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Two missense mutations, E123Q and K151E, identified in the *ERG11* allele of an azole-resistant isolate of *Candida kefyr* recovered from a stem cell transplant patient for acute myeloid leukemia

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ABSTRACT

We report on the first cloning and nucleotide sequencing of an *ERG11* allele from a clinical isolate of *Candida kefyr* cross-resistant to azole antifungals. It was recovered from a stem cell transplant patient, in an oncohematology unit exhibiting unexpected high prevalence of *C. kefyr*. Two amino acid substitutions were identified: K151E, whose role in fluconazole resistance was already demonstrated in *Candida albicans*, and E123Q, a new substitution never described so far in azole-resistant *Candida* yeast.

1. Introduction

Candida kefyr is an ubiquituous yeast that is usually considered as making part of the yeast microflora in dairy products and cheeses [1]. Accordingly, most of *C. kefyr* were isolated from gastrointestinal tract in humans, and it can be supposed that its carriage tightly depends on eating habits. *C. kefyr* has been described also as an emerging opportunistic pathogen, particularly in patients with oncohematological diseases [2–4]. Intriguingly, the prevalence of *C. kefyr* seems to be unexpectedly high in some oncohematology units: for example, in three French teaching hospitals, its frequency was reported to be twice that of all other wards [3]. Even though such a high prevalence remains rationally unexplained, it may pose specific problems of resistance in oncohematology where antifungal prophylaxis and empirical treatment are commonly used. It is probable that clinical isolates of *C. kefyr* can exist majoritarily under the form of haploid cells, as do its teleomorphic form *Kluyveromyces marxianus* [5]. Haploidy could contribute to the development of antifungal resistance, notably by mutations in the genes encoding the target of antifungals [6]. Recently, the first clinical isolate of *C. kefyr* resistant to caspofungin was described [4]. In this study, we report the isolation of a clinical isolate cross-resistant to azole-antifungals in a patient who had undergone a stem cell transplantation for an acute myeloid leukemia. Taking advantage of the recent nucleotide sequencing of the genome of *K. marxianus* [7], we retrieved the sequence of the *ERG11* gene, and designed primers for the cloning and sequencing of the *ERG11* allele from the azole-resistant isolate. This allowed us to identify two missense mutations leading to the amino acid substitutions E123Q and K151E, whose combination could account for the high level of resistance to fluconazole and voriconazole, and to azole cross-resistance phenotype in *C. kefyr*.

2. Case

A 32-year-old woman was diagnosed in 1997 with JAK2 mutation-negative essential thrombocythemia (ET) treated by pipobroman the first 7 years, then hydroxyurea and anagrelide for 4 and 3 years, respectively. In 2012, the patient began complaining about asthenia, with a gradual decrease in hemoglobin level. Anagrelide was stopped and a bone marrow biopsy was performed in December 2012, which showed the occurrence of

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a grade 2 myelofibrosis. A few weeks later, hematologic tests revealed more than 20% of blastic cells in peripheral blood, indicating that ET with myelofibrosis had evolved into acute myeloid leukemia.

The patient had one HLA-identical sister, and received an allogeneic bone marrow hematopoietic stem-cell transplantation (HSCT) on 27 March 2013. On Day 0 (D0) of HSCT, the patient was admitted to intensive care unit (ICU) for an acute respiratory distress syndrome (RDS). Four blood cultures (BacT/Alert 3D, BioMérieux, Organon Teknika, USA) yielded *Staphylococcus aureus* and *Escherichia coli*. A broad-spectrum antibiotherapy (tazobactam/piperacillin, amikacin and vancomycin) associated with furosemide for the acute pulmonary edema led to a rapid improvement of the patient condition. An oral fluconazole prophylaxis (400 mg/day) was also started. On Day 12, the patient was readmitted to ICU for an acute RDS and a septic shock with no microbiological evidence of infection. The patient became afebrile after an empirical antibiotherapy (ceftazidime and linezolid).

On Day 37, the patient presented with the first symptoms of a Graft-versus-Host Disease (GvHD), that developed to the skin, the gastro-intestinal tract and the liver. She received corticosteroids, inolimomab, sirolimus and basiliximab but digestive disorders remained chronic with hemorrhagic manifestations.

On Day 63, during another admission to ICU for similar respiratory symptomatology, a new antibiotic regimen (imipenem, ciprofloxacin and subsequently ceftriaxone) associated to intravenous caspofungin (50 mg/day) was started. Routine microbiological surveillance cultures were performed weekly. During two months, June and July, *Candida albicans* was isolated from oropharynx swab, rectal swab or stools and urine. Then, from 12 August, *C. albicans* was no longer isolated butcultures documented a colonization of the gastrointestinal and urinary tracts by *C. kefyr* (teleomorph, *K. marxianus*), with an increasing fungal load.

Caspofungin stopped after 2 months and switched to oral fluconazole (400 mg/day) was restarded on 17 August.The neutropenic patient suffered from an unexplained fever that led to perform a thoracic computed tomography (CT) on 24 September. CT imaging showed pulmonary nodules suspected to be a pulmonary aspergillosis. Antifungal therapy with caspofungin was switched to voriconazole. Serum galactomannan (GM) assay (Platelia® Aspergillus Ag Kit, Bio-Rad), performed twice a week, was constantly negative. On 26 September, GM assay index measured on bronchoalveolar lavage (BAL) was negative. The BAL culture yielded no *Aspergillus* but heavy growth of *C. kefyr*. Blood cultures remained negative. No other pathogen was found.

On 2 October, measurement of (1→3)-β-D-glucan antigenemia (Fungitell® Assay, Associates of Cape Cod, Inc., East Falmouth, MA) revealed a significantly elevated serum concentration of 411 pg/mL (positive cutoff $\geq$80 pg/mL). A possible deep candidiasis was suspected by the clinicians and treatment with caspofungin was resumed [8]. Follow-up CT showed the increase in size and number of pulmonary lesions. The patient was readmitted to ICU with an acute renal failure and died of multiple organ failure one week later.

Identification of *C. kefyr* was performed with matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Microflex, Bruker Daltonik). Identification was confirmed by amplifying and sequencing the internal transcribed spacer (ITS) region of rDNA and a segment of the 18S rDNA gene, using the universal primer pair ITS1 and ITS4, and the primer pair NS3 and NS4, respectively (Table 1). PCR products were synthesized using HiFidelity Taq-polymerase (QIAGEN, Hamburg, Germany), and purified with QIAquick PCR purification kit (QIAGEN). ITS1, ITS4, NS3, NS4 were used individually as primers to sequence both strands of each PCR product with ABIPrism Dye Terminator Cycle Sequencing Ready Reaction v1.1 Kit (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. The nucleotide sequences of ITS (GenBank accession number KF964549) and of the 18S rDNA gene segment (GenBank accession number KF964550) were compared with those of the databases of the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool [9].

Antifungal susceptibility testing was performed by *E*-test (BioMérieux) on RPMI medium. The MICs were measured after 24 h and 48 h of incubation, and tested twice from different *C. kefyr* isolates. The MIC values of azole, amphotericin B and echinocandin antifungals for the strain isolated in September, so called the *C. kefyr* PAZ isolate, are given in Table 2. The PAZ isolate exhibited high MIC values for all azole antifungals tested, and was fully resistant to fluconazole and voriconazole. The MIC values of two other strains were determined as control: one routinely recovered from an unrelated patient (isolate TEM) and the reference strain *K. marxianus* CBS 6556, the teleomorph of *C. kefyr*. Both strains were fully susceptible to all antifungals tested.

The *ERG11* gene encoding lanosterol 14-α-demethylase of *C. kefyr* was identified from the raw database of the genome sequence of the strain CBS 6556 (ATCC 26548) of *K. marxianus* [7]. The nucleotide sequence of *ERG11* was retrieved from the scaffold 2 (Genbank accession number JH924897) by the dot-plot DNA matrix analysis tool [10] embedded in the MacVector software (v12.7), using as query the *ERG11* nucleotidic sequence of the *Kluyveromyces lactis* strain NRRL Y-1140 (Genbank accession CR382125, locus tag KLLA0_E03653g, [11]). The *ERG11* gene was then amplified by PCR from the clinical isolates PAZ and TEM, and from the reference strain CBS 6556, using the primer pair FergCk0 and RergCk0 (Table 1). The nucleotide sequence of the 1581 bp coding region was determined for the three alleles with the aim to identify mutations that could be responsible for the resistance to azole antifungals in the isolate PAZ. The sequence data were assembled and compared each other, allowing the identification of several single nucleotide polymorphisms (SNP) (Table 3). Most SNP corresponded to silent mutations with no amino acid changes. Interestingly, the PAZ *ERG11* allele possessed two missense mutations, E123Q and K151E that were not present in the *ERG11* alleles of the azole susceptible control strains. Genbank accession numbers for the nucleotide sequences of the TEM, PAZ and CBS 6556 *ERG11* alleles are KF964548, KF964547 and KF964546, respectively.

3. Discussion

C. kefyr may be considered as an emerging pathogen, particularly in patients of oncohematology units [2–4]. In Bordeaux University Hospital Center (Bordeaux, France), during an 8-year period (2005–2012), a total of 3701 *Candida* spp. were isolated from routine mycological cultures performed from oncohematological patients. Non-*albicans Candida* represented 1858 isolates (50.2%), of which 359 isolates of *C. kefyr* (9.7% of total *Candida* isolates). This unexpected prevalence of *C. kefyr* is two-fold higher than that previously reported (4.8%) from oncohematology wards of three other French teaching hospitals [3]. The incidence of *C. kefyr* in the 612 candidemia diagnosed over the period 2005–2012 from all wards of our institution was 1.8%. This is similar to the data reported from the YEASTS surveillance program in another French area during the period 2002–2010 (1.7% of candidemia caused by *C. kefyr* [12]). However, in Bordeaux, two-third of candidemia due to *C. kefyr* were diagnosed in adult patients hospitalized in oncohematology, making *C. kefyr* responsible for 15% of the candidemia in the adult oncohematology ward.

The reasons of the high incidence of *C. kefyr* in the gastrointestinal flora of patients with hematologic malignancies are not