



MSH2 Gene Point Mutations Are Not Antifungal Resistance Markers in *Candida glabrata*

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ABSTRACT The high rates of antifungal resistance in *Candida glabrata* may be facilitated by the presence of alterations in the *MSH2* gene. We aimed to study the sequence of the *MSH2* gene in 124 invasive *C. glabrata* isolates causing incident episodes of candidemia ($n = 81$), subsequent candidemia episodes ($n = 9$), endocarditis ($n = 2$), and *in vitro*-generated echinocandin-resistant isolates ($n = 32$) and assessed its relationship with genotypes, acquisition of antifungal resistance *in vivo* and *in vitro*, and patient prognosis. The *MSH2* gene was sequenced, and isolates were genotyped using six microsatellite markers and multilocus sequence typing (MLST) based on six housekeeping genes. According to EUCAST, isolates causing candidemia ($n = 90$) were echinocandin susceptible, and four of them were fluconazole resistant (MIC ≥ 64 mg/liter). One isolate obtained from a heart valve was resistant to micafungin and anidulafungin (MICs, 2 mg/liter and 1 mg/liter, respectively). *MSH2* gene mutations were present in 44.4% of the incident isolates, the most common being V239L. The presence of *MSH2* mutations was not correlated with *in vitro* or *in vivo* antifungal resistance. Microsatellite and MLST revealed 27 genotypes and 17 sequence types, respectively. Fluconazole-resistant isolates were unrelated. Most *MSH2* mutations were found in cluster isolates; conversely, some mutations were found in more than one genotype. No clinical differences, including previous antifungal use, were found between patients infected by wild-type *MSH2* gene isolates and isolates with any point mutation. The presence of *MSH2* gene mutations in *C. glabrata* isolates causing candidemia is not correlated with specific genotypes, the promotion of antifungal resistance, or the clinical outcome.

KEYWORDS *Candida glabrata*, *MSH2* gene, antifungal resistance

C*andida* bloodstream infections have been increasing in recent years (1), posing a substantial economic burden for hospitals and high mortality among patients (2, 3). Since fluconazole resistance is becoming more common, echinocandins are currently preferred as first-line treatment (4). However, strains with resistance to echinocandins and/or to multiple antifungals have been sporadically reported (5).

The incidence of infections due to *Candida glabrata* has notoriously grown in Spain (1). In comparison to other *Candida* species, *C. glabrata* is characterized by the ability to develop fluconazole resistance and a certain degree of resistance to echinocandins in some hospitals (1, 6–9). In a previous work, we showed that *C. glabrata* can acquire *in vitro* resistance to echinocandins after exposure to low and/or increasing echinocandin concentrations (10–12). There is little information on why *C. glabrata* acquires resistance more rapidly to multiple antifungal classes than other *Candida* species. The potential for acquisition of resistance is unclear; however, organisms with a haploid

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genome, e.g., *C. glabrata*, might accumulate a number of mutations in key genes, such as *FKS*, during genome replication (13). DNA polymerase mismatches coupled with failures in DNA repair can lead to multiple antifungal resistance in *Candida* spp. (14, 15). An *MSH2* gene mismatch repair in *C. glabrata* and *Candida albicans* may lead to a hypermutable phenotype prone to developing antifungal resistance (14, 15). Furthermore, certain *C. glabrata* genotypes seem to harbor specific polymorphisms in the *MSH2* gene (16).

There are other aspects that require attention. First, only a small number of invasive isolates have been studied (16, 17). Second, studies have been reported without genotyping procedures (15, 17). Third, very few cases of *MSH2* gene sequences from antifungal-susceptible isolates that have shifted to resistant have been studied (15). Finally, the studies include scarce clinical data from infected patients. Here, we aim to study the sequence of the *MSH2* gene of a collection of *C. glabrata* isolates obtained in Spain causing fungemia and to assess the potential relationship with genotypes, possible acquisition of antifungal resistance *in vivo* and *in vitro*, and patient prognosis.

RESULTS

Antifungal susceptibility and *MSH2* gene sequencing. As per EUCAST breakpoints, the 90 blood culture isolates were echinocandin susceptible (geometric mean MICs of micafungin and anidulafungin, 0.015 and 0.023 mg/liter, respectively), and four of them (4.9%) were fluconazole resistant (patients 11, 20, 44, and 63; Fig. 1). Only one of the isolates, which was collected from the heart valve, was resistant to micafungin and anidulafungin (2 mg/liter and 1 mg/liter, respectively) and harbored an S663P mutation in hot spot 1 of the *FKS2* gene. The 21 *in vitro*-generated echinocandin-resistant colonies carried the following *FKS2* mutations: Δ F658, S663P, W715L, and E655A (12).

Thirty-six incident isolates from 81 patients with candidemia (44.4%) had one or more of the following *MSH2* gene point mutations: V239L, P208S, A942T, S653F, A313V, E456D, E459K, E7K, L588V, N890I, and/or L810H. Here, we report the S653F mutation for the first time. Mutation frequencies were 33.3% for V239L, 25.6% for P208S/N890I, 13.9% for V239L/A942T, 8.3% for E459K, and 2.7% for each of the following: S653F, A313V, E456D, E7K, L588V, N890I, and L810H. We observed a match between *MSH2* sequences of incident isolates and those causing second episodes. Two point mutations in the gene (P208S/N890I) were found in the four isolates from a patient who suffered two episodes of candidemia and endocarditis.

In our study, only two out of the four fluconazole-resistant isolates (from patients 11 and 20) harbored *MSH2* gene mutations (P208S/N890I and E456D, respectively) (Fig. 1). The sequence of the *in vitro*-generated echinocandin-resistant *MSH2* gene and the respective parent isolates were identical. Eight (38.1%) out of the 21 colonies that acquired *FKS2* mutations harbored *MSH2* gene mutations (P208S/N890I [$n = 4$], V239L [$n = 3$], and L810H [$n = 1$]). Nine (81.82%) out of 11 echinocandin-resistant colonies (i.e., those with an *FKS* mutation) had the same *MSH2* gene mutation (V239L) and were sourced from a single isolate, whereas the remaining two isolates were from different isolates and the *MSH2* wild type (Fig. 2).

Genotyping analysis and *MSH2* gene sequencing. Figure 1 shows the genotyping of the 81 incident isolates. Isolates responsible for second episodes, the case of endocarditis, and *in vitro*-generated isolates were isogenic to parent strains, regardless of the used method. Microsatellite markers revealed 27 genotypes (CG-1 to CG-27), 13 of which were clusters involving 82.7% of the isolates. Multilocus sequence typing (MLST) revealed 17 sequence types (STs), including six newly described ones (Table 1), and 11 STs being clusters that involved 92.6% of the isolates. The clusters with the highest number of isolates were microsatellites (CG-24 [30.86%], CG-6 [9.88%], and CG-23 [8.64%]) and MLST (ST3 [43.21%], ST34 [9.88%], ST10 [8.64%], and ST6 [7.41%]). In general, clusters detected by any method mostly matched, although there was a tendency for the microsatellites to split up ST clusters into clonally related genotypes

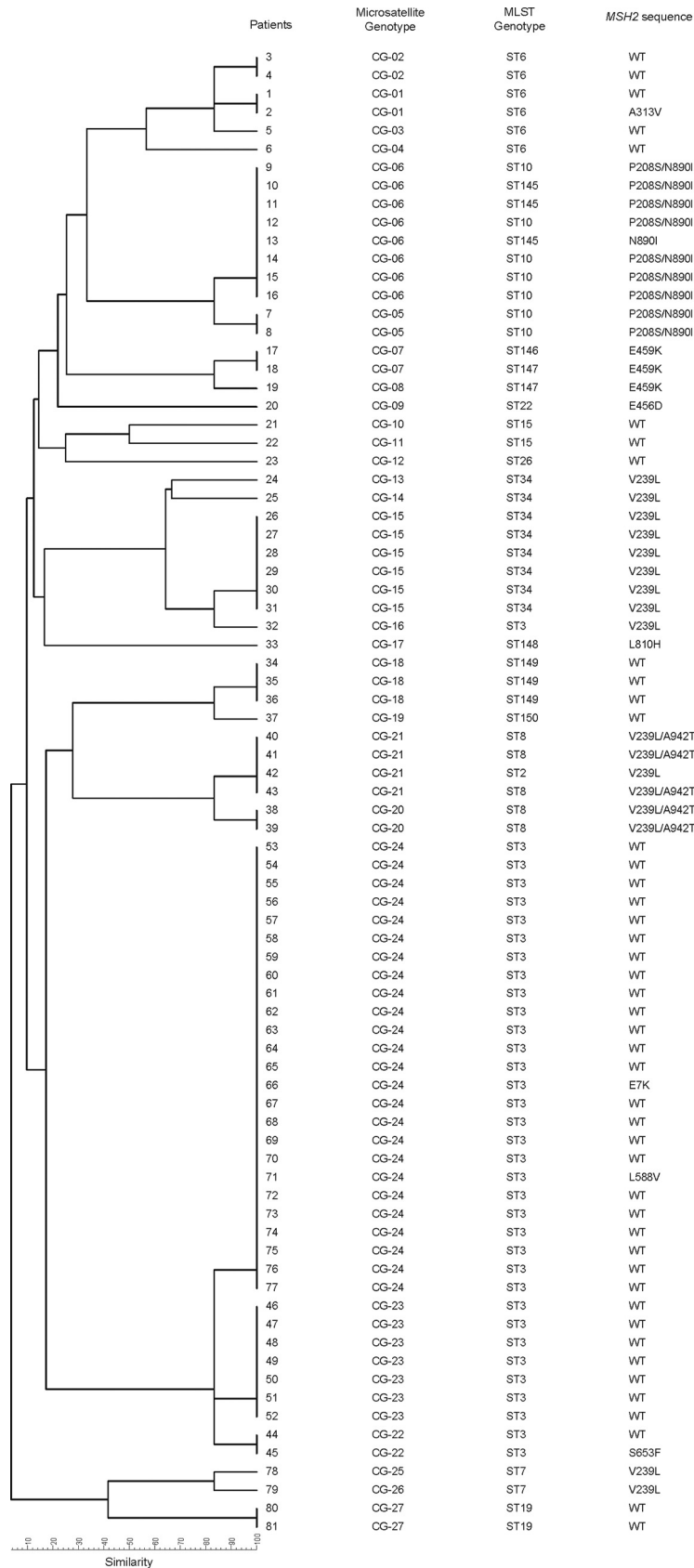


FIG 1 Genetic relationship with microsatellite markers between the incident 81 *Candida glabrata* isolates with the *MSH2* sequence and the sequence type (ST). WT, wild type.

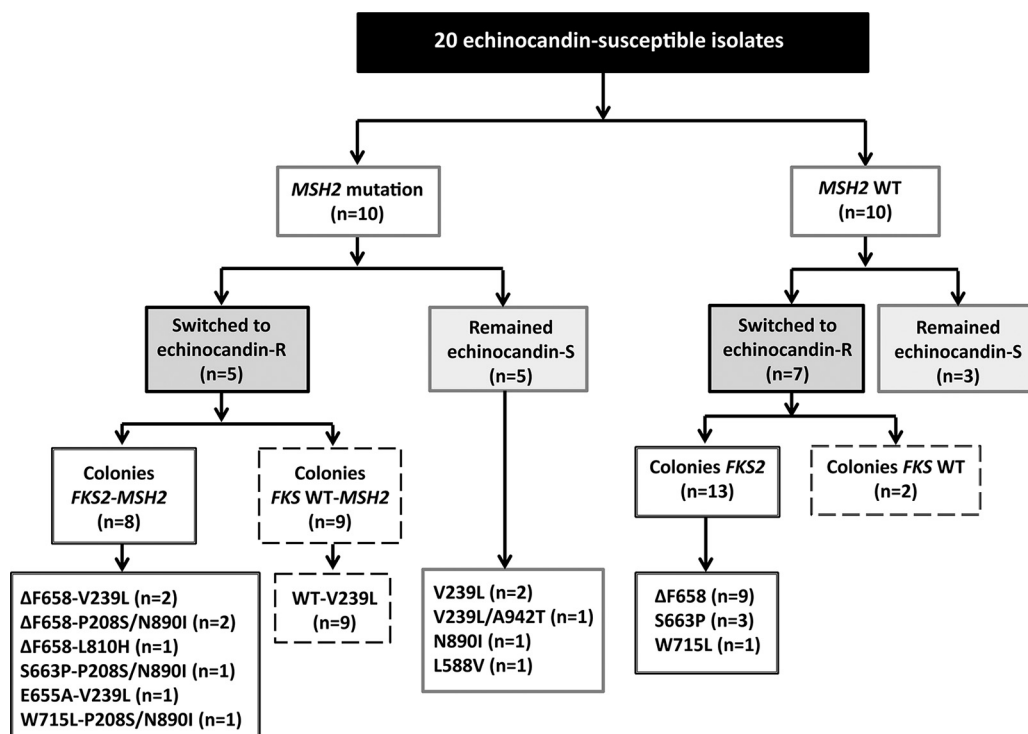


FIG 2 Description of the 20 echinocandin-susceptible isolates previously exposed *in vitro* to echinocandins and the generated colonies phenotypically resistant to echinocandins. R, resistant; S, susceptible; WT, wild type.

and, exceptionally, few microsatellite clusters were broken into clonally related sub-STs (Fig. 1). Fluconazole-resistant isolates were unrelated.

Most *MSH2* mutations were found in cluster isolates. In general, the bulk of isolates within each cluster showed the same *MSH2* polymorphism, although a few genotypes (CG-1, CG-22, and CG-24) involved wild-type and *MSH2* mutant isolates. Conversely, some mutations were found in more than one genotype (Fig. 1).

Association of *MSH2* mutations and genotypes with clinical characteristics of the patients. Table 2 summarizes the characteristics of the study patients. There were no differences in demographics, underlying conditions, risk factors for candidemia, or sources of candidemia between patients infected by wild-type *MSH2* isolates and those with any point mutation. Most patients infected with *MSH2* mutants were adults (94.4%), with a mean age of 71 years, and 63.9% were male. Gastrointestinal disease was the most frequent comorbidity in 50% of patients, and the most frequent department of admission was a medical ward (47.2%) (Table 2). Most cases had an intra-abdominal source (38.9%) or were central venous catheter-related infections (25%).

TABLE 1 Newly characterized STs and description of the alleles in the six housekeeping genes^a

Patient(s)	New ST	Allele by gene					
		<i>FKS</i>	<i>LEU2</i>	<i>NMT1</i>	<i>TRP1</i>	<i>UGP1</i>	<i>URA3</i>
10, 11, 13	145	8	5	3	5	1	2
17	146	51	24	49	7	51	6
18,19	147	51	24	49	30	51	6
33	148	3	9	26	4	17	4
34, 35, 36	149	7	25	51	7	51	4
37	150	7	25	51	30	51	4

^aST 145 is a new combination of previously identified alleles. STs 146 to 150 are new combinations of previously identified alleles or the following new alleles (reported in this work for the first time).

TABLE 2 Demographics and clinical status of patients based on *MSH2* gene sequence^a

Patient characteristic ^b	Total (n = 81)	Wild-type <i>MSH2</i> gene isolates (n = 45)	Isolates with <i>MSH2</i> gene mutations (n = 36)	P value
Demographics				
Age (median [IQR]) (yr)	71 (0–91)	72 (0–90)	71 (0–91)	0.556
Male	50 (61.7)	27 (60.0)	23 (63.9)	0.819
Neonate (≤28 days)	4 (4.9)	2 (4.4)	2 (5.6)	1
Department				
Medical	36 (44.4)	19 (42.2)	17 (47.2)	0.822
Surgical	21 (25.9)	12 (26.7)	9 (25.0)	1
Onco-hematology	3 (3.7)	1 (2.2)	2 (5.6)	0.582
ICU	21 (25.9)	13 (28.9)	8 (22.2)	0.612
Underlying disease				
Cardiovascular disease	32 (39.5)	18 (40.0)	14 (38.9)	1
Neurological disorder	15 (18.5)	10 (22.2)	5 (13.9)	0.398
Chronic renal failure	22 (27.2)	11 (24.4)	11 (30.6)	0.619
Diabetes mellitus	18 (22.2)	13 (28.9)	5 (13.9)	0.178
Pulmonary disease	19 (23.5)	9 (20.0)	10 (27.8)	0.440
Liver disease	13 (16.0)	8 (17.8)	5 (13.9)	0.765
Gastrointestinal disease	38 (46.9)	20 (44.4)	18 (50.0)	0.659
Solid-organ malignancy	28 (34.6)	14 (31.1)	14 (38.9)	0.490
Hematologic malignancy	2 (2.5)	1 (2.2)	1 (2.8)	0.694
Solid-organ transplant	1 (1.2)	0	1 (2.8)	0.444
HIV	5 (6.2)	2 (4.4)	3 (8.3)	0.651
Charlson index (median [IQR])	6 (0–12)	6 (0–10)	6 (0–12)	0.388
Risk factor				
Central venous catheter	64 (79.0)	35 (77.8)	29 (80.6)	0.791
Total parenteral nutrition	44 (54.3)	24 (53.3)	20 (55.6)	1
Chemotherapy	9 (11.1)	5 (11.1)	4 (11.1)	1
Neutropenia (<500 cells/mm ³)	2 (2.5)	1 (2.2)	1 (2.8)	1
Corticosteroids (prior mo)	15 (18.5)	8 (17.8)	7 (19.4)	1
Immunosuppressive therapy	4 (4.9)	3 (6.7)	1 (2.8)	0.625
Surgery	33 (40.7)	16 (35.6)	17 (47.2)	0.364
Abdominal surgery	26 (32.1)	13 (28.9)	13 (36.1)	0.633
Previous antimicrobial use	76 (93.8)	42 (93.3)	34 (94.4)	1
Antifungal use				
Previous AF used	17 (22.4)	9 (22.0)	8 (22.9)	1
Voriconazole	2 (2.5)	2 (4.4)	0	0.5
Fluconazole	15 (18.5)	7 (15.6)	8 (22.2)	0.567
Echinocandins	4 (4.9)	1 (2.2)	3 (8.3)	0.318
Amphotericin B	1 (1.2)	1 (2.2)	0	1
Duration of AF (days)	15 (3–63)	21 (9–44)	14.5 (3–63)	0.933
Time from previous AF to episode (days)	1 (–11–73)	0 (–6–73)	2.5 (–11–73)	0.755
Breakthrough fungemia	7 (8.6)	3 (6.7)	4 (11.1)	0.694
Fluconazole resistance	4 (4.9)	2 (4.4)	2 (5.6)	1
Clinical manifestation				
Septic shock	24 (29.6)	18 (40.0)	6 (16.7)	0.028
Pitt score (median [IQR])	1 (0–12)	2 (0–10)	0 (0–12)	0.093
Infection source				
Primary	14 (17.3)	9 (20.0)	5 (13.9)	0.562
Central venous catheter	27 (33.3)	18 (40.0)	9 (25.0)	0.235
Urinary tract	4 (4.9)	3 (6.7)	1 (2.8)	0.625
Abdomen	28 (34.6)	14 (31.1)	14 (38.9)	0.490
Other	3 (3.7)	0	3 (8.3)	0.084
Unknown	5 (6.2)	1 (2.2)	4 (11.1)	0.166
Therapeutic management				
No antifungals	14 (17.3)	9 (20.0)	5 (13.9)	0.562
Echinocandin as initial AF	29 (43.3)	15 (41.7)	14 (45.2)	0.809
Adequate targeted AF	62 (76.5)	34 (75.6)	28 (77.8)	1
Infection source control	56 (81.2)	31 (81.6)	25 (80.6)	1

(Continued on following page)

TABLE 2 (Continued)

Patient characteristic ^b	Total (n = 81)	Wild-type <i>MSH2</i> gene isolates (n = 45)	Isolates with <i>MSH2</i> gene mutations (n = 36)	P value
Outcomes				
Metastatic complications	13 (18.1)	6 (15.0)	7 (21.9)	0.543
ICU due to candidemia	10 (12.3)	6 (13.3)	4 (11.1)	1
7-day mortality	13 (16.0)	10 (22.2)	3 (8.3)	0.129
30-day mortality	24 (29.6)	18 (40.0)	6 (16.7)	0.028
Mortality >30 days	11 (13.6)	5 (11.1)	6 (16.7)	0.526
Overall mortality	35 (43.2)	23 (51.1)	12 (33.3)	0.121
Hospital length of stay (days)	26 (0–165)	25 (0–165)	26.5 (0–112)	0.711

^aAll data reported as number (%), unless otherwise specified.

^bIQR, interquartile range; ICU, intensive care unit; HIV, human immunodeficiency virus; AF, antifungal.

Only one out of the four patients infected by fluconazole-resistant isolates was previously treated with fluconazole and caspofungin. The patient with endocarditis caused by the echinocandin-resistant isolate had previously received caspofungin, micafungin, and fluconazole. *In vitro* echinocandin-resistant-generated isolates came from 12 patients, of whom only four had received previous antifungal treatment. Eight patients with alterations in the *MSH2* gene isolates received systemic antifungals before the diagnosis, including fluconazole ($n = 8$) and echinocandins ($n = 3$). No statistical differences were determined in the median time of exposure to previous antifungals between patients infected by *MSH2* mutants and patients infected by wild-type isolates (14.5 and 21 days, respectively). Adequate targeted treatment and appropriate source control were equally ensured in both groups. Concerning clinical outcomes, there were no differences between groups with respect to metastatic complications, intensive care unit admission due to the severity of the candidemia, hospital length of stay, and early 7-day mortality. However, there was a higher proportion of patients with septic shock and 30-day mortality in those infected by wild-type *MSH2* gene isolates ($P = 0.028$) (Table 2). The same trend was observed when evaluating the mortality rates among patients with less severe clinical presentations (Pitt score, ≤ 1), although no statistical significance was found (data not shown). Furthermore, the ST3 genotype, which included mostly wild-type *MSH2* gene isolates (31/35), was detected more frequently in patients with septic shock (40% versus 21.7%; $P = 0.09$) and poor outcome (40% versus 21.7%; $P = 0.09$).

DISCUSSION

The analysis of a large number of *C. glabrata* isolates causing candidemia showed that the presence of *MSH2* gene mutations is not correlated with the previous use of antifungal agents, with specific genotypes (either defined using MLST or microsatellite markers), or with the promotion of antifungal resistance *in vitro* and in the patients.

Defects in DNA mismatch repair in multiple fungal species, including *C. glabrata*, have been reported to be associated with the acquisition of antifungal resistance (hypermutable phenotype) (14, 15, 18). A recent study identified a prevalent mutator *C. glabrata* phenotype caused by a mismatch repair defect in the *MSH2* gene (15). This phenotype may acquire resistance to multiple antifungals with the consequent added complication in patient care; the detection of *MSH2* point mutations was thus proposed as a marker of potential development of resistance. We did not find any correlation between the presence of *MSH2* gene mutations and the acquisition of antifungal resistance either *in vitro* or in clinical settings. This is in line with previous studies in which this lack of correlation was also highlighted (16, 17, 19).

A limitation of previous similar studies is the low number of resistant isolates. To solve this, in this study, we analyzed echinocandin-susceptible isolates that switched to a resistant phenotype after being exposed to candins *in vitro* ($n = 20$) (12) and naturally occurring in a patient with candidemia and endocarditis who had previously undergone treatment with echinocandins. We observed the following (Fig. 2): first, parent echinocandin-susceptible isolates and their respective resistant isolates had the same *MSH2* gene sequence. Second, echinocandin-susceptible isolates with either *MSH2*

gene mutations or a wild-type *MSH2* gene were able to switch to a resistant phenotype following drug exposure. Third, isolates with *MSH2* gene mutations were able to yield both echinocandin-resistant and echinocandin-susceptible isolates, as shown in the patient with endocarditis. Fourth, the *MSH2* gene sequence did not affect the acquired mechanism of resistance to echinocandins, as shown by the three isolates that were exposed to candins and yielded echinocandin-resistant isolates (*FKS2* mutants and wild-type *FKS*). Finally, mutations in the *MSH2* gene did not influence fluconazole resistance. The *MSH2* gene sequences of incident isolates and isolates causing second episodes were identical. Moreover, mutations were commonly found in incident susceptible isolates that did not turn resistant in further episodes. Here, the rate of isolates harboring mutations in the *MSH2* gene (44.4%) is similar to that reported in some other studies (15, 17) but lower than the 77.2% reported by Hou et al. (19). Certain mutations, such as V239L and P208S/N890I, have been associated with resistance (15, 17), whereas others that are less common, E7K, E456D, or E459K, have not (15–17).

The results of the two genotyping techniques are comparable, although a higher number of genotypes was obtained with microsatellite markers (19). Previous studies have tried to correlate specific genotypes with *MSH2* gene mutations (16, 19). Dellière and colleagues found a correlation between some microsatellite genotypes and *MSH2* gene mutations; however, the mutations were not genotype specific, as some of them were found in more than one genotype (16). Hou et al. reported a correlation between ST7 and ST10 and the presence of V239L and/or K583N and P208S/N890I mutations in the *MSH2* gene, respectively; however, the authors did not disclose if the same mutations were also found in other STs (19). Finally, the results described by Deshpande and Castanheira are in line with those of Hou et al., except that the V239L mutation was also found in ST2 and ST7, whereas ST3 isolates carried a wild-type *MSH2* gene (20). In considering the results of all these studies, including ours, there seems to be a relationship between STs and the *MSH2* gene sequence; e.g., ST7 and ST10 are found in different geographic places, including our isolates, and harbor specific mutations. However, there is no genotype specificity regarding the mutations; for example, we found that V239L was conspicuously present in ST2, ST3, ST7, ST8, and ST34 (Fig. 1). This suggests that a number of mutations may be potential *MSH2* gene constitutive polymorphisms rather than resistance-causing mutations. A recent study using whole-genome sequencing data suggests that these *MSH2* mutations are natural genetic variations rather than a mutator phenotype (21). Furthermore, *MSH2* gene sequences were mostly identical in the isolates belonging to a given ST or microsatellite genotype, with some exceptions (Fig. 1) (19, 22).

We report not only on the microbiological characterization of *C. glabrata* isolates but also the clinical description of the infected patients, which is a strength of our study. Patients infected by *C. glabrata* carrying *MSH2* gene mutations or with the wild-type *MSH2* gene presented similar demographic and clinical characteristics. Moreover, no differences in terms of previous antifungal use between groups were found. Our observations are in line with those from a previous study (16) but diverge with results from another work (15). Despite the comparable host characteristics and therapeutic management, a higher number of severe cases (higher median Pitt scores) was detected in the group of patients infected by strains with the wild-type *MSH2* gene, resulting in a higher incidence of septic shock and 30-day mortality. In fact, such differences could be related to the remarkable prevalence of ST3 genotype isolates among these patients. Other authors have reported that the most frequently found ST3 and ST7 may be correlated with a poor outcome (23), supporting our findings. In fact, our observations show that patients infected by ST3 show higher 30-day mortality than do patients infected by any of the other STs (40% versus 21.7%, respectively; $P = 0.09$). Further studies, including a higher number of episodes, should be carried out to evaluate the clinical impact of infections by ST3 isolates.

The low numbers of *FKS* mutant and fluconazole-resistant isolates found in the patients are in line with previous studies and the main limitation of our study (1, 24, 25).

However, our conclusions are supported by a number of *C. glabrata* resistant isolates *in vitro* generated from a set of incident isolates.

We conclude that the presence of *MSH2* gene mutations in *C. glabrata* isolates causing candidemia does not correlate with the previous use of antifungal agents by the patients, with specific genotypes, or with the promotion of antifungal resistance. Furthermore, not differences in clinical outcomes were observed.

MATERIALS AND METHODS

Yeast isolates, antifungal susceptibility, and patients. One hundred twenty-four *C. glabrata* isolates were studied. The distribution of isolates was as follows: isolates causing candidemia in 81 patients ($n = 81$ incident isolates and $n = 9$ second episodes; $n = 90$), isolates from the heart valve of one patient who developed endocarditis (patient no. 15, Fig. 1; $n = 2$), and a group of phenotypically echinocandin-resistant isolates that had been exposed *in vitro* (12) to echinocandins and were either *FKS2* mutants ($n = 21$) or wild-type *FKS* ($n = 11$) (Fig. 2). Second episode was defined as the isolation of *C. glabrata* from blood cultures taken ≥ 7 days after the diagnosis. Patients were admitted to Gregorio Marañón Hospital (Madrid, Spain) between January 2007 and December 2016, and isolates were molecularly identified and tested for susceptibility to micafungin, anidulafungin, and fluconazole following the EUCAST 7.3.1 microdilution procedure (26, 27). General characteristics (demographic, department of admission, underlying diseases, risk factors, and antifungal use) and clinical condition (clinical manifestation, source of infection, therapeutic management, and outcome) were collected, as well as the use of antifungals in the six months before the diagnosis.

***MSH2* sequencing and genotyping.** The *MSH2* gene of the 124 isolates was amplified and sequenced as described by Dellière et al. (16), with some modifications: 1.25 U of AmpliTaq gold (Applied Biosystems), 0.2 mM deoxynucleoside triphosphates, 2 mM MgCl₂, and 100 ng of the extracted DNA were used (12). *MSH2* sequences were compared with the reference wild-type *MSH2* gene sequence of *C. glabrata* (GenBank accession number XM_447585.1) (16).

The isolates were further genotyped by two previously described methods, a panel of six microsatellite markers (*MT1*, *ERG3*, *GLM4*, *CG10*, *CG7*, and *CG4*) (28–30) and MLST based on six housekeeping genes, including the loci *FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3* (31). PCR products of the six housekeeping gene loci were sequenced in both directions. The combination of the alleles of the six loci defined an MLST genotype or sequence type (ST) according to the MLST database (<http://pubmlst.org/cglabrata>). Singleton genotypes were defined as those found only once, whereas a cluster was defined as the presence of ≥ 2 strains showing identical genotypes.

Data analysis. Normally distributed clinical variables are expressed as means \pm standard deviation. Variables with nonnormal distribution are expressed as medians and interquartile ranges. Categorical variables are expressed as percentages and compared using Fisher's exact test or the χ^2 test, as appropriate. Continuous variables were compared with the Mann-Whitney U test. The comparisons were considered statistically significant with *P* values of < 0.05 (IBM SPSS Statistics for Windows, version 21.0; Armonk, NY, USA).

Ethical considerations. This study was approved by the ethics committee of Hospital Gregorio Marañón (CEIC-A1; study no. 203/18).

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