The Role of FKS1 and ERG11 Gene Variation in Multidrug Resistance among Candida spp. Isolated from Immunocompromised Patients

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Abstract

Candidiasis is an infection caused by Candida species, mostly by Candida albicans. Some strains of Candida are resistant to the antifungals used to treat them. The current study was aimed to investigate the genetic variations in FKS1 and ERG11 genes that correlates with Candida spp. isolates resistance to antifungal drugs. with cancer, diabetes, and breastfeeding infants.

The study included a collection of 157 clinical samples from immunocompromised patients, these samples were subjected to several steps of diagnosis techniques to identify the isolates to the species level. The antifungal drug sensitivity was determined by disk division method, and the genetic variation and mutations correlated with the drug resistance were detected by analyzing the DNA sequences of the FKS1 and ERG11 genes, by amplifying those genes from the drug resistance and sensitive isolates with a polymerase chain reaction (PCR) and performing a Sanger sequencing technique for the PCR amplicons which followed by conducting an alignment analysis to investigate the posable gene variation and mutations. The culture results detected a positive Candida species growth in 53 out of 157 samples (33.7%), and the drug susceptibility test results for these isolates to seven antifungal drugs included in the study detected a variable drug resistance among these isolates for each drug tested. The highest percentage of resistant isolates was 16.98% for Caspofungin followed by 11.32% for Fluconazole, 5.66% for Ketoconazole and 3.77% for Clotrimazole. While there was no resistant isolate for Itraconazole, Nystatin and Amphotericin-B. The results of the DNA sequencing analysis of the HS1 region of FKS1gene detected the presence of amino acid substitution F640L mutation in in the Caspofungin resistant isolates. And S642L mutation in all of the resistant and susceptible isolates. While the sequencing analysis of the ERG11 gene detected 7 different amino acid substitution mutations, F126L, I131F, C134F, F145I, A149S and V159F were observed only in resistant isolates, while E266D was observed in sensitive and resistant isolates. All mutations were previously reported except for I131F, C134F, A149S and V159F were reported only in the present study. In conclusion the amino acids substitution mutations in FKS1 and ERG11 genes were responsible for the reduced susceptibility of Candida isolates to Caspofungin and Azoles respectively.

Keywords: FKS1, ERG11, Candidiasis, Multidrug Resistant Candida

1. Introduction

Candida species are among the most common etiological agents of fungal infections in humans [1]. The main types of Candida spp. that causes clinical infections are Candida albicans and then Candida glabrata. The rest of the other types isolated from the bloodstream that cause candidemia include C. parapsilosis, C. krusei, C. tropicalis, C. dubliniensis, C. lusitaniae, and the most recent C. auris, which is considered the most dangerous species because of its multidrug resistance to antifungal drugs, C. auris described cases increased by three folds between 2015 and 2018 [2].

The most susceptible are immunologically compromised patients, such as HIV-infected, Patients with Diabetes Mellitus, oral cavities of breastfeeding infants, and cancer patients especially for those undergoing chemotherapy and radiotherapy [3-5].

When Candida acquires mutation or intrinsic mutations in some genetic regions that are encode for the subunits of the β-(1,3)-D-glucan synthase (Fks), that are responsible for the synthesis of β-(1,3)-D-glucan, which is one of the most important components in the wall of fungi, these mutations in genetic regions causes a reduce in binding affinity and, thus, causes Echinocandin resistance [6].

Echinocandins are lipopeptides that mostly fungicidal class of antifungals, include caspofungin, micafungin and anidulafungin. These drugs targeting β 1-3 glucan synthase, an enzyme important for cell wall biosynthesis localized to the plasma membrane. The enzyme is encoded by the homologs FKS1 and FKS2 [7-9].

The mechanism of echinocandin resistance in Candida species involves genetic acquisition of mutations in FKS genes, which encode the catalytic subunits of glucan synthase. Echinocandin resistance is associated with amino acid substitutions in two narrow hot spot regions of Fks1 for all Candida specie [9].

Ergosterol that is essential component of candida plasma membrane that maintains of integrity of fungal cell wall, ER11 gene is encode lanosterol 14α-demethylase that important in the synthesis of ergosterol. This enzyme is the target of fluconazole which causes inhibit it with a resulting impairment of ergosterol synthesis and decrease its quantity. When a mutation occurs in ER11 gene causes over-production of these enzyme in increase in the synthesis of ergosterol which enables Candida to resist fluconazole [10, 11].

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This substitution is the only mechanism to produce clinical breakthrough infection during therapy. The echinocandins are not substrates for multidrug transporters, and other mechanisms causing azole resistance are not cross-resistant with echinocandins [12]. Certain Candida species are intrinsically resistant to particular anti-fungals, meaning that all isolates of this species show elevated minimum inhibitory concentrations (MIC) to a drug compared with other Candida species. Here we will use MIC to refer to MIC50, which is defined as the lowest drug concentration that inhibits 50% fungal growth after 24 h [13, 14].

Multidrug-resistant(MDR) was defined as acquired non susceptibility to at least 1 agent in 3 or more antimicrobial categories [15]. This definition cannot be directly accepted for resistance in Candida because that only four drug classes are existing for systemic treatment of Candidiasis including the Azoles, Polyenes, Echinocandins, and the end is Pyrimidine analogue. Among these drug classes, only members of the first 3 are allowed for monotherapy against Candidiasis among them fluconazole and Echinocandins are only recommended as first line agents for invasive candidiasis. So, the appropriate definition for MDR Candida as an isolate non susceptible to ≥1 agent in ≥2 different drug classes [13].

2. Materials and Methods

Yeast Isolates and Phenotypic Identification

A total of 157 clinical samples from patients with cancer from Euphrates cancer Hospital in Iraq. The diagnose of clinical sample done by some phenotypic, biochemical characteristics of yeasts according to Morphological Characteristics (Culture Examination, Microscopic Examination), Biochemical tests (API 20C AUX Yeast Identification System, HiCrome™ Candida Differential Agar Test, HiCrome™ Candida Differential Agar supplemented with Pal's Medium Test, Tobacco Test).

Antifungal Susceptibility Test with Disk Diffusion Method

Seven types of antifungal drug discs were used in current study: Fluconazole (FLC 10µg), Ketoconazole (KT 10µg), Itraconazole (IT 10µg), Clotrimazole (CC 10µg), Nystatin (NS 100IU), Amphotericin-B (AMB 10 µg) and Caspofungin (CA 5 µg). Disk diffusion testing was performed as described by CLSI document M44, 3rd ed. (2018).

FKS1 and *ERG11* Genes Sequence Analyses

A fragment sized 450bp of FKS1contains the hot-spot region 1 (HSR1) in which mutations are known to cause reduced susceptibility to Echinocandins including the Caspofungin antifungal drug was amplified with a previously published [16] primer pair FKS1-F 5'-GAAATCGGCATATGCTGTGTC-3' and FKS1-R 5'-AATGAACGACCAATGGAGAAG-3'.

For ERG11 genes, the entire coding region was amplified using the primers pair ERG11-F: 5'-CAAGAAGATCATAACTCAAT-3' and ERG11-R: 5'-AGAACACTGAATCGAAAG-3' [17] for the investigating of the mutations that cause drug resistance to Azoles and Polyenes antifungal agents.

The PCR products were sequenced at MACROGEN sequencing company, Seoul, Korea using the automated sequencer 23 ABI 3730xl DNA analyzer (by the dideoxynucleotide chain termination reaction). The informativity of the genetic variation was investigated by comparing these sequences with the sequences published in GenBank sequence database, which is part of the National Center for Biotechnology Information (NCBI), that taken as a reference through BLAST (Basic Local Alignment Search Tool) searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were analyzed with BioEdit sequence alignment editor version 7.2.5 (Isis Therapeutics, Carlsbad, CA, USA).

3. Results and Discussion

Isolation of *Candida* species and Distribution according to *Candida* species

From the total 157 clinical samples included in the present study 53 (33.7%) samples were positive for Candida spp. growth. according to the morphological characteristics and the biochemical and molecular study results. the most predominant species among the clinical isolates included in this study was C. albicans at 26 out of 53 clinical isolates (49.05%) followed by C. glabrata 7 (13.21%), C. parapsilosis 6 (11.32%), C. dubliensis 6 (11.32%), C. tropicalis 4 (7.54%), C. auris 3(5.67%), and finally, C. haemulonii 1(1.8%).

The Antifungal Susceptibility Test

This study was carried out by disk diffusion method which conducted and interpreted according to the CLSI document M44, 3rd ed. (2018) standard method, the results were demonstrated as a susceptibility pattern composed of three categories resistant, intermediate and susceptible for each drug.

The susceptibility pattern for Caspofungin was 9 (16.98%) isolates resistant, 12 (22.64%) intermediate and 32 (60.37%) susceptible. While for Fluconazole was 6 (11.32%) resistant and 47 (88.67%) susceptible with no intermediate isolate. The susceptibility pattern for Ketoconazole was 3 (5.66%) resistant, 7 (13.20%) intermediate and 43 (81.13%) susceptible, which differed slightly from that of Clotrimazole which was 2 (3.77%) resistant, 6 (11.32%) intermediate and 45 (84.90%) susceptible. The susceptibility pattern of Itraconazole and Amphotericin-B were 11 (20.75%), 8 (15.09%) intermediate and 42 (79.25%), 45 (84.90%) susceptible respectively with no resistant isolates. While for Nystatin all of the 53 (100%) isolates were susceptible (Table 1).

Table 1: The antifungal drugs susceptibility patterns of Candida spp. isolates

Antifungal disk	Sensitive (%)	Intermediate (%)	Resistance (%)
Caspofungin (5µg)	32 (60.37%)	12 (22.64%)	9 (16.98%)
Fluconazole (10 µg)	47 (88.67%)	0 (0%)	6 (11.32%)
Ketoconazole (10µg)	43 (81.13%)	7 (13.20%)	3 (5.66%)

Clotrimazole (10 µg)	45 (84.90%)	6 (11.32%)	2 (3.77%)
Itraconazole (10 µg)	42 (79.25%)	11 (20.75%)	0 (0%)
Amphotericin-B (10µg)	45 (84.90%)	8 (15.09%)	0 (0%)
Nystatin (100 µg)	53 (100%)	0 (0%)	0 (0%)

Multidrug Resistant Isolates among *Candida spp.*

Multidrug-resistant (MDR) isolate was defined as an isolate non susceptible to one or more antifungal agent in at least two different drug classes [13]. According to that and depending on the results of the antifungal susceptibility test, Five MDR isolates were detected among Candida spp. isolated in the current study (Table 2), Three C. auris MDR isolates resistant to Ketoconazole and Caspofungin, one C. albicans MDR isolates resistant to Ketoconazole and Caspofungin, and one C. dubliensis MDR isolate resistant to Caspofungin and Fluconazole.

Table 2: Multidrug Resistant (MDR) Candida spp. isolates.

Candida spp.	No. of MDR Isolate	Antifungal agent	Antifungal Class
C. auris	3	Caspofungin,	Echinocandin
		Ketoconazole	Azole
C. albicans	1	Caspofungin	Echinocandin
		Ketoconazole	Azole
C. dubliensis	1	Caspofungin,	Echinocandin
		Fluconazole	Azole
Total	5		

FKS1 Sequence Analysis for Caspofungin resistance Isolates

The DNA sequencing analysis were done by comparing the sequence of the 450 bp PCR products fragment of the FKS1gene (Figure 1) containing the hot-spot region 1 (HS1) of ten randomly selected isolates having different Caspofungin susceptibility patterns (6 resistant and 4 susceptible) with the a wild-type drug sensitive strain (Fks1p, accession number D88815.1), published in GenBank sequence database of NCBI as a reference through BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by using BioEdit sequence alignment editor (Isis Therapeutics, Carlsbad, CA, USA).

The results detected the presence of a point mutation in HS1 region of FKS1gene in the 2625 location (base T to A) in the isolates that previously detected by antifungal disk diffusion method as Caspofungin resistant (samples 1, 2, 3, 4,5 and 6) this mutation caused a substitution of the amino acid Phenylalanine by Leucine (F640L), while there was no point mutation in the sequences of the susceptible isolates (samples 7,8,9 and 10). The results also detected another point mutation in HS1 region of FKS1gene in the 2632 location (base C to T) in all of the resistant and susceptible isolates causing substitution of the amino acid Serin by Leucin (S642L).

The present study revealed the associated of the amino acid substitution F640L in the 1,3 β-glucan synthases with the acquisition of Caspofungin resistance, in three isolates of C. auris and one isolate of each C. albicans and C. dubliensis, there is no previous reports of detecting this mutation in these species except one study of Lockhart et al. [18] who reported that only a single C. lusitaniae isolate with a reduced susceptibility to Anidulafungin, Caspofungin and Micafungin had an F640L substitution.

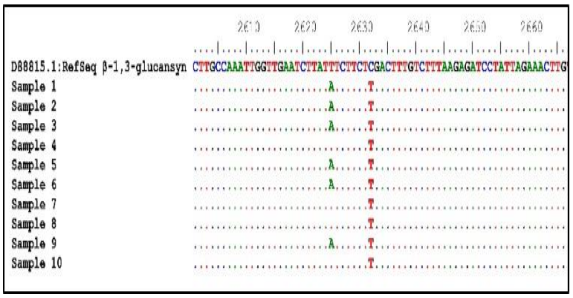

Figure 1: DNA sequence alignment of the PCR amplicons FKS1gene

ERG11 Sequence Analysis for Azoles Resistance Mutations

The genetic variation was investigated by comparing the entire open reading frame (ORF) of ERG11 gene (Figure 2) sequence of eight randomly selected isolates having different Azoles susceptibility patterns (6 resistant and 2 susceptible) with the sequence of the fluconazole susceptible strain SC5314 (GenBank accession number X13296.1), that it is published in GenBank sequence database, which is part of the National Center for Biotechnology Information (NCBI), that taken as a reference through BLAST (Basic Local Alignment Search Tool) searches (http://blast.ncbi.nlm. nih.gov/Blast.cgi). Sequences were analyzed with BioEdit sequence alignment editor version 7.2.5 (Isis Therapeutics, Carlsbad, CA, USA).

Sequence analysis detected 7 different amino acid substitution mutations: F126L, I131F, C134F, F145I, A149S and V159F were observed only in resistant isolates (Figure 4-16), these results agreed with [19] and Wang et al. [20], while E266D was observed in sensitive and resistant isolates as reported by Wang et al. [20]. All mutations were previously reported except for I131F, C134F, A149S and V159F were reported only in the present study.

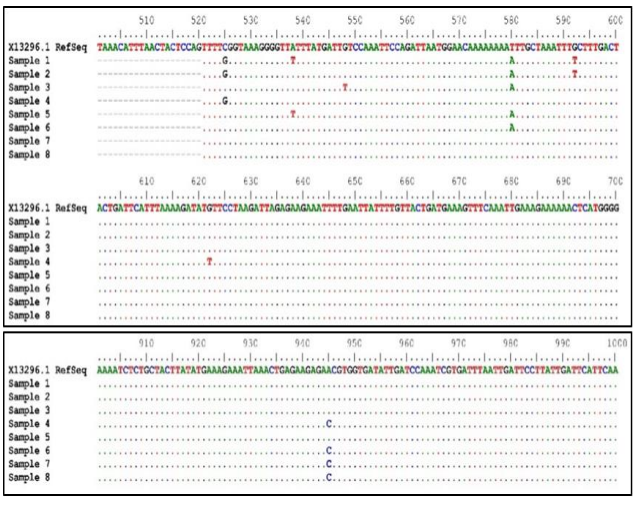

Figure 2: DNA sequence alignment of the PCR amplicons ERG11 gene

4. Conclusions

The present study concluded that the amino acids substitution mutations in FKS1 and ERG11 genes were responsible for the reduced susceptibility of Candida isolates to Caspofungin and Azoles respectively.

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