 (26.0)      | 1.0        |                          |         |
| Not done[b]                      | 29 (45.3)    | 46 (35.1)      |            |                          |         |
| Intracranial pressure            |              |                |            |                          |         |
| Opening pressure ≥250 mmH2O      | 16 (25.0)    | 37 (28.2)      | 1.0        | 0.4–2.6                  | 0.92    |
| Opening pressure <250 mmH2O      | 12 (18.8)    | 29 (22.1)      | 1.0        |                          |         |
| Not done or not available[b]     | 36 (56.3)    | 65 (49.6)      |            |                          |         |
| Neurosurgical intervention       | 9 (14.1)     | 13 (9.9)       | 1.5        | 0.6–3.7                  | 0.43    |

Abbreviation: CSF: cerebrospinal fluid.
[a]“Others” included 19 patients with cryptococcemia died and 12 patients with cryptococcemia lived.
[b]Data which were not done or not available were excluded from statistical analysis.

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French cohort [19] and 18% in Mexican [20]. Only 15% patients were no underlying condition in Taiwan (this study). This was very different from reports in China (68%) [16] and Vietnam (81%) [11]; and yet was close to a study in Korea (19%) [15], USA (22%) [10] and results of another review from China (16%) [17].

Regarding the distribution of underlying conditions and their impact on 10-week mortality, this study showed that HIV infection was the most common underlying condition (25%), but not a risk factor associated with mortality of cryptococcosis (Table 4). Liver diseases (either HBV carrier or cirrhosis) were the most common underlying conditions among HIV-negative patients in Taiwan (30%, Table 3) and in China (12%) [17]. Furthermore, cirrhosis of liver was an independent predictor of mortality in this study (Table 4) and our previous single center study of cryptococcemia [21]. High CSF antigen titers have been associated with death at 10 weeks in a cohort of Italian HIV-positive patients [22] and HIV uninfected patients in Vietnam [11] and our previous study [23]. Our current study confirmed this finding as well. Thus, a threshold of 1:512 or higher should help monitor patients with cryptococcosis, regardless of their HIV status.

In this study, we found clinical presentation of patients with C. gattii infection were more likely than those with C. neoformans infection to have meningoencephalitis, were younger, and were less likely to have underlying conditions (Table 2), which was concordant with an Australian study [18]. The past studies from a center in northern Taiwan (i.e. NTUH) revealed that clinical cases of C. gattii decreased from 59% (17/29) during 1982–1994 to 13% (4/30) during 1995–1997 [24], and 1% (1/100) during 1999–2004 [25]. Another report from a center in southern Taiwan showed 15% (5/34) clinical cases during 1998–2002 were C. gattii [26]. Although the ecological niches of C. gattii are poorly defined in Taiwan [27], Chaturvedi V. et al. suggested a hypothetical lifecycle of C. gattii whereby it cycles through plants, soil, air, and water [28]. Loss of tree coverage in mountainous areas following numerous landslides washed into the estuaries in recent years might explain part of the reason why there has been a decrease in C. gattii in Taiwan. We speculate that the global distribution of C. gattii, as shown in Table 5, might be related to ocean circulation to allow distribution and thriving of C. gattii propagules into new ecological niches.

Recently, Espinel-Ingroff A. et al. suggested the epidemiologic cutoff values (ECVs) (highest wild type susceptibility endpoint) of antifungal susceptibility for reference [6,7] as the Clinical and Laboratory Standards Institute (CLSI) does not provide clinical breakpoints (CBPs) for Cryptococcus species [9]. While CBPs predict the clinical outcome of therapy, the ECVs could monitor the emergence of strains with reduced susceptibility (due to mutation) to the agent being evaluated. In the current study, only nine of 219 isolates had MICs higher than ECVs (Table 1). Of them, seven isolates (3.4%) of the VNI genotype had amphotericin B MIC levels higher than ECV, while the global study showed 2.8% [6]. Regarding fluconazole MIC, the values of MIC50 and MIC90 in

### Table 5. The global distribution of clinical isolates of Cryptococcus gattii by genotype in the literature reviewed.

| Report year | Collection year | Region          | No. of isolates | Reference |
|-------------|-----------------|-----------------|-----------------|-----------|
|             |                 |                 | Total           | VGI       | VGII  | VGIII | VGIV       |
| 1996        | 1965–1994       | Australia       | 48              | 44        | 3     | 1     | 0          |
| 2003        | 1961–2001       | South American  | 33              | 3         | 13    | 16    | 1          |
| 2004        | 1999–2002       | Canada, BC      | 21              | 1         | 20    | 0     | 0          |
| 2005        | NA              | Papua New Guinea| 37              | 31        | 2     | 4     | 0          |
| 2005        | NA              | Australia, NT   | 21              | 9         | 12    | 0     | 0          |
| 2005        | NA              | India           | 5               | 0         | 5     | 0     | 0          |
| 2006        | 1987–2004       | Colombia        | 16              | 1         | 14    | 0     | 0          |
| 2006        | 1998–2003       | Hong Kong       | 3               | 1         | 2     | 0     | 0          |
| 2007        | 2004–2005       | USA, Northwest  | 5               | 1         | 4     | 0     | 0          |
| 2008        | 1994–2006       | China, 16 provinces | 9       | 9         | 0     | 0     | 0          |
| 2008        | 1981–2005       | China, Southeastern| 9      | 8         | 1     | 0     | 0          |
| 2009        | 2006–2008       | USA, Northwest  | 14              | 0         | 14    | 0     | 0          |
| 2009        | 1994–2004       | Mexico          | 8               | 2         | 2     | 2     | 2          |
| 2009        | 2007            | USA, Southeastern| 1          | 1         | 0     | 0     | 0          |
| 2010        | 2003–2004       | Malaysia        | 11             | 4         | 4     | 0     | 0          |
| 2010        | 1998–2007       | Vietnam         | 10              | 9         | 1     | 0     | 0          |
| 2010        | 1990–2008       | Korea           | 2               | 0         | 1     | 1     | 0          |
| 2010        | 2007            | Japan           | 1               | 0         | 1     | 0     | 0          |
| 2012        | 2005–2007       | India           | 4               | 0         | 0     | 0     | 4          |
| 2012        | 2011            | USA, Southeastern| 1         | 1         | 0     | 0     | 0          |
| 2012        | 1997–2010       | Taiwan          | 9               | 3         | 6     | 0     | 0          |

Abbreviations: NT: Northern Territory; BC: British Columbia; NA: not available.

*Mating type a.

11 strains with mating type a were included.

Three untyped C. gattii were included.

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this study (Table 1) and ECVs in global studies [7] were higher for VGI than for VGI, VNI, and VNII. This indicates antifungal susceptibility for Cryptococcus should be species-specific and molecular type-specific [6,7]. It seems likely that the differences seen among the C. neoformans-C. gattii species complex are due to intrinsic heteroresistance to fluconazole [29], chromosome duplication during prolonged azole therapy [30], and possible involvement of phosphoinositide-dependent kinase (PDK1), protein kinase C (PKC), and target of rapamycin (TOR) signaling pathways in basal fluconazole tolerance [31].

The strengths of this study are the large number of cryptococcal clinical isolates collected from hospitals representative of all regions of Taiwan during a 13 year period, the use of molecular methods for genotyping, assessment of antifungal susceptibility, and characterization of the risk factors for 10-week mortality. The weaknesses inherent in a study of this kind were the inability to collect sufficient isolates of rare genotypes or those with MICs higher than ECV to determine the impact on outcome. Generally only one isolate per infection is tested, although it has been revealed that 20% of patients with cryptococcosis can be infected by multiple strains or molecular types [32]. The geographic distribution according to hospital location might not represent the places where exposure to Cryptococcus occurred. Besides, we could not evaluate treatment responses of an individual drug because antifungal regimens and dosages were modified in many of the patients and confounded by the underlying conditions.

In conclusion, the major genotype of Cryptococcus clinical isolates in Taiwan was VNI. Only nine of 219 patients were infected by C. gattii. Isolates with antifungal MICs higher than ECVs were rare. HIV infection was the most common underlying condition and all except one such patient was infected by the VNI genotype. Liver diseases were the most common underlying conditions in HIV-negative patients. Cirrhosis of liver and high CSF cryptococcal antigen levels were independent predictors of 10-week mortality.

Supporting Information

Figure S1 Details of dendrogram of M13 PCR fingerprint analysis of 219 clinical isolates of Cryptococcus neoformans- Cryptococcus gattii species complex collected in Taiwan during 1997 to 2010 and 12 reference strains. (TIF)

Table S1 Microbiological, epidemiological, and clinical characteristics and outcomes of cryptococcosis due to VNII genotype in Taiwan, 1997 to 2010. (DOC)

Table S2 Microbiological, epidemiological, and clinical characteristics and outcomes of Cryptococcus gattii in Taiwan, 1997 to 2010. (DOC)

Table S3 Microbiological, epidemiological, and clinical characteristics and outcomes of cryptococcosis due to Cryptococcus VNI isolates with antifungal minimum inhibition concentration above epidemiologic cutoff values in Taiwan, 1997 to 2010. (DOC)

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Author Contributions

Conceived and designed the experiments: YCC. Performed the experiments: YCC HKT. Analyzed the data: HKT YCC WLC. Contributed reagents/materials/analysis tools: YCC CPT MWH YHL PLL HJL. Wrote the paper: HKT YCC.

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